Asymmetrische Zellteilung und Zellpolarität von humanen hämatopoietischen Stamm- und Vorläuferzellen

Inaugural – Dissertation

zur

Erlangung des Doktorgrades der

Mathematisch-Naturwissenschaftlichen Fakultät

der Heinrich-Heine-Universität Düsseldorf

vorgelegt von

Julia Beckmann

aus Bergisch Gladbach

Mai 2008

Aus dem Institut für Transplantationsdiagnostik und Zelltherapeutika
der Universitätskliniken der Heinrich-Heine-Universität Düsseldorf

Gedruckt mit der Genehmigung der
Mathematisch-Naturwissenschaftlichen Fakultät der
Heinrich-Heine-Universität Düsseldorf

Referent: PD Dr. M. Uhrberg

Koreferent: Prof. Dr. J. H. Hegemann

Tag der mündlichen Prüfung: 27.06.2008

1 Zusammenfassung

Für die lebenslange Aufrechterhaltung der Blutbildung ist es erforderlich, dass sich hämatopoietische Stammzellen (HSZ) über einen langen Zeitraum hinweg selbst erneuern und gleichzeitig Vorläufer hervorbringen können, die sich zu reifen Blutzellen entwickeln. Wie das Gleichgewicht zwischen Selbsterneuerung und Differenzierung bei HSZ kontrolliert wird, ist nicht geklärt. Vergleichende Proliferations- und Zellschicksalsanalysen von Nachkommen einzelner primitiver hämatopoietischer Zellen deuten darauf hin, dass die Entscheidung bei hämatopoietischen Stamm- und Vorläuferzellen (HSZ/HVZ) möglicherweise durch asymmetrische Zellteilungen reguliert wird. Um auszuschließen, dass die Unterschiede zwischen den Geschwisterzellen erst nach der Teilung etabliert werden, habe ich in dieser Arbeit nach Faktoren gesucht, die in mitotischen Zellen asymmetrisch auf die beiden Tochterzellen verteilt werden.

Da asymmetrisch segregierende Faktoren vor Stammzellteilungen bei Modellorganismen polar angeordnet werden, wurde zunächst in HSZ/HVZ nach polar lokalisierten Faktoren gesucht, um potenzielle Kandidaten zu identifizieren. Die Untersuchungen zur Zellpolarität ergaben, dass kultivierte HSZ/HVZ eine polarisierte Zellform annehmen können. Es zeigte sich, dass dieser Prozess von der Aktivität der Phosphatidyl-Inositol-3-Kinase, der atypischen Proteinkinase C, der Rho-GTPasen sowie von der Integrität des Zytoskeletts abhängt. Die morphologische Polarisierung ging weiterhin mit einer Umverteilung bestimmter Oberflächenmoleküle zu den Zellpolen einher. Desweiteren beoabachteten wir, dass diese Faktoren ihre polare Verteilung auch während der Mitose beibehalten, die Aufteilung auf die Tochterzellen jedoch symmetrisch erfolgt.

Im Folgenden identifizierten wir mehrere Proteine, deren Expessionsmuster nach der ersten Zellteilung eine Unterscheidung von Subfraktionen innerhalb der $CD34^+$ Zellen zuließ. Da diese Subfraktionen möglicherweise infolge einer asymmetrischen Verteilung der Proteine auf die Tochterzellen entstehen und darüber hinaus funktionelle Unterschiede aufwiesen, wurde die Verteilung von vier dieser Proteine in mitotischen Zellen analysiert. Dabei fanden wir, dass die untersuchten Proteine tatsächlich in einem Teil der Mitosen asymmetrisch auf die Tochterzellen verteilt werden. Somit konnten wir erstmals zeigen, dass sich primitive hämatopoietische Zellen asymmetrisch teilen können.

2 Summary

For the maintenance of the production of all blood cell lineages throughout life hematopoietic stem cells (HSC) have to self renew over a long period of time. At the same time, they generate progenitor cells which differentiate into mature blood cells during their further development. It is currently not well understood how the balance between self-renewal and differentiation of HSC is controlled. Comparative studies of the cell fate and proliferation behaviour of progenies from single primitive hematopoietic cells suggest that the observed differences between daughter cells originate from asymmetric cell divisions. To rule out that other mechanisms like e.g. post-mitotic decision processes account for the differences between the sibling cells, molecules should be identified that are distributed asymmetrically onto the daughter cells during cell division.

Since asymmetrically segregating factors are arranged in a polarized manner prior to stem cell divisions in model organisms we first looked for molecules with a polarized localization in hematopoietic stem and progenitor cells (HSC/HPC) to identify potential candidates for asymmetric segregation. These studies on cell polarity revealed that during cultivation HSC/HPC can acquire a polarized morphology. We could show that this process depends on the activity of the phospho-inositol-3-kinase, atypical kinase C, Rho-GTPases and the integrity of the cytoskeleton. Additionally, the morphologic polarization coincides with a redistribution of several surface molecules to the arising cell poles. Furthermore, we found that these antigens maintain their polarized localizations during mitosis but are distributed in equal amounts among the daughter cells.

Furthermore, we identified several proteins that allow the flow cytometric discrimination of two subfractions within $CD34^+$ cells after their first cell division during cultivation. As the subfractions might arise from asymmetric protein segregation into the daughter cells during cell division and besides showed differences in functional assays, we analyzed the distribution of four candidate proteins in mitotic cells. We found that these proteins are frequently distributed asymmetrically between the daughter cells during mitosis. Thus we could confirm the occurrence of asymmetric cell divisions within the HSC/HPC compartment for the first time.

3 Abkürzungsverzeichnis

AGM	Aorta-Gonad-Mesonephros
aPKC	atypische Proteinkinase C
bFGF	*basic fibroblast growth factor;* basischer Fibroblasten-Wachstumsfaktor
bzw.	beziehungsweise
CB	*umbilical cord blood*, Nabelschnurrestblut
CD	*cluster of differentiation;* Differenzierungscluster
CFU-GEMM	*Colony-Forming-Unit Granulocyte, Erythrocyte, Macrophage, Megakaryocyte;* Zelle mit dem Potenzial zur Bildung von Granulozyten-, Erythrozyten-, Makrophagen- und Megakaryozyten-Kolonien
CLP	*Common lymphoid progenitor*, gemeinsame lymphatische Vorläuferzelle
CMP	*Common myeloid progenitor*, gemeinsame myeloische Vorläuferzelle
EPO	Erythropoietin
Flt3-L	*macrophage-colony-stimulating factor receptor (FMS)-like tyrosine kinase 3 ligand*, Makrophagen-Kolonie stimulierender Faktor (FMS)-ähnliche Tyrosinkinase-3-Ligand
G-CSF	*granulocyte-colony-stimulating factor;* Granulozyten-Kolonie-stimulierender Faktor
HSZ	hämatopoietische Stammzelle
HVZ	hämatopoietische Vorläuferzelle
ICAM	*intercellular adhesion molecule;* interzelluläres Adhäsionsmolekül
IGF	*Insulin-like Growth Factor;* Insulinähnlicher Wachstumsfaktor
IL	Interleukin

KM	Knochenmark
LAMP-3	*lysosomal-membrane-associated glycoprotein 3;* Lysosomen-Membran assoziiertes Glykoprotein-3
Lin	*lineage;* Entwicklungslinie
LTC-IC	*long-term-culture initiating cell;* Zelle mit Koloniebildungspotenzial nach Langzeitkultur
ML-IC	*myeloid-lymphoid-initiating cell;* Zelle, die das Potenzial besitzt, Zellen myeloischer und lymphatischer Abstammung hervorzubringen
NB	Neuroblasten
NK-Zelle	natürliche Killerzelle
NK-IC	*natural-killer-cell initiating cell;* Zelle, die das Potenzial besitzt, natürliche Killerzellen hervorzubringen
NOD/SCID	*non-obese-diabetic/ severe combined immundeficiency;* Nicht-fettleibig-diabetisch/ schwerer kombinierter Immundefekt
PB	peripheres Blut
PI3K	Phosphatidyl-Inositol-3-Kinase
SCF	*Stem cell factor/ steel factor/ c-kit-Ligand;* c-Kit-Ligand
SCID	*severe combined immundeficiency;* schwerer kombinierter Immundefekt
SDF-1α	*stroma-cell derived factor 1-α;* CXC-Ligand 12
SRC	*SCID repopulating cell;* Zelle mit SCID(-Maus) Wiederbesiedelungspotenzial
SZ	Stammzelle
TPO	Thrombopoietin
VZ	Vorläuferzelle
z.B.	zum Beispiel

4 Einleitung

4.1 Stammzellen

Für die Aufrechterhaltung eines multizellulären Organismus ist es erforderlich, dass Zellen, die durch Krankheiten, Verletzung oder Alterung verloren gegangenen sind, ständig erneuert und ersetzt werden. Für diese Regeneration sind somatische (adulte) Stammzellen verantwortlich, die in zahlreichen Geweben vorkommen. Diese adulten Stammzellen sind definiert als undifferenzierte Zellen, die sich über einen langen Zeitraum hinweg selbst erneuern und Vorläuferzellen hervorbringen können, die im weiteren Verlauf ihrer Entwicklung differenzieren. Während die differenzierten Zellen der Regeneration von Geweben dienen, bleibt durch die Selbsterneuerung ein Reservoir von Stammzellen erhalten.

Damit eine ausreichende Anzahl Vorläuferzellen hervorgebracht wird und es dabei nicht zu einer kontinuierlichen Abnahme des Stammzellreservoirs kommt, ist ein kontrolliertes Gleichgewicht zwischen Selbsterneuerung und Differenzierung der Stammzellen erforderlich. Reduziert sich der Stammzellvorrat oder die Stammzellaktivität, führt dies zu einer verminderten Regenerationsfähigkeit, die im Extremfall zur Degeneration des betroffenen Gewebes führen kann (Taupin und Gage, 2002; Anversa et al., 2005; Wagers und Conboy, 2005; Kamminga und de Haan, 2006). Auch Alterungsprozesse scheinen teilweise mit einer verringerten Funktionalität gewebespezifischer Stammzellen assoziiert zu sein (Rando, 2006; Ruzankina und Brown, 2007). Ein Beispiel für solche Alterungserscheinungen sind graue Haare, die durch eine verringerte Selbsterneuerung der Melanozyten-Stammzellen hervorgerufen werden (Nishimura et al., 2005).

Andererseits kann aber auch die Verschiebung des Gleichgewichts zugunsten der Selbsterneuerung der Stammzellen fatale Folgen für einen multizellulären Organismus haben. Eine Deregulation der Signaltransduktionskaskaden, die an der Kontrolle der Selbsterneuerung beteiligt sind, kann die maligne Transformation der Zellen und damit die Krebsentstehung fördern (Reya et al., 2001; Beachy et al., 2004; Al-Hajj, 2007). Eine wichtige Frage im Bereich der Stammzellbiologie ist daher, durch welche Mechanismen die Entscheidung zwischen Selbsterneuerung und Differenzierung kontrolliert wird.

Diese Fragestellung wird in unserer Arbeitsgruppe an humanen hämatopoietischen Stammzellen (HSZ) untersucht, und zum besseren Verständnis der Thematik werden in den folgenden Abschnitten zunächst die Eigenschaften von HSZ, ihre Lokalisation und die allgemein verwendeten Nachweisverfahren beschrieben. Im Anschluss daran wird speziell am Beispiel der HSZ die Frage nach der Kontrolle zwischen Selbsterneuerung und Differenzierung erneut aufgegriffen. In diesem Zusammenhang werden die Befunde dargestellt, die zur Formulierung der Fragestellung für die vorliegende Arbeit geführt haben.

4.2 Hämatopoietische Stammzellen

Zu den meist untersuchten adulten Stammzellen gehören die hämatopoietischen Stammzellen (HSZ), die von besonderer medizinischer Relevanz sind. Sie werden seit über 30 Jahren für die Behandlung von Leukämiepatienten nach einer Strahlen- oder Hochdosischemotherapie verwendet (Weissman, 2000; Shizuru et al., 2005). HSZ sind in der Lage, über verschiedene Vorläuferzelltypen alle zellulären Bestandteile des Bluts hervorzubringen. Aufgrund dieser Eigenschaft können sie die Funktionsfähigkeit eines zuvor durch Strahlen- oder Chemotherapie zerstörten hämatopoietischen Systems nach Transplantation wiederherstellen (Korbling und Anderlini, 2001; Ballen, 2005; Ringden und Le Blanc, 2005). Dem klassischen Modell der Hämatopoiese zufolge unterscheidet man die myeloische Entwicklungslinie mit Monozyten, Granulozyten, Megakaryozyten und Erythrozyten von der Entwicklungslinie der lymphatischen Zellen wie B- und T-Lymphozyten und Natürlichen Killer (NK)-Zellen (Abbildung 4-1). Dendritische Zellen können sowohl myeloischen als auch lymphatischen Ursprungs sein. In diesem Modell wird postuliert, dass sich zu einem frühen Zeitpunkt in der Hämatopoiese die myeloische und die lymphatische Entwicklungslinie unwiderruflich voneinander abspalten. Aus multipotenten Vorläuferzellen entwickeln sich dabei sogenannte gemeinsame lymphatische Vorläuferzellen, die im Englischen als *Common-Lymphoid Progenitor*s (CLP) bezeichnet werden, bzw. gemeinsame myeloische Vorläuferzellen oder *Common-Myeloid Progenitors* (CMP) (Kondo et al., 1997; Akashi et al., 2000; Reya et al., 2001). Aufgrund neuer Erkenntnisse, die auf die Existenz von Vorläuferzellen hindeuten, die zusätzlich zum lymphatischen Entwicklungspotenzial noch ein eingeschränktes myeloisches Entwicklungspotenzial besitzen, wird dieses

klassische Hämatopoiese-Modell jedoch modifiziert werden müssen (Brown et al., 2007; Buza-Vidas et al., 2007; Bell und Bhandoola, 2008; Wada et al., 2008) (Giebel und Punzel, 2008; im Druck).

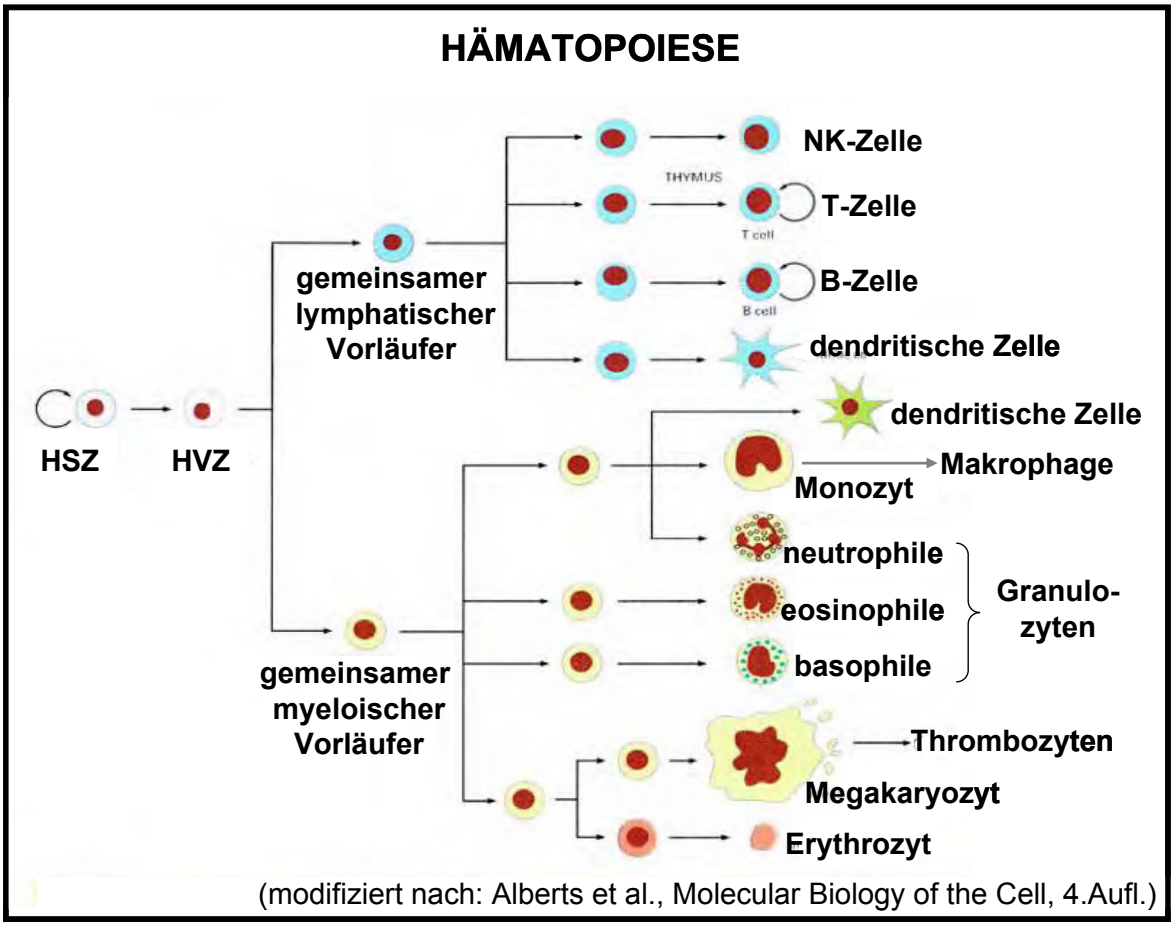


Abbildung 4-1: Schematische Darstellung des klassischen Modells der Hämatopoiese. Hämatopoietische Stammzellen (HSZ) können sich selbst erneuern und alle reifen Blutzelltypen bilden. Die Bildung reifer Blutzellen erfolgt, indem HSZ multipotente hämatopoietische Vorläuferzellen (HVZ) hervorbringen. Aus diesen HVZ entstehen gemeinsame myeloische und gemeinsame lymphatische Vorläuferzellen, die ein eingeschränktes Entwicklungspotenzial aufweisen. So können aus gemeinsamen lymphatischen Vorläuferzellen nur noch Zellen der lymphatischen Entwicklungslinie (NK-, T-, B- und dendritische Zellen) entstehen, während gemeinsame myeloische Vorläuferzellen ausschließlich Zellen myeloischen Ursprungs (Makrophagen, Granulozyten, Thombozyten, Erythrozyten und dendritische Zellen) hervorbringen.

4.2.1 Entstehungsorte hämatopoietischer Stamm- und Vorläuferzellen

Während der embryonalen Entwicklung werden HSZ in der Aorta-Gonaden-Mesonephros(AGM)-Region, dem Dottersack und der Plazenta gebildet (Dzierzak, 2005; Mikkola et al., 2005) und besiedeln im weiteren Verlauf die fetale Leber und

das Knochenmark. Im Kindes- und Erwachsenenalter findet die Blutbildung dann hauptsächlich im Knochenmark statt (Dzierzak, 2005; Mikkola et al., 2005).

Entsprechend ihres ontogenetischen Ursprungs lassen sich HSZ aus der fetalen Leber, dem Knochenmark (KM) und aus dem Nabelschnurrestblut, das im Englischen als *cord blood* (CB) bezeichnet wird, isolieren. Auch das periphere Blut (PB) von Personen, die zuvor mit Granulozyten-Kolonie-stimulierendem Faktor (G-CSF) behandelt wurden, eignet sich für die Gewinnung von HSZ, da G-CSF die Auswanderung der HSZ aus dem Knochenmark ins PB fördert (Nervi et al., 2006).

4.2.2 Nachweis hämatopoietischer Stamm- und Vorläuferzellen unter Verwendung funktioneller Analyseverfahren

In der klinischen Anwendung hat sich gezeigt, dass sich vor allem Knochenmark, Nabelschnurrestblut und peripheres Blut G-CSF stimulierter Spender für die Rekonstitution des hämatopoietischen Systems myeloablatierter Patienten eignen (Bensinger et al., 1997; Korbling und Anderlini, 2001; Rocha et al., 2001; Ballen, 2005; Ringden und Le Blanc, 2005). Dabei kann die erfolgreiche Wiederherstellung eines funktionsfähigen hämatopoietischen Systems im Empfänger durch Spenderzellen als Nachweis für das Vorkommen von HSZ im Transplantat angesehen werden. Da sich humane HSZ bis heute aufgrund des Fehlens eines spezifischen HSZ Phänotyps nicht eindeutig identifizieren und spezifisch isolieren lassen, sind im Laufe der Zeit verschiedene Nachweisverfahren entwickelt worden, mit denen das Entwicklungspotenzial primitiver hämatopoietischer Zellen analysiert werden kann.

Für die *in vivo* Untersuchung von primitiven humanen hämatopoietischen Zellen sind xenogene Transplantationsmodelle entwickelt worden, da entsprechende Experimente beim Menschen aus ethischen Gründen nicht durchgeführt werden können. Für xenogene Transplantationen werden oftmals immundefiziente NOD/SCID (*non-obese-diabetic/severe-combined-immundeficient*) Mäuse verwendet, die transplantierte Zellen nicht abstoßen. Anhand dieses Transplantationsmodells wird ermittelt, ob die transplantierten Zellen eine menschliche Hämatopoiese in den Empfängertieren initiieren können (Kamel-Reid und Dick, 1988; Lapidot et al., 1992; Larochelle et al., 1996).

Hierbei ist anzumerken, dass ein Teil der transplantierten Zellen nicht in das Knochenmark, sondern in die Lunge einwandert, wo sie ihr hämatopoietisches Potenzial nicht entfalten können (Krause et al., 2001; Dooner et al., 2004). Somit wird in diesem Transplantationsmodell die tatsächliche Anzahl der Zellen mit rekonstituierendem Potenzial, die im Englischen als SCID-*repopulating cells* (SRC) bezeichnet werden, möglicherweise unterbewertet.

Auch wenn SRC humanen HSZ aufgrund ihrer Eigenschaften sehr nahe stehen, ist nicht geklärt, ob sie auch die Fähigkeit zur Langzeit-Rekonstitution im Menschen besitzen. In Versuchen mit hämatopoietischen Zellen aus Affen konnte gezeigt werden, dass für die Repopulation von NOD/SCID Mäusen und die Langzeitbesiedelung in Affen unterschiedliche Zellpopulationen verantwortlich sind. Daher sollten Zellen, die im xenogenen Transplantationsmodell Repopulationspotenzial zeigen, nicht mit HSZ gleichgesetzt werden (Horn et al., 2003; Horn und Blasczyk, 2007).

Neben den Transplantationsversuchen stehen für die funktionelle Analyse primitiver hämatopoietischer Zellen auch diverse *in vitro* Verfahren zur Verfügung. Hier wird das Koloniebildungspotenzial entsprechend ausgesäter Zellen ermittelt, das in Abhängigkeit der zugesetzten Zytokine steht. Im so genannten CFU-GEMM (*Colony-Forming-Unit Granulocyte, Erythrocyte, Macrophage, Megakaryocyte*)-Ansatz wird das myeloische Entwicklungspotenzial von eingebrachten Zellen analysiert. Hierbei wird die Entwicklung der Zellen zu Erythrozyten, Megakaryozyten, Monozyten und Granulozyten in semisolidem Medium unterstützt. Anhand der Koloniebildung kann in diesem Versuchsansatz das Entwicklungspotenzial der ursprünglich eingesetzten Zellen retrospektiv beurteilt werden. Vorläuferzellen, die verschiedene Zelltypen hervorbringen, werden als besonders primitiv eingestuft.

Der CFU-GEMM-Versuchsansatz eignet sich nicht zur Identifizierung sehr früher Vorläuferzellen, weil diese mehr Zeit und den Kontakt mit Stromazellen benötigen, um differenzierte Nachkommen hervorzubringen (Whitlock und Witte, 1982; Kierney und Dorshkind, 1987; McGlave et al., 1994; Miller et al., 1994; LeBien, 1998; Coulombel, 2004). Daher sind zum Nachweis von primitiven myeloischen und lymphatischen Vorläuferzellen Langzeitversuche entwickelt worden, in denen die Zellen über mehrere Wochen in Gegenwart von Stromazellen kultiviert werden.

Diese Stromazellen unterstützen das Überleben und die Proliferation primitiver Vorläuferzellen sowie die Differenzierung von spezifizierten Vorläuferzellen (Sutherland et al., 1990; Hao et al., 1998; Miller et al., 1999; Punzel et al., 1999; Punzel et al., 1999; Nolta et al., 2002). Nach mehreren Wochen können daher nur Nachkommen von zu Beginn sehr primitiven hämatopoietischen Zellen noch Kolonien in einem nachgeschalteten Koloniebildungsansatz hervorbringen. In solchen Langzeitversuchen kann man, je nach verwendetem Zytokingemisch, einerseits im *Long-term-culture-intiating cell* (LTC-IC)-Versuchsansatz den Gehalt an frühen myeloischen oder andererseits mithilfe des *Natural-Killer-cell-initiating cell* (NK-IC)-Versuchsansatzes den der unreifen lymphatischen Vorläuferzellen bestimmen. Das kombinierte myeloische-lymphatische Entwicklungspotenzial kann im so genannten *Myeloid-Lymphoid Initiating Cell* (ML-IC)-Ansatz ermittelt werden. Hier werden einzeln abgelegte Zellen zunächst 14 Tage auf Stromazellen expandiert, bevor ihre Nachkommen aufgeteilt und in LTC-IC- bzw. NK-IC-Ansätze überführt werden (Punzel et al., 1999; Punzel et al., 2002; Giebel et al., 2006). Zellen, deren Nachkommen sowohl Zellen der myeloischen (LTC-IC) als auch der lymphatischen Entwicklungslinie (NK-IC) hervorbringen, werden als ML-IC bezeichnet. Zellen mit ML-IC-Potenzial gelten derzeit als die primitivsten *in vitro* detektierbaren Zellen.

4.2.3 Phänotypische Charakterisierung und Anreicherung HSZ-enthaltender Zellfraktionen

Im humanen System sind bisher noch keine Marker beschrieben worden, die ausschließlich auf HSZ exprimiert werden und so eine direkte Identifikation und Isolierung ermöglichen würden. Über die Anreicherung von primitiven Zellen, die sich in spezifischen phänotypischen Eigenschaften von reifen hämatopoietischen Zellen unterscheiden, lassen sich aber Zellfraktionen gewinnen, die HSZ enthalten.

Die Unterscheidung zwischen primitiven und reiferen hämatopoietischen Zellen erfolgt anhand von bestimmten, mit der Differenzierung von Zellen assoziierten Zelloberflächenproteinen, die eine *Cluster of Differentiation* (CD)-Bezeichnung tragen. Das Expressionsmuster dieser Antigene auf der Zelloberfläche hämatopoietischer Zellen lässt Rückschlüsse auf den Entwicklungszustand der Zellen zu. So gelten z.B. CD3, CD4 und CD8 als T-Lymphozyten-spezifische

Oberflächenproteine, während CD19 typisch für B-Lymphozyten ist. Primitive humane hämatopoietische Zellen unterscheiden sich von reifen Blutzellen durch das Fehlen solcher Entwicklungslinien-spezifischen Antigene, die im Englischen als *lineage* (lin)-Marker bezeichnet werden. Im Gegensatz zu reiferen Zellen exprimieren sie die Zelloberflächenproteine CD34 und CD133 (Civin et al., 1984; Katz et al., 1985; Andrews et al., 1986; Miraglia et al., 1997; Yin et al., 1997). Beide Oberflächenmoleküle werden zur Charakterisierung einer HSZ-enthaltenden Fraktion verwendet, und auf einem Großteil der primitiven humanen hämatopoietischen Zellen werden CD34 und CD133 überlappend exprimiert (Miraglia et al., 1997; Yin et al., 1997; de Wynter et al., 1998; Matsumoto et al., 2000; Handgretinger et al., 2003). Bei der Analyse der Expression weiterer Oberflächenantigene zeigt sich jedoch, dass die Fraktion der $CD34^+$ Zellen äußerst heterogen ist. So finden sich Zellen, die CD38 stark exprimieren ($CD38^+$), und andere, die nur schwach positiv für CD38 sind ($CD38^{low/-}$). Allerdings gibt es aufgrund der graduellen CD38-Expression zwischen den beiden Populationen $CD34^+CD38^+$ und $CD34^+CD38^{low/-}$ keine scharfe Abgrenzung, die eine objektive Unterscheidung der beiden Fraktionen erlaubt.

Nach einer Separation der Zellen in eine $lin^-CD34^+CD38^+$ und eine $lin^-CD34^+CD38^{low}$ Fraktion konnte belegt werden, dass die $lin^-CD34^+CD38^{low}$-Zellen verglichen mit der $lin^-CD34^+CD38^+$-Fraktion sehr viel stärker für primitive hämatopoietische Zellen mit LTC-IC Potenzial (Terstappen et al., 1991; Hao et al., 1995; Hao et al., 1996; Petzer et al., 1996) und auch für Zellen mit rekonstituierendem Potenzial (SRC) angereichert sind (Bhatia et al., 1997; Dick et al., 1997). Während Zellen mit LTC-IC-Potenzial in beiden Fraktionen vorkommen, wurden SRC ausschließlich in der $lin^-CD34^+CD38^-$ Fraktion gefunden. Dabei liegt die SRC Frequenz innerhalb dieser $lin^-CD34^+CD38^-$ Fraktion mit etwa 0.2% (Larochelle et al., 1996; Bhatia et al., 1997) deutlich unter der LTC-IC Frequenz von etwa 10% (Terstappen et al., 1991; Hao et al., 1995; Hao et al., 1996; Petzer et al., 1996).

Auch in der $lin^-CD34^-CD38^-$ Fraktion gibt es Zellen mit rekonstituierendem Potenzial (Bhatia et al., 1998; Zanjani et al., 1998), die sich weiterhin durch die Expression von CD133 und das Fehlen des lymphatischen Entwicklungsmarkers CD7 auszeichnen (Gallacher et al., 2000). Lin^-CD34^- Zellen sind außerdem auch in der Lage, $CD34^+$ Zellen hervorzubringen und repräsentieren möglicherweise

eine primitivere Vorstufe der $CD34^+$ Zellen (Bhatia et al., 1998; Nakamura et al., 1999). Es konnte gezeigt werden, dass die beiden Stammzellsurrogatmarker CD34 und CD133 in einem Großteil der Zellen coexprimiert werden (Miraglia et al., 1997; Yin et al., 1997; de Wynter et al., 1998; Matsumoto et al., 2000; Handgretinger et al., 2003). Da CD133 aber auch in $CD34^-$ Zellen mit Repopulationseigenschaften exprimiert ist, die wiederum $CD34^+$ Zellen hervorbringen können (Gallacher et al., 2000; Kuci et al., 2003), läßt sich anhand der CD133 Expression auch eine Subpopulation von Zellen charakterisieren, die primitivere Zellen enthält als die Fraktion der $CD34^+$ Zellen.

4.2.4 Entscheidung zwischen Selbsterneuerung und Differenzierung von Stammzellen

Wie unter 4.1 beschrieben, spielt die Frage nach den Mechanismen, die die Entscheidung zwischen Selbsterneuerung und Differenzierung von Stammzellen regulieren eine zentrale Rolle im Bereich der Stammzellbiologie.

Wie diese Entscheidung bei HSZ kontrolliert wird, ist noch weitgehend unbekannt. Untersuchungen an Modellorganismen haben jedoch dazu beigetragen, Mechanismen, die die Entscheidung zwischen Selbsterneuerung und Differenzierung von Stammzellen beeinflussen, besser zu verstehen. Hierbei zeigte sich, dass dabei sowohl äußere (extrinsische) Einflüsse als auch intrinsische Faktoren eine wichtige Rolle spielen können. Im folgenden Abschnitt werden Beispiele solcher Zellschicksalsentscheidungen aus *Drosophila melanogaster* und *Caenorhabditis elegans* näher erläutert. Hieraus lassen sich Ansatzpunkte für die Erforschung der entsprechenden Fragestellung bei HSZ entwickeln.

4.2.4.1 Zellschicksalsentscheidungen bei Modellorganismen

Bei Stammzellteilungen in *Drosophila melanogaster* wird die Entscheidung zwischen Selbsterneuerung und Differenzierung im Wesentlichen durch zwei Mechanismen kontrolliert: durch die direkte räumliche Umgebung der Stammzellen, die als Stammzellnische bezeichnet wird (Abbildung 4-2), und durch asymmetrische Zellteilungen, in denen intrinsische Faktoren ungleich auf die entstehenden Tochterzellen verteilt werden (Abbildung 4-3).

Untersuchungen der Keimbahnstammzellen in *Drosophila melanogaster* zeigten, dass die 2 Keimbahnstammzellen einer Ovariole in engem Kontakt mit Zellen somatischen Ursprungs stehen (Xie et al., 2005; Kirilly und Xie, 2007).

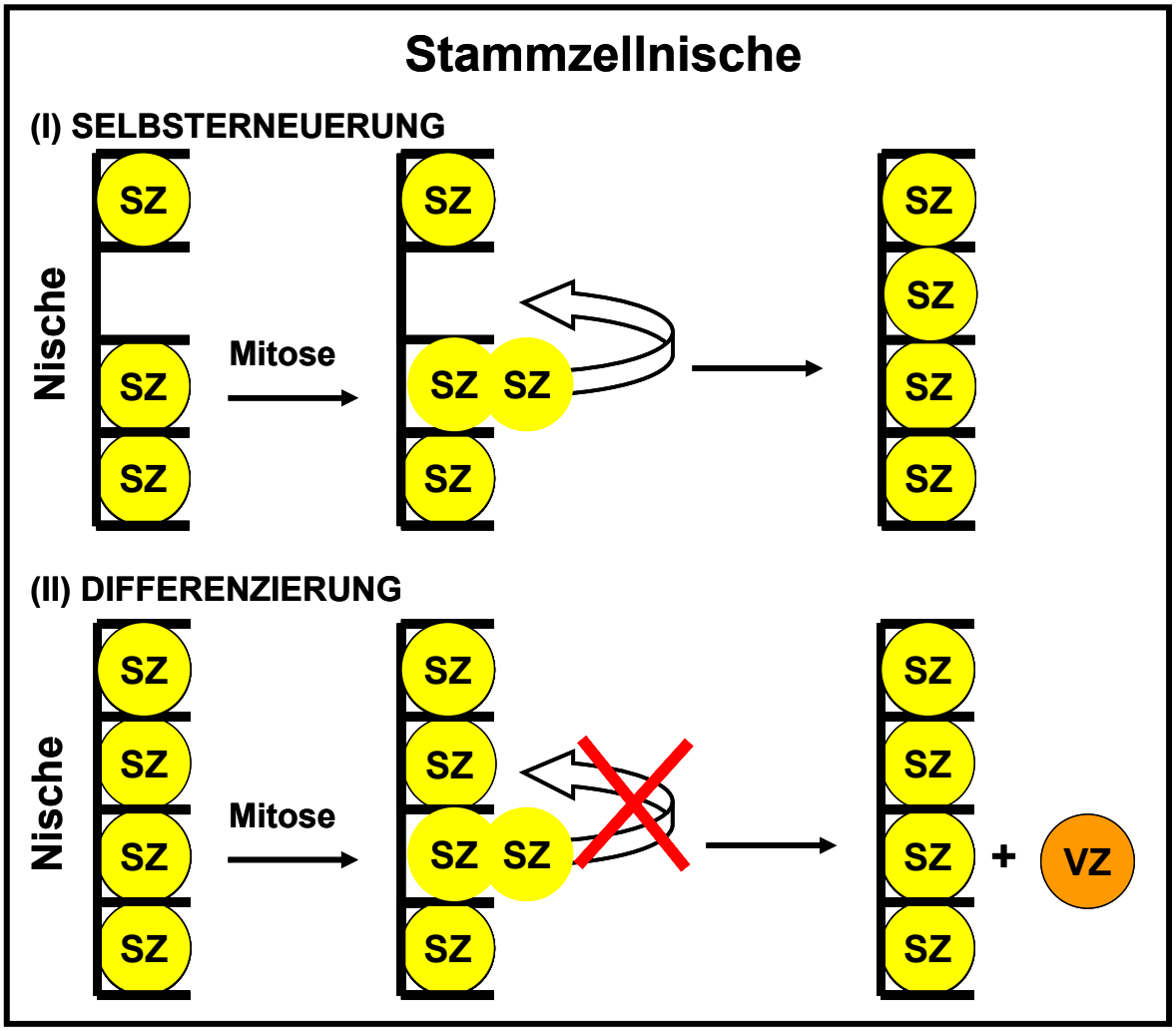


Abbildung 4-2: Modell der Stammzellnische. Das Zellschicksal der Tochterzellen hängt von extrinsischen Faktoren in Form einer Stammzellnische ab. Für den Erhalt der Stammzellen als solcher ist der Kontakt mit der Nische entscheidend. (I) Selbsterneuerung: sind nach einer Stammzellteilung ausreichend Nischen vorhanden, können beide Tochterzellen eine Nische besetzen und das Stammzellpotenzial realisieren. (II) Differenzierung: sind nach einer Zellteilung keine weiteren Nischen frei, besetzt eine Tochterzelle die Nische der Mutterzelle, während die andere aus der Nische verdrängt wird und sich zur Vorläuferzelle weiterentwickelt. SZ: Stammzelle; VZ: Vorläuferzelle.

Bei einer Teilung der Keimbahnstammzellen verlässt eine Tochterzelle diese räumliche Umgebung und entwickelt sich zur Oozyte, während die andere in der Nische als Stammzelle verbleibt (Abbildung 4-2II). Kommt es zum Verlust einer der beiden Keimbahnstammzellen, kann sie durch eine Teilung der zweiten Stammzelle ersetzt werden, indem die eine der beiden Tochterzellen die

benachbarte Nische besetzt und ebenfalls den Stammzellcharakter beibehält (Abbildung 4-2I). Für das Schicksal der Stammzellen scheint daher der Kontakt mit den umgebenden Zellen entscheidend für den Erhalt der Stammzellen als solcher zu sein (Xie und Spradling, 2000; Spradling et al., 2001; Lin, 2002; Kai und Spradling, 2003).

Im Gegensatz zum Einfluss extrinsischer Faktoren in Form der Stammzellnische spielen bei asymmetrischen Zellteilungen intrinsische Faktoren eine entscheidende Rolle für das Schicksal der entstehenden Tochterzellen. Abbildung 4-3 zeigt eine schematische Darstellung einer asymmetrischen Stammzellteilung. Faktoren, die das Entwicklungsschicksal der Zellen beeinflussen, werden auch als Zellschicksalsdeterminanten bezeichnet. Je nach Orientierung der Teilungsebene, die wiederum von der Ausrichtung der mitotischen Spindel abhängt, können Zellschicksalsdeterminanten bei einer Zellteilung asymmetrisch auf die Tochterzellen verteilt werden. Infolgedessen entwickeln sich die beiden Tochterzellen unterschiedlich voneinander weiter. Während die eine Tochterzelle als Stammzelle verbleibt, differenziert die andere Tochterzelle im weiteren Verlauf ihrer Entwicklung. Welche Mechanismen an der asymmetrischen Verteilung intrinsischer Faktoren beteiligt sind, konnte durch die Untersuchung asymmetrischer Zellteilungen bei Modellorganismen gezeigt werden.

Asymmetrische Zellteilungen kommen z.B. bei der Teilung neuraler Stammzellen (Neuroblasten) von *Drosophila melanogaster* und auch bei der *Caenorhabditis elegans* Zygote vor (Doe und Bowerman, 2001).

Bei der Teilung der Neuroblasten (NB) während der Entwicklung des Nervensystems von *Drosophila melanogaster* entstehen zwei Tochterzellen, von denen die eine als neurale Stammzelle erhalten bleibt, während die andere Tochterzelle (Ganglionmutterzelle) nach einer weiteren Teilung Nerven- und/oder Gliazellen hervorbringt. Die NB weisen aufgrund ihres epithelialen Ursprungs eine apikal-basale Polaritätsachse auf, die sich in der spezifischen Lokalisation bestimmter Faktoren am apikalen bzw. basalen Pol der Zellen manifestiert. Zu Beginn einer Zellteilung ist die mitotische Spindel zunächst so ausgerichtet, dass apikale und basale Faktoren symmetrisch auf die beiden Tochterzellen verteilt werden. Aufgrund einer Rotation der Spindel verläuft die Teilungsebene dann jedoch senkrecht zu der apikal-basalen Polaritätsachse. Aus diesem Grund

segregieren basal lokalisierte Faktoren, die als Zellschicksalsdeterminanten fungieren, überwiegend in die eine Tochterzelle, die sich infolgedessen zur Ganglionmutterzelle entwickelt. Die andere Tochterzelle bleibt dagegen als NB erhalten (Jan und Jan, 2001; Wodarz und Huttner, 2003; Wodarz, 2005; Yu et al., 2006; Chia et al., 2008).

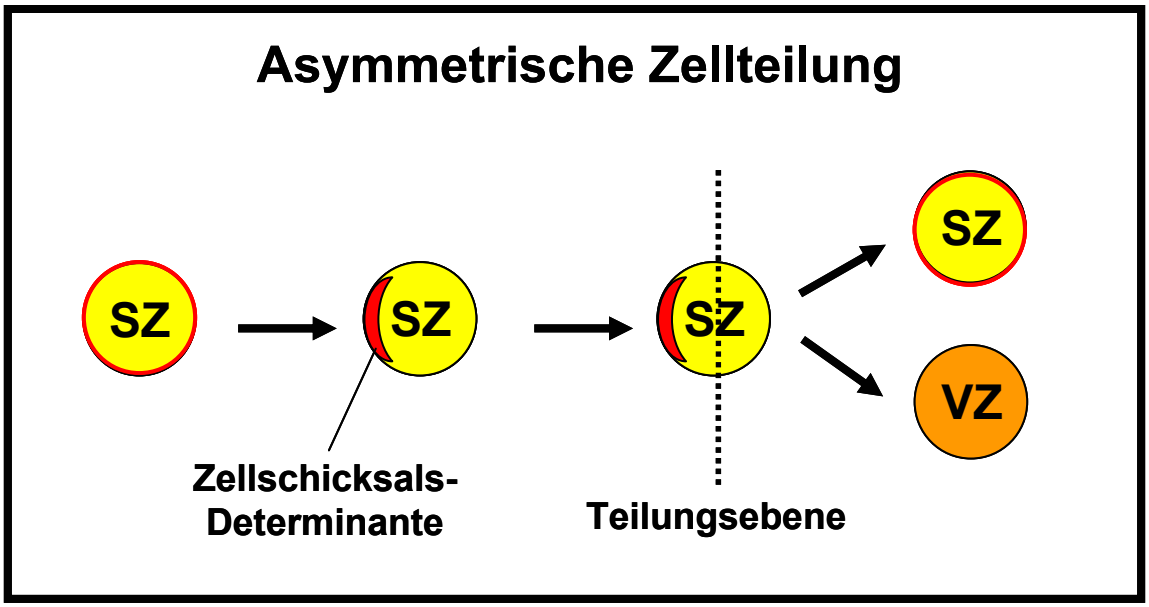


Abbildung 4-3: Modell einer asymmetrischen Stammzellteilung. Das Entwicklungspotenzial der Tochterzellen, die bei einer Stammzellteilung entstehen, ist abhängig von der Verteilung intrinsischer Faktoren, den sogenannten Zellschicksalsdeterminanten. Vor der Mitose werden die Zellschicksalsdeterminanten an einem Zellpol lokalisiert. Aufgrund der polaren Anordnung und einer entsprechend orientierten Teilungsebene gelangen die Zellschicksalsdeterminanten bei einer Zellteilung nur in eine der Tochterzellen. Infolgedessen entwickeln sich die beiden Tochterzellen unterschiedlich voneinander weiter. Während die eine Tochterzelle als Stammzelle (SZ) verbleibt, entwickelt sich die andere zu einer Vorläuferzelle (VZ).

Auch bei der Zygote von *Caenorhabditis elegans* wird vor der ersten Zellteilung eine Polaritätsachse ausgebildet, die eine anterior-posteriore Orientierung aufweist. Da die Teilungsebene aufgrund einer Spindelrotation senkrecht zu dieser Polaritätsachse liegt, werden anterior und posterior lokalisierte Faktoren asymmetrisch auf die beiden Tochterzellen verteilt, die sich infolgedessen unterschiedlich weiterentwickeln. Die posterior gelegene Tochterzelle (P1-Zelle) erhält unter anderem die P-Granula, die Determinanten der Keimbahn enthalten. Im weiteren Verlauf der Entwicklung bringt die P1-Zelle neben Keimbahnzellen auch mesodermale und endodermale Zellen hervor. Aus der anterior gelegene AB-Zelle entstehen im Gegensatz dazu hauptsächlich Zellen ektodermalen

Ursprungs (Kemphues et al., 1988; Guo und Kemphues, 1995; Boyd et al., 1996; Watts et al., 1996; Suzuki und Ohno, 2006).

Zusammenfassend wurden bei asymmetrischen Zellteilungen in den Modellorganismen folgende Beobachtungen gemacht: Zellen, die sich asymmetrisch teilen, erfüllen drei Kriterien, (I) sie sind polarisiert, (II) Proteine, die als Zellschicksalsdeterminanten fungieren, werden ungleichmäßig auf die entsprechenden Zellpole verteilt, und (III) eine korrekte Orientierung der mitotischen Spindel sorgt für die asymmetrische Verteilung der Zellschicksalsdeterminanten auf die Tochterzellen (Betschinger und Knoblich, 2004). Somit spielt die Organisation der Zellpolarität eine entscheidende Rolle bei der asymmetrischen Zellteilung und steht in direktem Zusammenhang mit dem Schicksal der entstehenden Tochterzellen.

Nach welchem Muster Stammzellteilungen im hämatopoietischen System ablaufen und welche Rolle intrinsische und/oder extrinsische Mechanismen dabei spielen, war zu Beginn dieser Arbeit noch nicht abschließend geklärt. Wie im folgenden Abschnitt erläutert, gibt es sowohl Hinweise auf die Existenz von HSZ Nischen als auch Befunde, die auf das Vorkommen asymmetrischer Zellteilungen bei HSZ hindeuten.

4.2.4.2 Teilungsmechanismen hämatopoietischer Stammzellen

Die Beobachtung, dass sich Zellen mit Langzeitrepopulationspotenzial *in vivo* expandieren können (Iscove und Nawa, 1997), jedoch *in vitro* in stromazellfreien Kulturen die Expansionsfähigkeit schnell verlieren (Bhatia et al., 1997; Conneally et al., 1997; Shimizu et al., 1998), deutet auf den Einfluss extrinsischer Faktoren auf das Schicksal von HSZ hin. Dies wird weiter unterstützt durch den Befund, dass adäquate Stromazellen den Erhalt von primitiven Zellen *in vitro* über einen längeren Zeitraum fördern (Moore et al., 1997; Thiemann et al., 1998; Punzel et al., 1999; Shih et al., 1999; Nolta et al., 2002). In diesem Zusammenhang wurde postuliert, dass es auch im hämatopoietischen System Stammzellnischen gibt.

Die Existenz von HSZ-Nischen konnte tatsächlich von mehreren Arbeitsgruppen nachgewiesen werden, die zeigen konnten, dass Osteoblasten zu den wichtigsten Bestandteilen der HSZ-Nischen im Endosteum des Knochemarks gehören (Calvi et al., 2003; Zhang et al., 2003). Weiterhin fanden Kiel und Kollegen, dass

sinusoidale Endothelzellen Komponenten der vaskulären HSZ-Nischen sind, die in der Milz und dem Knochenmark gefunden wurden (Kiel et al., 2005). Beide Nischenarten sind an der Regulation der Größe des Stammzellreservoirs *in vivo* beteiligt, und dabei scheint der Notch-Signalweg eine wichtige Rolle zu spielen (Leary et al., 1985; Milner und Bigas, 1999; Calvi et al., 2003; Suzuki und Chiba, 2005).

Vor der Entdeckung der HSZ-Nischen wurde allgemeinhin angenommen, dass sich HSZ asymmetrisch teilen. Dies begründete sich auf folgende experimentelle Beobachtungen: bei der Analyse der Differenzierung muriner und humaner hämatopoietischer Vorläuferzellen (HVZ) unter stromazellfreien Kulturbedingungen wurden Tochterzellen von HVZ unmittelbar nach der Teilung separiert und getrennt voneinander weiterkultiviert. Dabei fand man, dass manche Geschwisterzellen Kolonien hervorbrachten, die sich entweder in der Art oder der Größe der Kolonien voneinander unterschieden (Suda et al., 1984; Suda et al., 1984; Leary et al., 1985). Weiterhin ergaben Studien an humanen Zellen, die für HSZ angereichert waren, dass die Nachkommen dieser Zellen bezüglich ihrer Funktion und Proliferationskinetik zum Teil sehr heterogen sind. In diesem Zusammenhang wurde gezeigt, dass primitivere Zellen langsamer proliferieren als weiter spezifizierte Zellen (Mayani et al., 1993; Brummendorf et al., 1998; Punzel et al., 2002).

Aufgrund der Beobachtung, dass unter konstanten Bedingungen etwa 20-30% der primitiven, langsam proliferierenden hämatopoietischen Zellen Tochterzellen mit unterschiedlichen Proliferationskinetiken und Zellschicksalen hervorbringen, wurde von verschiedenen Arbeitsgruppen postuliert, dass diese Ergebnisse auf asymmetrische Zellteilungen zurückzuführen sind (Brummendorf et al., 1998; Huang et al., 1999; Punzel et al., 2002).

Neuere Studien des Entwicklungspotenzials von HSZ/HVZ-Tochterzellen auf Einzelzellebene haben weiterhin ergeben, dass der größte Teil der primitivsten *in vitro* detektierbaren humanen hämatopoietischen Zellen (ML-IC) Tochterzellen hervorbringt, die unterschiedliche Zellschicksale annehmen (Giebel et al., 2006). Dabei behält die eine dieser Tochterzellen das Entwicklungspotenzial der Mutterzelle, während die andere nur ein eingeschränktes Entwicklungspotenzial realisiert. Im Gegensatz dazu realisieren bei der Zellteilung reiferer Vorläuferzellen

häufiger beide Tochterzellen das Schicksal ihrer Mutterzelle. Das deutet darauf hin, dass primitivere Zellen unter den verwendeten Versuchsbedingungen eine größere Tendenz dazu haben, Tochterzellen mit unterschiedlichem Schicksal hervorzubringen als reifere Zellen, was ebenfalls mit einer asymmetrischen Zellteilung vereinbar wäre (Giebel et al., 2006).

4.3 Fragestellung und Zielsetzung dieser Arbeit:

Die oben beschriebenen Befunde zum unterschiedlichen Koloniebildungs- und Proliferationsverhalten von Tochterzellen primitiver hämatopoietischer Zellen sind alle mit dem Modell der asymmetrischen Zellteilung vereinbar. Sie könnten aber auch hypothetisch - wie schon von der Arbeitsgruppe um Lansdorp diskutiert (Brummendorf et al., 1998) - durch postmitotische Entscheidungsprozesse zustande kommen. Hierbei könnten sich zunächst identische Tochterzellen nach der Mitose gegenseitig beeinflussen und infolgedessen unterschiedlich weiterentwickeln (Giebel et al., 2006) (Abbildung 4-4).

Ein Beispiel hierfür ist der Prozess der lateralen Inhibition während der Entwicklung des Nervensystems von *Drosophila melanogaster*, bei dem der Notch-Signalweg eine zentrale Rolle spielt. Die neuralen Vorläuferzellen entstehen in kleinen Zellgruppen, den so genannten proneuralen Äquivalenzgruppen. Zunächst exprimieren alle benachbarten Zellen proneurale Gene und besitzen das Potenzial, sich zu neuralen Vorläuferzellen zu entwickeln. Sobald sich in einer der Zellen eine für die neurale Determinierung ausreichende Menge proneuraler Faktoren angesammelt hat, inhibiert sie die umliegenden Zellen, dasselbe Schicksal anzunehmen. Diese laterale Inhibition erfolgt über die Aktivierung des Notch-Signalwegs in den benachbarten Zellen, die zu einer Suppression der proneuralen Genfunktion in diesen Zellen führt. Als Folge davon nehmen benachbarte Zellen mit ursprünglich identischem Entwicklungspotenzial unterschiedliche Zellschicksale an (Giebel, 1999; Martinez Arias et al., 2002; Castro et al., 2005).

Da die im hämatopoietischen Kompartiment beobachteten Unterschiede im Proliferationsverhalten und der Koloniebildung der Geschwisterzellen sich nicht eindeutig auf eine asymmetrische Zellteilung zurückführen, sondern sich auch

anders erklären lassen, sollte im Rahmen der vorliegenden Arbeit untersucht werden, ob HSZ/HVZ tatsächlich in der Lage sind, sich asymmetrisch zu teilen.

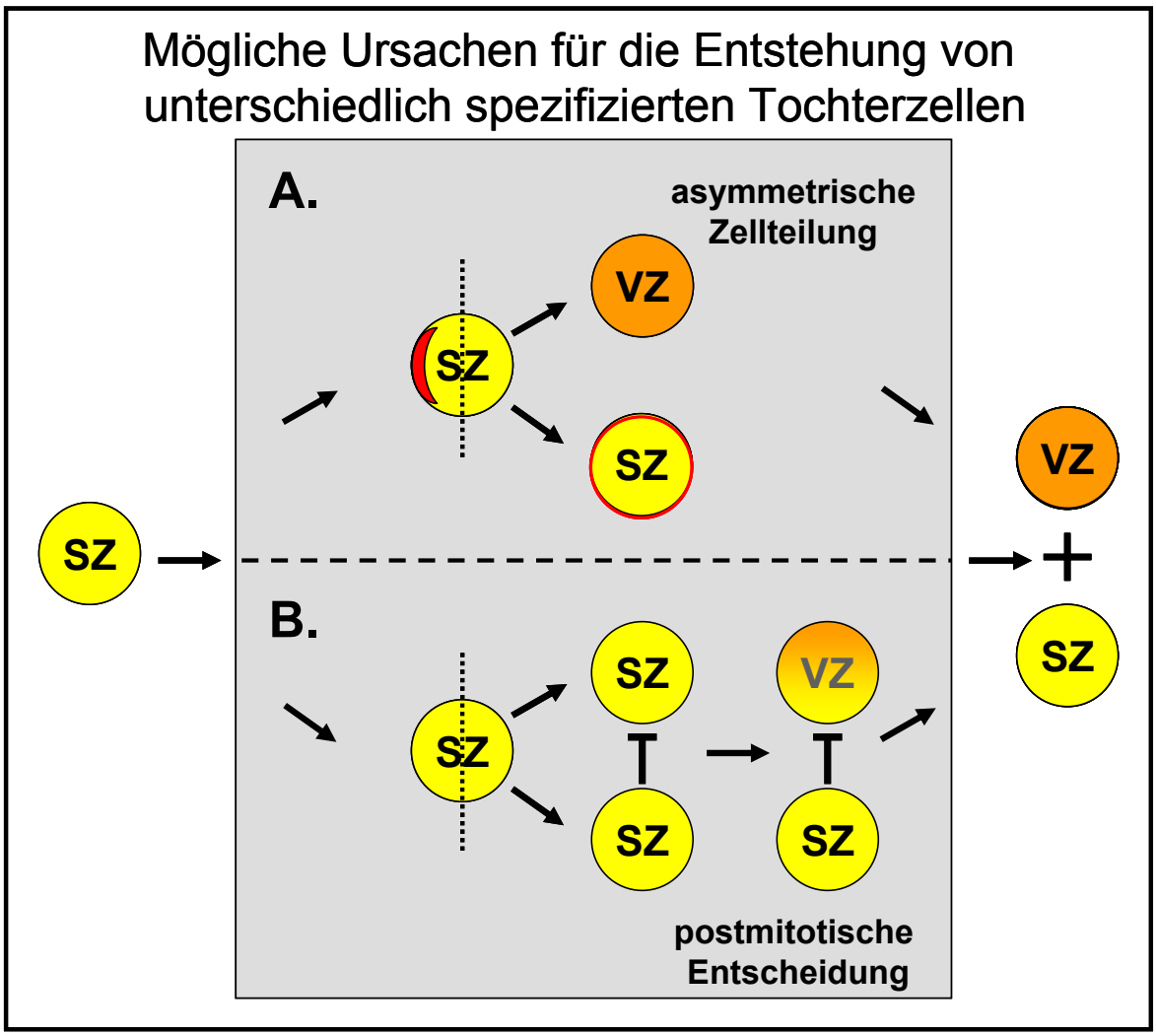


Abbildung 4-4: Unterschiedliche Mechanismen ermöglichen eine Diversität der Zellschicksale. Die Entstehung von unterschiedlich spezifizierten Geschwisterzellen bei einer Stammzellteilung lässt sich mit zwei Modellen erklären. A: Zellschicksalsdeterminanten werden während der Zellteilung asymmetrisch verteilt, sodass die beiden Tochterzellen ein unterschiedliches Entwicklungspotenzial besitzen. B: Bei der Teilung entstehen zunächst zwei identische Tochterzellen, die sich nach der Mitose gegenseitig beeinflussen, z.B. durch laterale Inhibition und daraufhin unterschiedliche Zellschicksale verfolgen. SZ: Stammzelle; VZ: Vorläuferzelle.

Um zwischen post-mitotischen Entscheidungsprozessen und asymmetrischen Zellteilungen als möglichen Ursachen für die Unterschiede zwischen Tochterzellen von HSZ/HVZ differenzieren zu können, sollte daher nach Faktoren gesucht werden, die während der Zellteilung asymmetrisch auf die Tochterzellen verteilt werden.

Da am Beispiel der Modellorganismen gezeigt werden konnte, dass der Aufbau von Polaritätsachsen eine zentrale Voraussetzung für asymmetrische Zellteilungen darstellt, bestand ein erstes Ziel dieser Arbeit darin, die Zellpolarität von HSZ/HVZ zu untersuchen. Hierbei sollten polar angeordnete Faktoren identifiziert werden, da sie aufgrund ihrer polaren Verteilung - ähnlich wie die polar lokalisierten Zellschicksalsdeterminanten bei *Drosophila melanogaster* – mögliche Kandidaten für eine asymmetrische Segregation in die beiden Tochterzellen darstellen. Um diese Annahme zu überprüfen, sollte im Anschluss daran ihre Lokalisation in sich teilenden Zellen untersucht werden.

5 Ergebnisse und Diskussion

Die zentrale Fragestellung dieser Arbeit war, ob sich HSZ/HVZ asymmetrisch teilen können. Da, wie eingangs beschrieben, die Etablierung von Polaritätsachsen eine essenzielle Voraussetzung für asymmetrische Zellteilungen darstellt, wurde zunächst die Zellpolarität primitiver hämatopoietischer ($CD34^+$) Zellen untersucht.

Zu Beginn meiner Arbeit gab es in der Arbeitsgruppe bereits erste Hinweise darauf, dass kultivierte $CD34^+$ Zellen einen polarisierten Phänotyp annehmen. Es wurde beobachtet, dass die frisch isolierten, runden Zellen unter Kulturbedingungen mit bestimmten, früh wirksamen Zytokinen wachsen, eine tropfenförmige Gestalt annehmen und zu wandern beginnen (Giebel et al., 2004).

Die polarisierte Zellform der kultivierten $CD34^+$ Zellen ähnelte dabei der Morphologie immunreaktiver Leukozyten, die am vorderen Ende eine Leitfront und am hinteren Ende einen pseudopodartigen Ausläufer ausbilden, der auch als Uropod bezeichnet wird (Sanchez-Madrid und del Pozo, 1999). Daher stellte sich die Frage, ob es weitere Parallelen zwischen den polarisierten Phänotypen gibt. Da für verschiedene Leukozyten-Oberflächenmoleküle eine spezifische Assoziation mit dem Vorder- oder Hinterende beschrieben wurde (Sanchez-Madrid und del Pozo, 1999), sollte untersucht werden, ob diese Membranproteine auch in kultivierten $CD34^+$ Zellen entsprechend lokalisiert sind. Nachdem die Expression der Proteine bereits bestätigt wurde, deuteten erste Färbeversuche in der Arbeitsgruppe darauf hin, dass bestimmte Zelladhäsionsmoleküle, die am hinteren Ende immunreaktiver Leukozyten angereichert sind, auch bei $CD34^+$ Zellen im Uropod lokalisiert sind.

Um die Lokalisation der leukozytenspezifischen Oberflächenantigene in $CD34^+$ Zellen untersuchen zu können, haben wir zu Beginn dieser Arbeiten zunächst Protokolle etablieren müssen, mit denen sich die polarisierte Morphologie der kultivierten $CD34^+$ Zellen erhalten lässt, und die sich für eine doppelte Immunfärbung der $CD34^+$ Zellen eignen. Eine Schwierigkeit hierbei war es, gleichzeitig die polarisierte Morphologie der Zellen durch die Fixierung möglichst gut zu konservieren, und trotz der zum Teil fixierungssensitiven Epitope reproduzierbare Färbeergebnisse zu erzielen. Da die meisten der kommerziellen Antikörper gegen Leukozyten-Antigene aus der Maus stammen und es unser Ziel

war, Doppelfärbungen mit einem murinen Antikörper gegen den Stammzellsurrogatmarker CD133 durchzuführen, haben wir unsere Protokolle entsprechend optimiert. Standardmäßig können wir nun $CD34^+$ Zellen mit zwei unterschiedlichen primären Antikörpern, die beide aus der Maus stammen, unter Verwendung fluoreszenzmarkierter Sekundärantikörper für die Fluoreszenzmikroskopie färben.

5.1 Identifizierung von polar verteilten Zelloberflächenproteinen in $CD34^+$ Zellen

Da unserer Arbeitshypothese entsprechend polar lokalisierte Faktoren potenzielle Kandidaten für eine asymmetrische Segregation während der Mitose sind, sollten Moleküle in $CD34^+$ Zellen identifiziert werden, die dieses Kriterium erfüllen. Dazu wurde mithilfe von Immunfärbungen an $CD34^+$ Zellen die Verteilung der aus immunreaktiven Leukozyten bekannten Uropodmarker ICAM-1/CD54, ICAM-3/CD50, CD43, CD44 sowie die des Leitfront-assoziierten Chemokinrezeptors CXCR4 und des Gangliosids GM3 untersucht (Sanchez-Madrid und del Pozo, 1999; Gomez-Mouton et al., 2001).

Bei der Analyse der gefärbten $CD34^+$ Zellen, die zuvor für zwei Tage in Gegenwart bestimmter, früh wirksamer Zytokine kultiviert wurden, fanden wir, dass die Membranproteine CD43, CD44, ICAM-3/CD50 und ICAM-1/CD54 in polarisierten Zellen spezifisch im Uropod lokalisiert sind. Dagegen sind der Chemokinrezeptor CXCR4 und das Gangliosid GM3 stark im Bereich der Leitfront angereichert (Giebel et al., 2004). Die Ähnlichkeiten zwischen peripheren Leukozyten und $CD34^+$ Zellen manifestierten sich daher auch in der Verteilung bestimmter Zelloberflächenantigene.

Da CD133/Prominin-1, das in hämatopoietischen Zellen als Stammzellsurrogatmarker fungiert, in anderen Zelltypen bevorzugt auf Plasmamembranausläufern zu finden ist (Corbeil et al., 1999; Corbeil et al., 2001; Mizrak et al., 2008), sollte analysiert werden, ob CD133 auch in $CD34^+$ Zellen eine polare Verteilung aufweist. Dabei zeigte sich, dass CD133 stark in der Spitze des Uropods angereichert ist (Abbildung 5-1). Im Gegensatz zu den Uropod- und Leitfront-assoziierten Membranproteinen waren der Stammzellsurrogatmarker

CD34 und der Leukozytenmarker CD45 gleichmäßig über die Zelloberfläche verteilt und wiesen keine Präferenz für einen der Zellpole auf (Giebel et al., 2004).

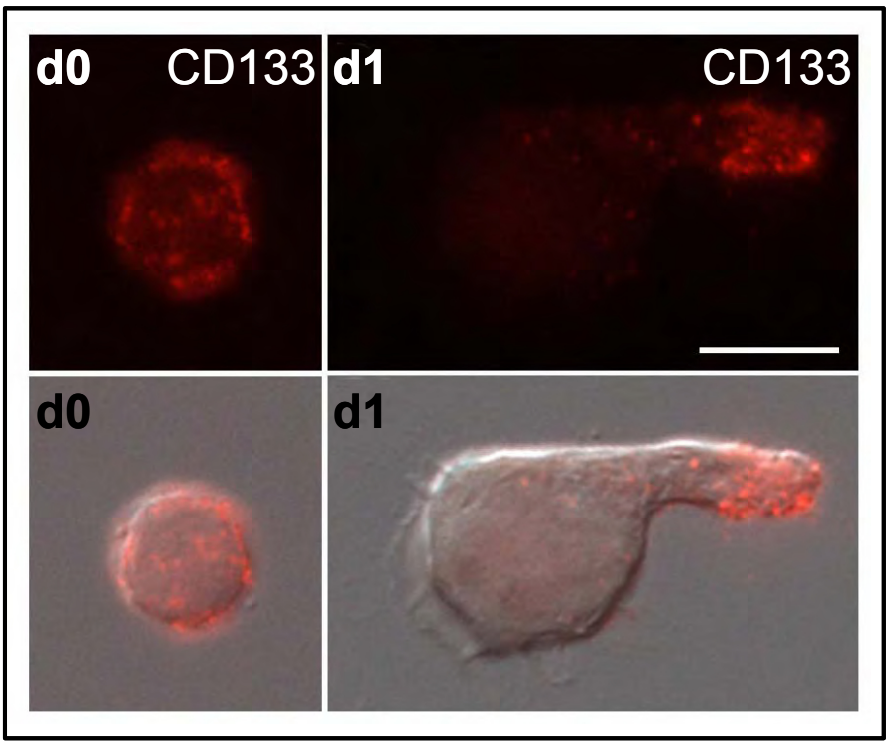


Abbildung 5-1: Verteilung von CD133 in frisch isolierten und in kultivierten CD34$^+$ Zellen. Frisch isolierte CD34$^+$ Zellen sind klein und rund (linke Spalte). Unter bestimmten Kulturbedingungen nehmen die meisten Zellen nach 1-2 Tagen eine polare Morphologie an (rechte Spalte). Während CD133 (rote Färbung) in frisch isolierten Zellen über die Zelloberfläche verteilt ist, kommt es in kultivierten, polarisierten Zellen zu einer Anreicherung von CD133 im Uropod der Zellen (rechte Spalte). Maßstab entspricht 5 µM. d: Tag.

Zusammenfassend konnten wir im Rahmen meiner Arbeiten anhand der Zellmorphologie und in Färbungen nachweisen, dass CD34$^+$ Zellen eine Polarität annehmen, die sich auch in der subzellulären Assoziation bestimmter Antigene mit den Zellpolen widerspiegelt. Damit haben wir erstmals gezeigt, dass CD34$^+$ Zellen in Kultur eine Zellpolarität aufbauen. Der Fragestellung dieser Arbeit entsprechend, ob sich HSZ/HVZ asymmetrisch teilen können, erfüllen diese Zellen demnach eine essenzielle Voraussetzung.

Die hier dargestellten Ergebnisse zur Zellpolarität primitiver humaner hämatopoietischer Zellen wurden unter dem Titel **„Segregation of lipid raft markers including CD133 in polarized human hematopoietic stem and progenitor cells“** (Giebel et al., 2004) (siehe 10.1) veröffentlicht.

5.2 Untersuchungen zur Zellteilung von CD34$^+$ Zellen

Nachdem wir Proteine identifizieren konnten, die in kultivierten CD34$^+$ Zellen polar verteilt sind, schloss sich die Frage an, wie diese Uropod- oder Leitfront-assoziierten Faktoren in mitotischen Zellen verteilt sind. Da wir uns gegen eine Synchronisation der Zellen entschieden hatten, war es unser Ziel, einen Zeitpunkt zu ermitteln, zu dem sich möglichst viele Zellen teilen, um dementsprechend ausreichend viele Mitosen analysieren zu können. Daher wurde zunächst das Teilungsverhalten von CD34$^+$ Zellen in Proliferationskinetiken untersucht.

5.2.1 Untersuchung des Proliferationsverhaltens von CD34$^+$ Zellen unter verschiedenen Kulturbedingungen

Mit dem Ziel, einen Zeitpunkt zu ermitteln, zu dem sich möglichst viele primitive Zellen teilen, wurden Proliferationskinetiken erstellt. Dazu wurden die CD34$^+$ Zellen für mehrere Tage mit zwei verschiedenen Zytokingemischen inkubiert. Für diese beiden Zytokinkombinationen haben Punzel und Kollegen eine unterschiedliche Wirkung auf das Proliferationsverhalten der Zellen beschrieben (Punzel et al., 2002). Weiterhin konnte gezeigt werden, dass sich dabei trotz der unterschiedlichen Proliferationsraten die Frequenz der Zellteilungen, aus denen voneinander unterschiedliche Tochterzellen hervorgehen, nicht ändert. Die Unterschiede zwischen den Tochterzellen wurden hierbei anhand des weiteren Teilungsverhaltens der Zellen ermittelt. Die initiale Zellteilung wurde als asymmetrisch bewertet, wenn die eine Tochterzelle weitere Nachkommen hervorbrachte, während die andere nicht weiter proliferierte oder starb (Punzel et al., 2002).

Ein Zytokingemisch enthielt die Zytokine *stem cell factor* (SCF), Makrophagen-Kolonie stimulierender Faktor (FMS)-ähnliche Tyrosinkinase-3-Ligand (Flt3-L) und Thrombopoietin (TPO), die auch als früh wirksame Zytokine bezeichnet werden. Punzel und Kollegen konnten zeigen, dass in Gegenwart dieser Zytokine die meisten Zellen, einschließlich der primitiven Zellen, relativ schnell beginnen sich zu teilen (Punzel et al., 2002). Außerdem wurde beschrieben, dass diese Zytokinkombination zur Erhaltung und einer leichten Expansion von humanen hämatopoietischen Zellen mit Repopulationspotenzial in Suspensionskultur beiträgt (Kobayashi et al., 1997; Ohmizono et al., 1997; Luens et al., 1998). Mit

der zweiten Kombination aus den sogenannten spät wirksamen Zytokinen Interleukin-3 (IL-3), Interleukin-6 (IL-6), Granulozyten-Makrophagen-Kolonie stimulierender Faktor (GM-CSF); basischer Fibroblasten Wachstumsfaktor (bFGF), Insulin-ähnlicher Wachstumsfaktor (IGF), *stem cell factor/* c-Kit-Ligand (SCF) und Erythropoietin (Epo) ließ sich im Vergleich zu früh wirksamen Zytokinen eine deutlich stärkere Gesamtexpansion der $CD34^+$ Zellen erzielen (Punzel et al., 2002). Da hierbei aber sehr primitive Zellen deutlich länger brauchten, bis sie ihre initiale Teilung absolvierten, scheint diese Zytokinkombination zunächst nur die Proliferation von reiferen Zellen zu begünstigen, die dann zu der beobachteten stärkeren Gesamtexpansion der Zellen führte (Punzel et al., 2002).

Um das Teilungsverhalten der Zellen verfolgen zu können, wurden frisch isolierte $CD34^+$ Zellen mit dem fluoreszierenden Lebendfarbstoff PKH2 gefärbt. PKH2 lagert sich in die Plasmamembran der Zellen ein (Slezak und Horan, 1989), und bei einer Zellteilung der gefärbten Zellen erhalten beide Tochterzellen gleiche Mengen des Farbstoffs. Dies hat zur Folge, dass sich bei jeder Zellteilung die Fluoreszenzintensität der Tochterzellen im Vergleich zur Mutterzelle halbiert (Lansdorp und Dragowska, 1993; Traycoff et al., 1995; Hendrikx et al., 1996; Punzel und Ho, 2001). Daher lässt die Fluoreszenzintensität der Zellen zu einem gegebenen Zeitpunkt einen Rückschluss auf die Anzahl der bis dahin absolvierten Teilungen zu.

Zur Dokumentation der Proliferation wurden in regelmäßigen Abständen Zellen aus der Kultur entnommen und im Durchflusszytometer bezüglich der PKH2-Färbung und ihrer CD34- und CD133-Expression untersucht (Giebel et al., 2006).

Dabei zeigte sich, dass sich unter beiden Kulturbedingungen die ursprünglich $CD34^+CD133^+$ doppeltpositiven Zellen ab Tag 3 in eine $CD34^+CD133^+$ ($CD133^{high}$) und eine $CD34^+CD133^-$ ($CD133^{low}$) Fraktion aufteilen (Abbildung 5-2). Dabei stieg der prozentuale Anteil der $CD34^+CD133^-$ Zellen mit der Länge der Kulturdauer (Giebel et al., 2006).

Anhand der PKH2-Fluoreszenzintensität der Zellen ließ sich ermitteln, dass sich in Gegenwart von spät wirksamen Zytokinen $CD34^+CD133^-$ Zellen deutlich häufiger teilen als $CD34^+CD133^+$ Zellen. Dagegen teilten sich unter Kulturbedingungen mit früh wirksamen Zytokinen alle $CD34^+$ Zellen über den gesamten Messzeitraum annähernd gleich häufig (Giebel et al., 2006).

Daher haben wir uns für die Kultur in Gegenwart von früh wirksamen Zytokinen entschieden, um möglichst viele mitotische Zellen in weiteren Analysen untersuchen zu können. Dabei wurden Tag 3 Zellen verwendet, da PKH2-Studien ergaben, dass die meisten Zellen ihre erste Teilung zwischen Tag 2 und 3 absolvieren und mit jeder weiteren Zellteilung das Entwicklungspotenzial der Zellen eingeschränkt werden kann (Beckmann et al., 2007).

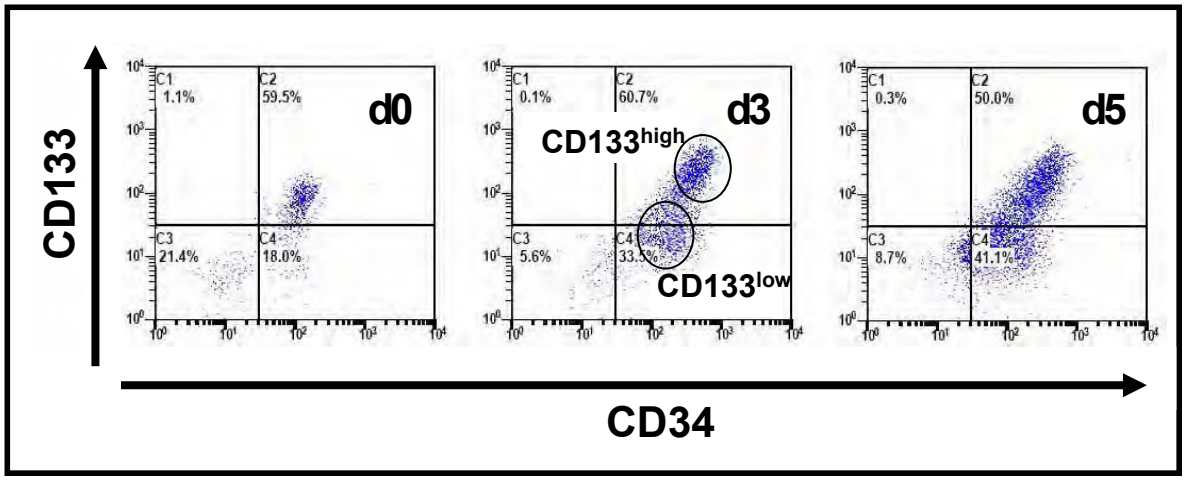


Abbildung 5-2: Verlauf der CD133-Expression bei CD34$^+$ Zellen. Die Punktediagramme zeigen die durchflusszytometrische Analyse der CD133- und CD34-Expression von frisch isolierten Tag 0 (d0) und für 3 (d3) und 5 Tage (d5) kultivierten CD34$^+$ Zellen. Die Zellen wurden an den entsprechenden Tagen mit fluoreszenz-konjugierten Antikörpern gegen CD133 und CD34 gefärbt und im Durchflusszytometer analysiert. Der größte Teil der frisch isolierten CD34$^+$ Zellen (d0) ist auch positiv für CD133. Im Verlauf der Kultur in Gegenwart von frühwirksamen Zytokinen treten ab Tag 3 (d3) neben den CD34$^+$CD133$^+$ Zellen (CD133^{high}-Region) auch Zellen auf, die wenig oder gar kein CD133 exprimieren (CD133^{low}-Region).

Der Vergleich der Kinetiken mit früh und spät wirksamen Zytokinen ist in dem Manuskript **„Primitive human hematopoietic cells give rise to differentially specified daughter cells upon their initial cell division“** (Giebel et al., 2006) (siehe 10.2) veröffentlicht worden. Der zeitliche Verlauf der Proliferation von CD34$^+$ Zellen, die mit früh wirksamen Zytokinen kultiviert wurden, ist in **„Asymmetric cell division within the human hematopoietic stem and progenitor cell compartment: identification of asymmetrically segregating proteins“** (Beckmann et al., 2007) (siehe 10.4) dargestellt.

Weiterhin haben wir mit Hilfe solcher Kinetiken zeigen können, dass die genetische Manipulation von CD34$^+$ Zellen mittels Nukleofektion keine Auswirkung auf die Expression von CD133 und CD34 und das Verhältnis zwischen CD34$^+$CD133$^+$ und CD34$^+$CD133$^-$ Zellen hat. Anhand dieser und weiterer

Ergebnissen zum Langzeitentwicklungspotenzial transfizierter Zellen konnten wir zeigen, dass die Methode der Nukleofektion zur Manipulation von $CD34^+$ Zellen geeignet ist. Diese Arbeiten haben wir unter dem Titel **„Nucleofection, an Efficient Nonviral Method to Transfer Genes into Human Hematopoietic Stem and Progenitor Cells“** (von Levetzow et al., 2006) (siehe 10.3) veröffentlicht.

5.2.2 Verteilung von Polaritätsmarkern in mitotischen Zellen

Aufgrund der Proliferationsanalysen haben wir uns entschieden, Zellen in Gegenwart von früh wirksamen Zytokinen für 3 Tage zu vermehren, um möglichst viele Mitosen analysieren zu können. In Immunfärbungen haben wir die subzelluläre Verteilung der zuvor identifizierten Polaritätsmarker untersucht. Ziel hierbei war es herauszufinden, wie diese Proteine in mitotischen Zellen verteilt sind.

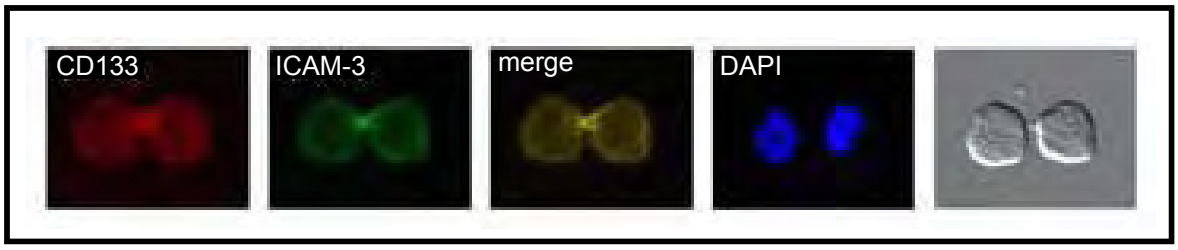


Abbildung 5-3: Verteilung des Uropod-assoziierten Proteins ICAM-3/CD50 in mitotischen $CD34^+$ Zellen. $CD34^+$ Zellen wurden in Gegenwart von früh wirksamen Zytokinen kultiviert und an Tag 3 mit Antikörpern gegen CD133 (rot) und gegen ICAM-3/CD50 (grün) gefärbt. Sowohl der Stammzellmarker CD133 als auch ICAM-3 wird in sich teilenden Zellen in der Zytoplasmabrücke zwischen den Tochterzellen angereichert. Die Zellkerne wurden mit DAPI (blau) angefärbt.

Es zeigte sich, dass CD43, CD44, ICAM-3/CD50, ICAM-1/CD54 sowie CD133 zuerst in der Teilungsfurche und später hauptsächlich im *Midbody*, einer Struktur der schmalen zytoplasmatischen Verbindung zwischen den beiden Tochterzellen (Otegui et al., 2005), angereichert werden (Beckmann et al., 2007). In Abbildung 5-3 ist exemplarisch eine mitotische $CD34^+$ Zelle dargestellt, die mit Antikörpern gegen CD133 und ICAM-3/CD50 gefärbt wurde, bei der eine Anreicherung von CD133 und ICAM-3/CD50 in der Zytoplasmabrücke zwischen den beiden Tochterzellen zu erkennen ist. Der Chemokinrezeptor CXCR4 war dagegen eher stärker in den äußeren, vom *Midbody* abgewandten Hälften der beiden Tochterzellen konzentriert (eigene, unveröffentlichte Beobachtung). Damit konnte

gezeigt werden, dass die für Zellen mit polarisierter Morphologie beschriebene polare Verteilung bestimmter Faktoren auch in der Mitose erhalten bleibt.

Diese Arbeiten sind als Teil der Publikation **„Asymmetric cell division within the human hematopoietic stem and progenitor cell compartment: identification of asymmetrically segregating proteins“** (Beckmann et al., 2007) (siehe 10.4) veröffentlicht.

Auch wenn wir zeigen konnten, dass mitotische $CD34^+$ Zellen eine Polarität aufweisen, fanden wir keine Hinweise auf eine asymmetrische Verteilung eines der untersuchten Proteine. Daraus haben wir die Schlussfolgerung gezogen, dass sich die $CD34^+$ Zellen unter den verwendeten Bedingungen entweder nicht asymmetrisch teilen oder andere Proteine existieren, die asymmetrisch verteilt werden.

Um solche Kandidaten zu identifizieren, haben wir verschiedene Überlegungen angestellt und daraus einen neuen Ansatz für die Suche nach asymmetrisch segregierenden Faktoren entwickelt. Auch wenn unsere Analyse von mitotischen Zellen dies nicht bestätigen konnte, lässt sich die differenzielle CD133 Expression bei kultivierten $CD34^+$ Zellen (Abbildung 5-2) hypothetisch dadurch erklären, dass sich ein Teil der ursprünglich $CD34^+CD133^+$ Zellen asymmetrisch teilt und dabei jeweils eine weitere primitive $CD34^+CD133^+$ und eine reifere $CD34^+CD133^-$ Zelle entstehen. Verschiedene Arbeitsgruppen haben beobachtet, dass sich etwa 30% der Tochterzellen primitiver hämatopoietischer Zellen unterschiedlich voneinander entwickeln, und allgemein wurden asymmetrische Zellteilungen als Ursache hierfür angenommen (Mayani et al., 1993; Brummendorf et al., 1998; Punzel et al., 2002). Wenn man infolgedessen annimmt, dass sich 30% der $CD34^+CD133^+$ Zellen asymmetrisch teilen und dabei $CD34^+CD133^+$ und $CD34^+CD133^-$ Zellen hervorbringen, würde man mit steigender Anzahl von Zellteilungen eine Abnahme des Anteils von $CD34^+CD133^+$ Zellen zugunsten des Anteils von $CD34^+CD133^-$ Zellen erwarten. Da die beobachtete Kinetik der CD133 Expression im Verlauf der Kultur $CD34^+$ Zellen mit diesem Modell der asymmetrischen Zellteilung vereinbar war (Beckmann et al., 2007), CD133 aber scheinbar unter den verwendeten Kulturbedingungen nicht asymmetrisch verteilt wird, haben wir angenommen, dass andere Proteine, die differenziell in der $CD34^+CD133^+$ und $CD34^+CD133^-$ Fraktion exprimiert werden, Kandidaten für eine asymmetrische Verteilung während der

Mitose sind. Daher haben wir uns entschlossen, nach solchen Faktoren zu suchen.

5.2.3 Identifizierung von asymmetrisch segregierenden Proteinen

Um Faktoren zu identifizieren, die differenziell auf der $CD34^{+}CD133^{high}$ und der $CD34^{+}CD133^{low}$ Population exprimiert sind, habe ich zusammen mit dem Diplomand Sebastian Scheitza entsprechende Kinetiken erstellt.

In einer durchflusszytometrischen Analyse mit 58 Antikörpern gegen unterschiedliche Leukozytenoberflächenantigene haben wir 39 Proteine gefunden, die auf kultivierten $CD34^{+}$ Zellen exprimiert sind. Davon waren 19 Antigene unterschiedlich stark in der $CD34^{+}CD133^{high}$ und $CD34^{+}CD133^{low}$ Fraktion exprimiert. Aus diesen 19 Proteinen wurden mit CD53, CD63/LAMP-3, CD62L/L-Selektin und CD71/Transferrinrezeptor 4 Oberflächenantigene für weitere Analysen ausgewählt, da ihr Expressionslevel den stärksten Kontrast zwischen den beiden Populationen aufzeigte (Beckmann et al., 2007).

5.2.3.1 Subzelluläre Verteilung von CD53, CD62L, CD63 und CD71 in polarisierten $CD34^{+}$ Zellen

Für die subzelluläre Lokalisation wurden kultivierte $CD34^{+}$ Zellen mit Antikörpern gegen CD53, CD62L, CD63 oder CD71 gefärbt. Dabei fanden wir, dass CD62L in Zellen mit polarisierter Morphologie in der Spitze des Uropods angereichert ist, während CD53 und CD63 in vesikelähnlichen Strukturen an der Basis des Uropods zu finden sind (Beckmann et al., 2007).

In dieser Region war auch der auf der gesamten Zelloberfläche vorkommende Transferrinrezeptor CD71 in vesikelähnlichen Strukturen angereichert (Beckmann et al., 2007).

5.2.3.2 Funktionelle Charakterisierung der neu identifizierten Subpopulationen von $CD34^{+}$ Zellen

Mit dem Ziel festzustellen, ob die differenzielle Expression der neu identifizierten Proteine innerhalb der beiden Subpopulationen von $CD34^{+}$ Zellen mit funktionellen Unterschieden korreliert, wurden funktionelle Analysen durchgeführt.

Dazu wurden die Subfraktionen der $CD34^+$ Zellen zunächst sortiert und anschließend in einem eingangs beschriebenen Langzeit-Analyse-Verfahren (LTC-IC) (siehe 4.2.2) bezüglich ihres myeloischen Entwicklungspotenzials charakterisiert. In Abbildung 5-4 ist die Strategie zur Zellsortierung am Beispiel von CD62L dargestellt.

Dabei zeigte sich, dass die in Kombination mit den neuen Markern isolierte $CD34^+CD133^+$ Fraktion signifikant mehr primitive Zellen enthält als die entsprechende $CD34^+CD133^{low}$ bzw. die $CD34^+$ Kontrollfraktion (Beckmann et al., 2007). Somit können CD53, CD63, CD62L und CD71 in Kombination mit CD133 für die Charakterisierung primitiver Zellen innerhalb der $CD34^+$ Fraktion dienen.

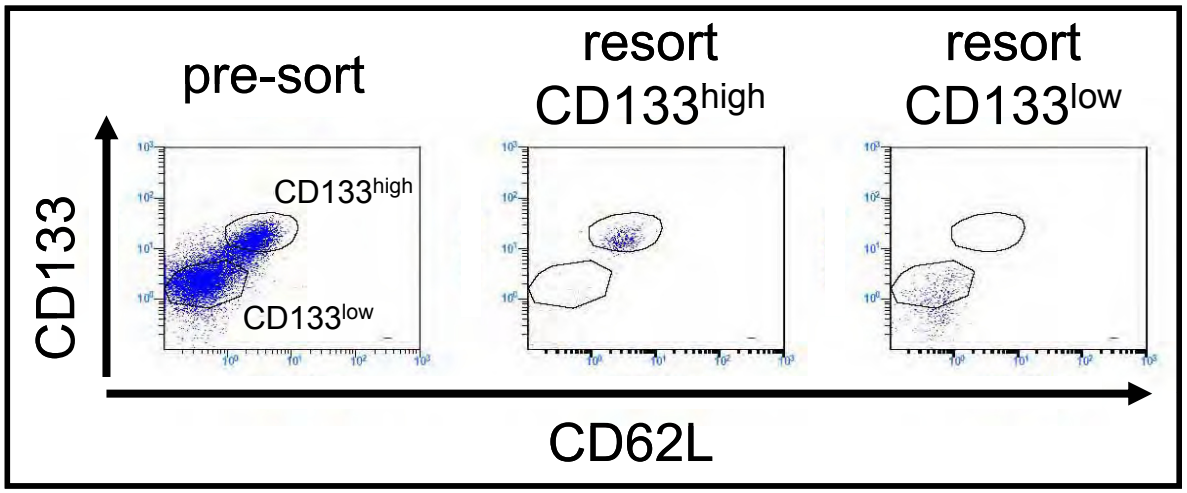


Abbildung 5-4: Strategie für die Zellsortierung anhand der Expression von CD62L. $CD34^+$ Zellen wurden an Tag 3 vor der Zellsortierung mit Antikörpern gegen CD133, CD62L und CD34 gefärbt. Das erste Punktediagramm (pre-sort) zeigt die durchflusszytometrische Analyse von lebenden $CD34^+$ Zellen in Bezug auf ihre CD133- und CD62L-Expression. Diese Zellen wurden nach den Kriterien $CD34^+CD133^{high}CD62L^{high}$ ($CD133^{high}$-Region) sowie $CD34^+CD133^{low}CD62L^{low}$ ($CD133^{low}$-Region) sortiert. Ein Teil der sortierten Zellfraktionen wurde anschließend zur Kontrolle der Reinheit reanalysiert (*resort*, zweites und drittes Punktediagramm).

Diese Befunde deuten auf einen Zusammenhang zwischen der Expression der neu identifizierten Proteine und dem Schicksal der Zellen hin. Daher sollte gezeigt werden, ob die Unterschiede zwischen den Subfraktionen in Bezug auf die Expression von CD53, CD62L, CD63 oder CD71 – wie eingangs angenommen - durch eine asymmetrische Verteilung dieser Faktoren während der Mitose zustande kommt.

5.2.3.3 Subzelluläre Verteilung der neu identifizierten Antigene in sich teilenden CD34$^+$ Zellen

Um diese Frage zu beantworten, haben wir im Anschluss daran die subzelluläre Verteilung der Antigene in sich teilenden CD34$^+$CD133$^+$ Zellen untersucht.

Dabei fanden wir sowohl in extrazellulären Färbungen mit Antikörpern gegen CD53, CD62L, CD63 als auch gegen CD71 Mitosen, bei denen die Tochterzellen unterschiedlich stark angefärbt waren, was auf eine unterschiedliche Verteilung der Proteine zwischen den Geschwisterzellen hindeutet (Beckmann et al., 2007). In Abbildung 5-5 sind am Beispiel von einer Färbung mit Antikörpern gegen CD62L (grün) zwei mitotische CD34$^+$ Zellen dargestellt. Dabei weist die sich teilende Zellen in der oberen Reihe eine symmetrische Verteilung von CD62L auf die beiden Tochterzellen auf, während die Verteilung bei der zweiten Mitose asymmetrisch ist.

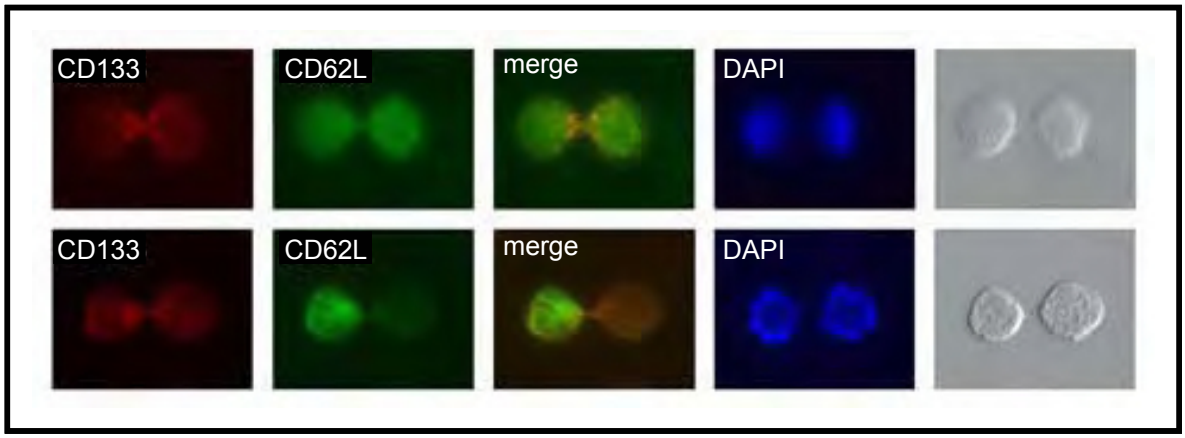


Abbildung 5-5: Verteilung von CD62L auf der Oberfläche von mitotischen CD34$^+$ Zellen. CD34$^+$ Zellen wurden in Gegenwart von früh wirksamen Zytokinen kultiviert und an Tag 3 mit Antikörpern gegen CD133 (rot) und gegen CD62L (grün) gefärbt. In der oberen Reihe ist eine Mitose dargestellt, bei der beide Tochterzellen ähnlich positiv für CD62L sind (symmetrische Verteilung von CD62L). Die untere Reihe zeigt eine Mitose, bei der die eine Tochterzelle deutlich stärker positiv für CD62L ist als die andere Tochterzelle (asymmetrische Verteilung von CD62L). Die Verteilung von CD133 (rot) auf die Tochterzellen ist dagegen in beiden Fällen gleichmäßig. Die Zellkerne wurden mit DAPI (blau) angefärbt.

Weiterhin wurde in den Färbungen mit Antikörpern gegen CD53, CD63 oder CD71 ein vesikelähnliches Färbemuster beobachtet. Da für CD53, CD63 und auch für den Transferrinrezeptor CD71 eine Assoziation mit Endozytose beschrieben wurde (Escola et al., 1998; Arribas und Cutler, 2000; Kobayashi et al., 2000; Qian

et al., 2002)., vermuteten wir, dass es sich bei den vesikelähnlichen Strukturen möglicherweise um abknospende Endosomen handelt.

Aufgrund dieser Verbindung zur Endozytose wurde untersucht, ob sich die asymmetrische Verteilung der identifizierten Proteine auch auf intrazellulärer Ebene manifestiert. In intrazellulären Doppelfärbungen mit Antikörpern gegen CD63 und CD71 wurden ebenfalls vesikelartige Strukturen angefärbt, und das Färbemuster deutete auf eine Colokalisation von CD63 und CD71 hin. Da auch in diesen Färbungen ein bestimmter Anteil von Mitosen gefunden wurde, bei denen die Tochterzellen unterschiedliche Mengen der $CD63^{+}CD71^{+}$ Strukturen enthielten, konnte die asymmetrische Verteilung von CD63 und CD71 auf intrazellulärer Ebene bestätigt werden (Beckmann et al., 2007).

Die in diesen Versuchen ermittelte Frequenz von Mitosen mit einer asymmetrischen Proteinverteilung liegt bei 20-30% (Beckmann et al., 2007). Damit entspricht sie in etwa der von verschiedenen Gruppen beschriebenen Häufigkeit, mit der sich Tochterzellen primitiver hämatopoietischer Zellen unterschiedlich voneinander entwickeln (Mayani et al., 1993; Brummendorf et al., 1998; Punzel et al., 2002).

Somit ist es uns gelungen, Faktoren zu identifizieren, die in einem Teil der Mitosen asymmetrisch auf die Tochterzellen verteilt werden. Wir konnten damit zum ersten Mal zeigen, dass HSZ/HVZ tatsächlich in der Lage sind, sich asymmetrisch zu teilen. Daher konnte die Frage nach der Existenz einer asymmetrischen Zellteilung, die die Grundlage für die in dieser Arbeit durchgeführten Versuche bildete, klar beantwortet werden.

Diese hier beschriebenen Ergebnisse haben wir unter dem Titel **„Asymmetric cell division within the human hematopoietic stem and progenitor cell compartment: identification of asymmetrically segregating proteins“** (Beckmann et al., 2007) (siehe 10.4) veröffentlicht.

5.2.4 Asymmetrische Zellteilung und Endozytose

In weiteren Versuchen wurde untersucht, ob das in Färbungen mit Antikörpern gegen CD53, CD63 und CD71 beobachtete vesikelähnliche Färbemuster an der Uropodbasis durch Vesikel entsteht, die in diesem Bereich abgeschnürt werden. Aufschluß hierüber sollte eine intrazelluläre Färbung von $CD34^{+}$ Zellen geben,

denen zuvor unter Kulturbedingungen die Gelegenheit gegeben wurde, gebundene Antikörper zu internalisieren. Dazu wurden lebende Zellen mit Antikörpern gegen CD71 gefärbt, gewaschen, für kurze Zeit weiterkultiviert und anschließend fixiert. Mithilfe von zwei unterschiedlichen Sekundärantikörpern habe ich gleichzeitig die extra- und intrazelluläre Verteilung des Primärantikörpers untersucht. Die in der extrazellulären Färbung beobachtete Anreicherung an der Basis des Uropods stimmte mit dem Färbemuster in Zellen überein, die vor der Färbung mit dem Primärantikörper bereits fixiert waren. Da auch die meisten der markierten intrazellulären vesikelartigen Strukturen in dieser Region zu finden waren, scheint deren Internalisierung hauptsächlich am Uropod stattzufinden (eigene, unveröffentlichte Beobachtungen). Diese Befunde deuten darauf hin, dass es sich bei den extrazellulär angefärbten Plasmamembranregionen tatsächlich um Bereiche handelt, die in Form von Vesikeln abgeschnürt werden können. Dies wird auch unterstützt durch Arbeiten von Hoffstein und Kollegen, die in elektronenmikroskopischen Untersuchungen von Neutrophilen ebenfalls eine Anreicherung von endozytotischen Vesikeln an der Basis des Uropods beschrieben haben (Hoffstein et al., 1982).

In neueren Untersuchungen bei Modellorganismen konnte ein Zusammenhang zwischen Endozytose und dem Zellschicksal der entstehenden Tochterzellen nachgewiesen werden (Shen und Temple, 2002; Bokel et al., 2006; Andrews und Ahringer, 2007). Auch für CD53, CD63 und CD71 wurde eine Assoziation mit intrazellulären Vesikeln beschrieben (Escola et al., 1998; Arribas und Cutler, 2000; Kobayashi et al., 2000; Qian et al., 2002). Da das Färbemuster in einem Teil der Mitosen auf eine asymmetrische Verteilung dieser Proteine auf die Tochterzellen hindeutete (Beckmann et al., 2007), ergibt sich möglicherweise im hämatopoietischen Kompartiment ein ähnlicher Zusammenhang zwischen Endozytose und dem Zellschicksal.

Einen Überblick über Befunde, die auf eine Verbindung zwischen dem endosomalen Kompartiment und Zellschicksalsentscheidungen hindeuten, haben wir unter dem Titel **„Asymmetric cell divisions of human hematopoietic stem and progenitor cells meet endosomes“** (Giebel und Beckmann, 2007) (siehe 10.5) veröffentlicht.

5.3 Funktionelle Analyse der Zellpolarität und der Migration bei CD34$^+$ Zellen

Nachdem wir erstmals die Polarität in kultivierten mitotischen und nicht-mitotischen Zellen darstellen konnten, haben wir begonnen, Mechanismen zu analysieren, die am Aufbau und dem Erhalt dieser Polarität beteiligt sind.

Zu Beginn haben wir uns dabei auf Signalwege konzentriert, die bei der zielgerichteten Rückkehr von HSZ aus der Blutbahn an ihren Entstehungsort im Knochenmark, dem sogenannten *Homing*, eine Rolle spielen. Die Überlegung hierbei war, dass viele Mechanismen, die am *Homing*-Prozess beteiligt sind, auch die Zellwanderung steuern. Da Zellpolarität wiederum eine Voraussetzung für Migration ist (Lauffenburger und Horwitz, 1996), haben wir untersucht, ob die aus dem *Homing* bekannten Signalwege bereits auf der Ebene des Polarisierungsprozesses von HSZ/HVZ eine Rolle spielen.

Im adulten Organismus enthält das periphere Blut unter physiologischen Bedingungen immer eine sehr geringe Anzahl von HSZ/HVZ. Diese zirkulierenden Zellen können in verschiedene Gewebe wandern und anschließend aufgrund ihrer *Homing*-Fähigkeit auch wieder in ihre funktionellen Nischen im Knochenmark oder in anderen Organen zurückkehren (Wright et al., 2001; Abkowitz et al., 2003). Um aus dem peripheren Blut wieder zurück ins Knochenmark zu gelangen, müssen HSZ/HVZ die Endothelschicht passieren, was man auch als Transmigration bezeichnet. Es konnte gezeigt werden, dass für den *Homing*-Prozess ein koordiniertes Zusammenspiel verschiedener Mechanismen und Signalwege erforderlich ist (Lapidot et al., 2005; Chute, 2006). Wichtige Signalmoleküle sind hierbei unter anderem der Chemokinrezeptor CXCR4 (Aiuti et al., 1997; Kawabata et al., 1999; Peled et al., 1999; Kollet et al., 2001; Bonig et al., 2004), die Phosphatidyl-Inositol-3-Kinase (PI3K) (Wang et al., 2000; Whetton et al., 2003), die atypische Proteinkinase C (aPKC) (Kollet et al., 2001; Petit et al., 2005) und GTPasen der Rho-Familie (Gu et al., 2003; Gottig et al., 2006; Yang et al., 2007).

Die Rolle einiger aus dem *Homing* bekannter Signalwege im Polarisierungsprozess von CD34$^+$ Zellen wurde untersucht, indem die Zellen vor der Analyse mit unterschiedlichen Inhibitoren und Toxinen behandelt wurden, die mit verschiedenen Wegen der Signaltransduktion interferieren. Da der Polarisierungsprozess und die Zellwanderung mit einer dynamischen

Reorganisation des Zytoskeletts verbunden sind, wurde zusätzlich überprüft, wie sich eine Manipulation des Aktin- und Mikrotubuli-Zytoskeletts auf die Polarität und die Wanderung der $CD34^+$ Zellen auswirkt.

Ziel hierbei war es herauszufinden, ob die Blockade einzelner Signalwege einen Einfluss auf die Zellform und die Polarität auf subzellulärer Ebene hat, die sich in Form von polar lokalisierten Faktoren manifestiert. Zusätzlich zur Zellpolarität kann durch die Analyse der Zellwanderung in Transmigrationsversuchen (*Transwellassays*) überprüft werden, ob sich die für die Rückkehr in das Knochenmark relevante Transmigrationsfähigkeit der Zellen infolge einer Inhibitor-Behandlung verändert. Somit kann unterschieden werden, ob die in der Literatur beschriebene reduzierte *Homing*-Fähigkeit der Zellen bereits durch eine Blockade auf der Ebene der intrinsischen oder morphologischen Polarisierung zustande kommt oder ob die Transmigration der Zellen gestört ist.

Um weiterhin unterscheiden zu können, ob bestimmte Signale oder Mechanismen wichtig für den Aufbau und/oder den Erhalt der Polarität sind, habe ich zwei verschiedene Versuchsansätze gewählt. Einerseits wurden frisch isolierte Zellen mit den Inhibitoren behandelt, um eine Beteiligung am initialen Aufbau der Polarität zu überprüfen. Inwieweit ein Signal andererseits wichtig für den Erhalt der Polarität ist, sollte durch die Behandlung bereits polarisierter Zellen geklärt werden.

Für die Auswertung wurde die Morphologie der Zellen beurteilt. Ob die Zellen auf subzellulärer Ebene Zeichen einer Polarität aufweisen, wurde in Immunfärbungen die Verteilung verschiedener Proteine untersucht. Diese auf eine polare Lokalisation von Proteinen bezogene Form der Zellpolarität haben wir im Folgenden auch als intrinsische Polarität bezeichnet.

In diesen Lokalisationsstudien wurde unter anderem die Verteilung der *lipid raft* assoziierten Flotillin-Proteine untersucht. Als *lipid rafts* werden bestimmte Mikrodomänen der Plasmamembran bezeichnet, die sich in ihrer Lipid- und Proteinzusammensetzung vom Rest der Membran unterscheiden. Aufgrund dieser Eigenschaften können *lipid rafts* die Interaktion bestimmter Signalproteine fördern oder verhindern. Ein Beispiel hierfür ist die Aktivierung von T-Zellen durch den Kontakt mit antigenpräsentierende Zellen, die mit einer Umverteilung von *lipid rafts* in die Kontaktzone einhergeht (Viola et al., 1999). In dieser Kontaktzone wird die

Assemblierung von Signalkomplexen und die Signalamplifikation unterstützt, indem bestimmte Signalmoleküle in *lipid rafts* rekrutiert werden (Alonso und Millan, 2001; Cheng et al., 2001; Sedwick und Altman, 2002). Flotillinproteine sind mit einer bestimmten Subklasse von *lipid rafts* assoziiert, die in nicht aktivierten Immunzellen im Gegensatz zu anderen *lipid raft*-Arten bereits eine polare Verteilung aufweisen (Rajendran et al., 2003). Da die polare Flotillinlokalisation zu den ersten detektierbaren Ereignissen im Polarisierungsprozess reifer hämatopoietischer Zellen gehört, die der polaren Anordnung anderer charakteristischer Polaritätsmarker vorausgeht (Rajendran et al., 2003), sollte untersucht werden, ob dies auch für primitive hämatopoietische Zellen zutrifft. Aus diesem Grund wurden die Flotillinproteine für den Lokalisationsvergleich mit dem Uropodmarker ICAM-3/CD50 ausgewählt, der ebenfalls mit *lipid rafts* assoziiert ist (Gomez-Mouton et al., 2001; Millan et al., 2002).

Abbildung 5-6 zeigt exemplarisch Aufnahmen von Zellen, die mit Antikörpern gegen Flotillin-1 und ICAM-3/CD50 gefärbt wurden. Die Untersuchung der Flotillinverteilung in unbehandelten $CD34^+$ Zellen ergab, dass Flotillin-1 (Abbildung 5-6A) und Flotillin-2 in den meisten der frisch isolierten, morphologisch nicht polarisierten Zellen über die ganze Zelloberfläche verteilt sind. Bei 25% der Zellen ließ sich eine einseitige, kappenähnliche Konzentration der Flotilline beobachten (Abbildung 5-6B).

In kultivierten, polarisierten Zellen waren die Flotilline mit der Spitze des Uropods assoziiert (Abbildung 5-6C). Im Gegensatz zu frisch isolierten Zellen wiesen auch die meisten der kultivierten, morphologisch runden Zellen eine polare Verteilung der Flotilline auf. Die Färbungen mit Antikörpern gegen Flotillin-2 lieferten ähnliche Ergebnisse (Beckmann et al., 2008, eingereicht).

Da die Färbemuster für ICAM-3/CD50 und Flotilline in polarisierten und auch in runden Zellen sehr ähnlich waren (Abbildung 5-6), lässt sich vermuten, dass die Verteilung der ICAM-3/CD50-positiven und Flotillin-positiven *lipid rafts* in ähnlicher Weise erfolgt oder dass sich ICAM-3/CD50 und Flotilline in denselben *lipid rafts* befinden.

Die Ergebnisse aus den Polaritätsanalysen deuten darauf hin, dass für den initialen Aufbau und die Aufrechterhaltung der morphologischen Polarität der $CD34^+$ Zellen vor allem die Aktivität der Phosphatidyl-Inositol-3-Kinase (PI3K), der

atypischen Protein Kinase C (aPKC) und Rho-GTPasen sowie ein intaktes Aktinzytoskelett benötigt wird, und dass in diesem Zusammenhang auch eine Proteinneusynthese stattfinden muss (Beckmann et al., 2008, eingereicht).

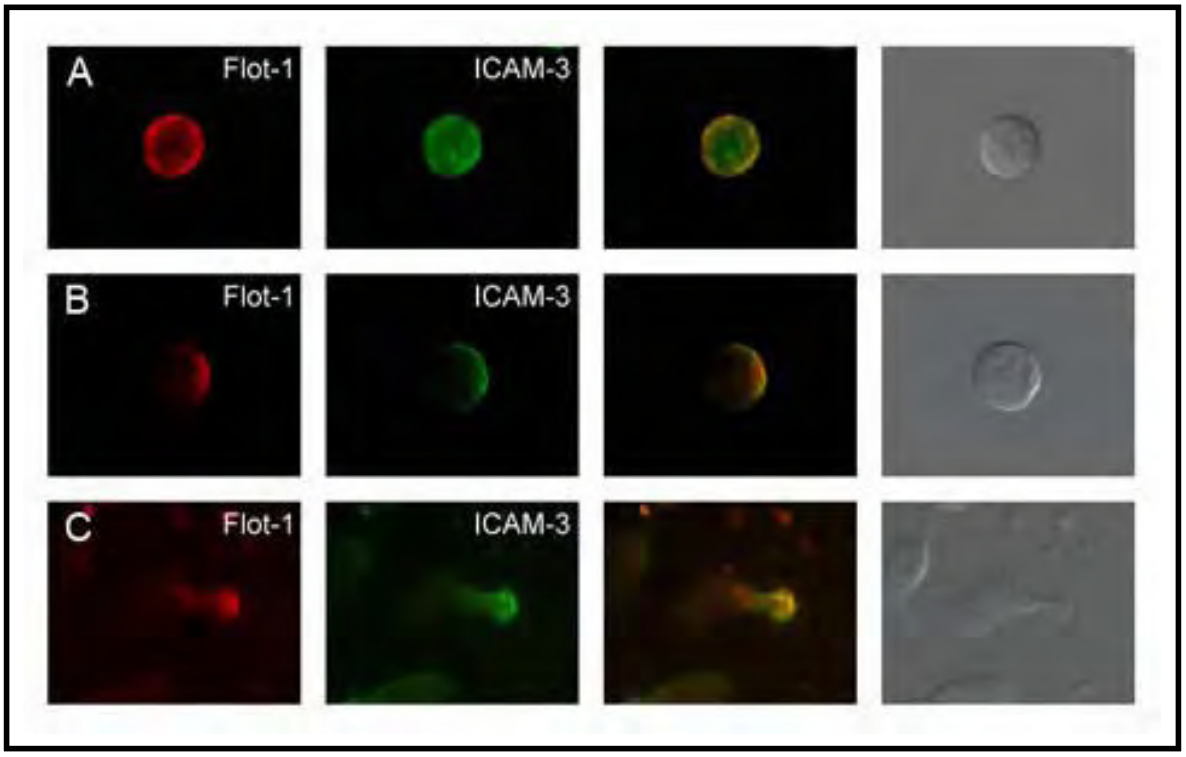


Abbildung 5-6: Verteilung von Flotillin-1 und ICAM-3/CD50 in runden und morphologisch polarisierten CD34$^+$ Zellen. CD34$^+$ Zellen wurden für 2 Tage in Gegenwart von früh wirksamen Zytokinen kultiviert und anschließend mit Antikörpern gegen Flotillin-1 (Flot-1, rot) und ICAM-3/CD50 (grün) gefärbt. Kultivierte CD34$^+$ Zellen lassen sich bezüglich ihrer Morphologie und Proteinverteilung in 3 Kategorien einteilen: (I) runde Zellen mit einer gleichmäßigen Oberflächenverteilung von Flotillin-1 und ICAM-3 (A), (II) runde Zellen mit einer polarisierten Lokalisation von Flotillin-1 und ICAM-3 (B) und (III) morphologisch polarisierte Zellen, in denen Flotillin-1 und ICAM-3/CD50 an der Spitze des Uropods angereichert sind (C).

Im Gegensatz dazu lassen die Untersuchungen zur Etablierung der intrinsischen Polarität darauf schließen, dass hierfür hauptsächlich die Aktivität der PI3K und der aPKC wichtig ist. Von den in dieser Arbeit verwendeten Inhibitoren beeinträchtigte nur die Behandlung der Zellen mit dem PI3K Inhibitor die Aufrechterhaltung der intrinsischen Polarität (Beckmann et al., 2008, eingereicht).

Die Integrität der Mikrotubuli war unseren Analysen zufolge nur für den Aufbau der morphologischen Polarität, nicht aber für die Wanderung sowie den Erhalt der polarisierten Zellform und der intrinsischen Polarität von bereits polarisierten CD34$^+$ Zellen wichtig (Beckmann et al., 2008, eingereicht).

Bei einer Kurzzeitbehandlung von kultivierten und größtenteils bereits polarisierten Zellen mit Nocodazol, das die Mikrotubuli-Depolymerisierung fördert, wurde sogar ein leichter Anstieg des Anteils an Zellen mit polarer Morphologie beobachtet. Die Transmigrationsrate der behandelten Zellen war außerdem deutlich höher als bei Kontrollzellen (Beckmann et al., 2008, eingereicht). Ähnliche Beobachtungen wurden auch bei einer Behandlung von immunreaktiven Leukozyten mit Nocodazol oder Colchicin gemacht, die beide zu einer Induktion der Polarisierung und Stimulation der Migration geführt haben (Keller et al., 1984; Niggli, 2003).

Es wurde gezeigt, dass das Chemokin SDF-1α eine zentrale Rolle im *Homing* Prozess spielt (Aiuti et al., 1997; Kawabata et al., 1999; Peled et al., 1999; Kollet et al., 2001; Bonig et al., 2004). Dazu passt die Beobachtung, dass $CD34^+$ Zellen, bei denen die Signaltransduktion des SDF-1α-Rezeptors durch die Behandlung mit Pertussistoxin beeinträchtigt wurde, in Transmigrationsversuchen fast keine Transmigrationsaktivität mehr aufwiesen. Im Gegensatz dazu ließ sich in den Polaritätsanalysen kein Effekt von Pertussistoxin auf die morphologische und intrinische Polarität beobachten (Beckmann et al., 2008, eingereicht). Interessanterweise war auch in Zeitrafferaufnahmen von Zellen in Suspensionskultur kein Unterschied zwischen der Fortbewegung von Pertussistoxin-behandelten und unbehandelten Zellen zu erkennen (eigene, unveröffentlichte Beobachtung).

Diese Ergebnisse deuten darauf hin, dass die SDF-1α vermittelte Signaltransduktion durch CXCR4 erst zu einem Zeitpunkt im *Homing*-Prozess wichtig ist, zu dem bereits eine Zellpolarität etabliert werden konnte, und die Zellen auch in der Lage sind zu wandern.

Zusammengefasst ergaben meine Analysen, dass die meisten Signalwege, die am *Homing* von HSZ/HVZ beteiligt sind, bereits im Prozess der Zellpolarisierung eine Rolle spielen. Da die Flotilline und ICAM-3/CD50 auch in morphologisch nicht polarisierten $CD34^+$ Zellen oftmals polar verteilt waren, besitzen diese Zellen, ähnlich wie es für Leukozyten gezeigt werden konnte, eine intrinsische Polarität, die der morphologischen Polarisierung vorausgeht (Rajendran et al., 2003). Die Ergebnisse zeigen auch, dass $CD34^+$ Zellen nur dann wandern, wenn sie eine polare Morphologie annehmen können.

Aufbauend auf den Analysemethoden und Ergebnissen zur Zellpolarität $CD34^+$ Zellen aus dem ersten Teil der vorliegenden Arbeit wurde durch die Untersuchung der Zellmorphologie und der intrinsischen Polarität der Einfluss einiger Signalwege und Mechanismen auf den Prozess der Zellpolarisierung analysiert. Die hier dargestellten Ergebnisse haben wir im Februar 2008 unter dem Titel **„Cell polarity is a prerequisite for migration and homing of primitive human hematopoietic cells“** (Beckmann et al., 2008) (siehe 10.6) bei Blood eingereicht.

5.4 Ausblick

In der vorliegenden Arbeit wurde untersucht, ob sich primitive hämatopoietische Zellen asymmetrisch teilen können. Mit der Identifizierung von CD53, CD62L, CD63 und CD71, die in 20-30% der untersuchten Mitosen asymmetrisch auf die beiden Tochterzellen verteilt werden, konnte diese Fragestellung klar beantwortet werden (Beckmann et al., 2007).

Für CD53, CD63 und CD71 wurde zudem eine Assoziation mit intrazellulärem Vesikeltransport beschrieben (Escola et al., 1998; Arribas und Cutler, 2000; Kobayashi et al., 2000; Qian et al., 2002). Dazu passt unsere Beobachtung, dass in Färbungen von $CD34^+$ Zellen mit den entsprechenden Antikörpern vesikuläre Strukturen angefärbt wurden (Beckmann et al., 2007).

Es bleibt zu zeigen, mit welchen endosomalen Strukturen CD53, CD63 und CD71 in mitotischen Zellen assoziiert sind. Die Spezifität des intrazellulären Vesikeltransports wird durch verschiedene Rab-GTPasen koordiniert, die die Vesikelbildung, ihr Andocken an bestimmte Zielmembranen und die Vesikelfusion regulieren (Schimmoller et al., 1998; Somsel Rodman und Wandinger-Ness, 2000; Jordens et al., 2005). So wird z.B. der Vesikeltransport von der Plasmamembran zu frühen Endosomen oder das Recyling von Vesikeln zur Plasmamembran von unterschiedlichen, membrangebundenen Rab-GTPasen kontrolliert. Doppelfärbungen an mitotischen $CD34^+$ Zellen mit Antikörpern gegen Rab5, Rab7 oder Rab11 könnten Aufschluss darüber geben, ob es sich bei den asymmetrisch verteilten Strukturen um frühe, späte bzw. um Recycling-Endosomen handelt. Diese Versuche können auch dazu beitragen zu klären, ob es einen Mechanismus gibt, der die Verteilung der Endosomen auf die Tochterzellen kontrolliert. In diesem Zusammenhang konnte bei Zellteilungen von *Drosophila melanogaster*

Flügelepithelzellen gezeigt werden, dass bestimmte Endosomen in der Mitose mit der Spindel assoziiert sind und so kontrolliert auf die Tochterzellen verteilt werden. Bei einer gestörten Verteilung dieser Endosomen wurden bei den Tochterzellen Veränderungen des Zellschicksals beobachtet (Bokel et al., 2006). Doppelfärbungen mit entsprechenden Antikörpern können Aufschluss darüber geben, ob die vesikelartigen Strukturen in mitotischen $CD34^+$ Zellen ebenfalls mit der Spindel oder mit anderen Zytoskelettkomponenten colokalisieren. Auch Versuche mit dominant-negativen bzw. konstitutiv-aktiven Rab-Varianten, die der Arbeitsgruppe zur Verfügung stehen, können zur Identifizierung von Mechanismen beitragen, die die Verteilung der vesikulären Strukturen organisieren. Hieran können sich gegebenenfalls Versuche zur Manipulation der Vesikelverteilung und deren Auswirkung auf das Zellschicksal anschließen.

Desweiteren stellt sich die Frage, inwieweit sich eine Überexpression oder eine RNAi-vermittelten Reduktion der Genexpression von CD53, CD62L, CD63 oder CD71 auf das Entwicklungspotenzial der Zellen auswirkt. Spielen diese Proteine möglicherweise eine ähnliche Rolle wie die Zellschicksalsdeterminanten in Modellorganismen oder gibt es andere, cosegregierende Faktoren, die einen entscheidenden Einfluss auf das Schicksal der Tochterzellen haben? Das Entwicklungspotenzial der manipulierten Zellen kann in Kurz- und Langzeitanalysen überprüft werden. Mit den entsprechenden Arbeiten wurde in der Arbeitsgruppe bereits begonnen, und sie sollen in einem gesonderten Projekt weitergeführt werden.

6 Literaturverzeichnis

Abkowitz, J. L., A. E. Robinson, S. Kale, M. W. Long and J. Chen (2003). Mobilization of hematopoietic stem cells during homeostasis and after cytokine exposure. Blood 102(4): 1249-53.

Aiuti, A., I. J. Webb, C. Bleul, T. Springer and J. C. Gutierrez-Ramos (1997). The chemokine SDF-1 is a chemoattractant for human CD34+ hematopoietic progenitor cells and provides a new mechanism to explain the mobilization of CD34+ progenitors to peripheral blood. J Exp Med 185(1): 111-20.

Akashi, K., D. Traver, T. Miyamoto and I. L. Weissman (2000). A clonogenic common myeloid progenitor that gives rise to all myeloid lineages. Nature 404(6774): 193-7.

Al-Hajj, M. (2007). Cancer stem cells and oncology therapeutics. Curr Opin Oncol 19(1): 61-4.

Alonso, M. A. and J. Millan (2001). The role of lipid rafts in signalling and membrane trafficking in T lymphocytes. J Cell Sci 114(Pt 22): 3957-65.

Andrews, R. and J. Ahringer (2007). Asymmetry of early endosome distribution in C. elegans embryos. PLoS ONE 2(6): e493.

Andrews, R. G., J. W. Singer and I. D. Bernstein (1986). Monoclonal antibody 12-8 recognizes a 115-kd molecule present on both unipotent and multipotent hematopoietic colony-forming cells and their precursors. Blood 67(3): 842-5.

Anversa, P., M. Rota, K. Urbanek, T. Hosoda, E. H. Sonnenblick, A. Leri, J. Kajstura and R. Bolli (2005). Myocardial aging--a stem cell problem. Basic Res Cardiol 100(6): 482-93.

Arribas, M. and D. F. Cutler (2000). Weibel-Palade body membrane proteins exhibit differential trafficking after exocytosis in endothelial cells. Traffic 1(10): 783-93.

Ballen, K. K. (2005). New trends in umbilical cord blood transplantation. Blood 105(10): 3786-92.

Beachy, P. A., S. S. Karhadkar and D. M. Berman (2004). Tissue repair and stem cell renewal in carcinogenesis. Nature 432(7015): 324-31.

Beckmann, J., S. Scheitza, P. Wernet, J. C. Fischer and B. Giebel (2007). Asymmetric cell division within the human hematopoietic stem and progenitor cell compartment: identification of asymmetrically segregating proteins. Blood 109(12): 5494-501.

Bell, J. J. and A. Bhandoola (2008). The earliest thymic progenitors for T cells possess myeloid lineage potential. Nature 452(7188): 764-7.

Bensinger, W. I., D. Buckner and G. Gahrton (1997). Allogeneic stem cell transplantation for multiple myeloma. Hematol Oncol Clin North Am 11(1): 147-57.

Betschinger, J. and J. A. Knoblich (2004). Dare to be different: asymmetric cell division in Drosophila, C. elegans and vertebrates. Curr Biol 14(16): R674-85.

Bhatia, M., D. Bonnet, U. Kapp, J. C. Wang, B. Murdoch and J. E. Dick (1997). Quantitative analysis reveals expansion of human hematopoietic repopulating cells after short-term ex vivo culture. J Exp Med 186(4): 619-24.

Bhatia, M., D. Bonnet, B. Murdoch, O. I. Gan and J. E. Dick (1998). A newly discovered class of human hematopoietic cells with SCID-repopulating activity. Nat Med 4(9): 1038-45.

Bhatia, M., J. C. Wang, U. Kapp, D. Bonnet and J. E. Dick (1997). Purification of primitive human hematopoietic cells capable of repopulating immune-deficient mice. Proc Natl Acad Sci U S A 94(10): 5320-5.

Bokel, C., A. Schwabedissen, E. Entchev, O. Renaud and M. Gonzalez-Gaitan (2006). Sara endosomes and the maintenance of Dpp signaling levels across mitosis. Science 314(5802): 1135-9.

Bonig, H., G. V. Priestley, L. M. Nilsson, Y. Jiang and T. Papayannopoulou (2004). PTX-sensitive signals in bone marrow homing of fetal and adult hematopoietic progenitor cells. Blood 104(8): 2299-306.

Boyd, L., S. Guo, D. Levitan, D. T. Stinchcomb and K. J. Kemphues (1996). PAR-2 is asymmetrically distributed and promotes association of P granules and PAR-1 with the cortex in C. elegans embryos. Development 122(10): 3075-84.

Brown, G., P. J. Hughes, R. H. Michell, A. G. Rolink and R. Ceredig (2007). The sequential determination model of hematopoiesis. Trends Immunol 28(10): 442-8.

Brummendorf, T. H., W. Dragowska, J. Zijlmans, G. Thornbury and P. M. Lansdorp (1998). Asymmetric cell divisions sustain long-term hematopoiesis from single-sorted human fetal liver cells. J Exp Med 188(6): 1117-24.

Buza-Vidas, N., S. Luc and S. E. Jacobsen (2007). Delineation of the earliest lineage commitment steps of haematopoietic stem cells: new developments, controversies and major challenges. Curr Opin Hematol 14(4): 315-21.

Calvi, L. M., G. B. Adams, K. W. Weibrecht, J. M. Weber, D. P. Olson, M. C. Knight, R. P. Martin, E. Schipani, P. Divieti, F. R. Bringhurst, L. A. Milner, H. M. Kronenberg and D. T. Scadden (2003). Osteoblastic cells regulate the haematopoietic stem cell niche. Nature 425(6960): 841-6.

Castro, B., S. Barolo, A. M. Bailey and J. W. Posakony (2005). Lateral inhibition in proneural clusters: cis-regulatory logic and default repression by Suppressor of Hairless. Development 132(15): 3333-44.

Cheng, P. C., A. Cherukuri, M. Dykstra, S. Malapati, T. Sproul, M. R. Chen and S. K. Pierce (2001). Floating the raft hypothesis: the roles of lipid rafts in B cell antigen receptor function. Semin Immunol 13(2): 107-14.

Chia, W., W. G. Somers and H. Wang (2008). Drosophila neuroblast asymmetric divisions: cell cycle regulators, asymmetric protein localization, and tumorigenesis. J Cell Biol 180(2): 267-72.

Chute, J. P. (2006). Stem cell homing. Curr Opin Hematol 13(6): 399-406.

Civin, C. I., L. C. Strauss, C. Brovall, M. J. Fackler, J. F. Schwartz and J. H. Shaper (1984). Antigenic analysis of hematopoiesis. III. A hematopoietic progenitor cell surface antigen defined by a monoclonal antibody raised against KG-1a cells. J Immunol 133(1): 157-65.

Conneally, E., J. Cashman, A. Petzer and C. Eaves (1997). Expansion in vitro of transplantable human cord blood stem cells demonstrated using a quantitative assay of their lympho-myeloid repopulating activity in nonobese diabetic-scid/scid mice. Proc Natl Acad Sci U S A 94(18): 9836-41.

Corbeil, D., K. Roper, C. A. Fargeas, A. Joester and W. B. Huttner (2001). Prominin: a story of cholesterol, plasma membrane protrusions and human pathology. Traffic 2(2): 82-91.

Corbeil, D., K. Roper, M. J. Hannah, A. Hellwig and W. B. Huttner (1999). Selective localization of the polytopic membrane protein prominin in microvilli of epithelial cells - a combination of apical sorting and retention in plasma membrane protrusions. J Cell Sci 112 (Pt 7): 1023-33.

Coulombel, L. (2004). Identification of hematopoietic stem/progenitor cells: strength and drawbacks of functional assays. Oncogene 23(43): 7210-22.

de Wynter, E. A., D. Buck, C. Hart, R. Heywood, L. H. Coutinho, A. Clayton, J. A. Rafferty, D. Burt, G. Guenechea, J. A. Bueren, D. Gagen, L. J. Fairbairn, B. I. Lord and N. G. Testa (1998). CD34+AC133+ cells isolated from cord blood are highly enriched in long-term culture-initiating cells, NOD/SCID-repopulating cells and dendritic cell progenitors. Stem Cells 16(6): 387-96.

Dick, J. E., M. Bhatia, O. Gan, U. Kapp and J. C. Wang (1997). Assay of human stem cells by repopulation of NOD/SCID mice. Stem Cells 15 Suppl 1: 199-203; discussion 204-7.

Doe, C. Q. and B. Bowerman (2001). Asymmetric cell division: fly neuroblast meets worm zygote. Curr Opin Cell Biol 13(1): 68-75.

Dooner, M., J. Cerny, G. Colvin, D. Demers, J. Pimentel, D. Greer, M. Abedi, C. McAuliffe and P. Quesenberry (2004). Homing and conversion of murine hematopoietic stem cells to lung. Blood Cells Mol Dis 32(1): 47-51.

Dzierzak, E. (2005). The emergence of definitive hematopoietic stem cells in the mammal. Curr Opin Hematol 12(3): 197-202.

Escola, J. M., M. J. Kleijmeer, W. Stoorvogel, J. M. Griffith, O. Yoshie and H. J. Geuze (1998). Selective enrichment of tetraspan proteins on the internal vesicles of multivesicular endosomes and on exosomes secreted by human B-lymphocytes. J Biol Chem 273(32): 20121-7.

Gallacher, L., B. Murdoch, D. M. Wu, F. N. Karanu, M. Keeney and M. Bhatia (2000). Isolation and characterization of human CD34(-)Lin(-) and CD34(+)Lin(-) hematopoietic stem cells using cell surface markers AC133 and CD7. Blood 95(9): 2813-20.

Giebel, B. (1999). The notch signaling pathway is required to specify muscle progenitor cells in Drosophila. Mech Dev 86(1-2): 137-45.

Giebel, B. and J. Beckmann (2007). Asymmetric cell divisions of human hematopoietic stem and progenitor cells meet endosomes. Cell Cycle 6(18): 2201-4.

Giebel, B., D. Corbeil, J. Beckmann, J. Hohn, D. Freund, K. Giesen, J. Fischer, G. Kogler and P. Wernet (2004). Segregation of lipid raft markers including CD133 in polarized human hematopoietic stem and progenitor cells. Blood 104(8): 2332-8.

Giebel, B., T. Zhang, J. Beckmann, J. Spanholtz, P. Wernet, A. D. Ho and M. Punzel (2006). Primitive human hematopoietic cells give rise to differentially specified daughter cells upon their initial cell division. Blood 107(5): 2146-52.

Gomez-Mouton, C., J. L. Abad, E. Mira, R. A. Lacalle, E. Gallardo, S. Jimenez-Baranda, I. Illa, A. Bernad, S. Manes and A. C. Martinez (2001). Segregation of leading-edge and uropod components into specific lipid rafts during T cell polarization. Proc Natl Acad Sci U S A 98(17): 9642-7.

Gottig, S., D. Mobest, B. Ruster, B. Grace, S. Winter, E. Seifried, J. Gille, T. Wieland and R. Henschler (2006). Role of the monomeric GTPase Rho in hematopoietic progenitor cell migration and transplantation. Eur J Immunol 36(1): 180-9.

Gu, Y., M. D. Filippi, J. A. Cancelas, J. E. Siefring, E. P. Williams, A. C. Jasti, C. E. Harris, A. W. Lee, R. Prabhakar, S. J. Atkinson, D. J. Kwiatkowski and D. A. Williams (2003). Hematopoietic cell regulation by Rac1 and Rac2 guanosine triphosphatases. Science 302(5644): 445-9.

Guo, S. and K. J. Kemphues (1995). par-1, a gene required for establishing polarity in C. elegans embryos, encodes a putative Ser/Thr kinase that is asymmetrically distributed. Cell 81(4): 611-20.

Handgretinger, R., P. R. Gordon, T. Leimig, X. Chen, H. J. Buhring, D. Niethammer and S. Kuci (2003). Biology and plasticity of CD133+ hematopoietic stem cells. Ann N Y Acad Sci 996: 141-51.

Hao, Q. L., A. J. Shah, F. T. Thiemann, E. M. Smogorzewska and G. M. Crooks (1995). A functional comparison of CD34 + CD38- cells in cord blood and bone marrow. Blood 86(10): 3745-53.

Hao, Q. L., E. M. Smogorzewska, L. W. Barsky and G. M. Crooks (1998). In vitro identification of single CD34+CD38- cells with both lymphoid and myeloid potential. Blood 91(11): 4145-51.

Hao, Q. L., F. T. Thiemann, D. Petersen, E. M. Smogorzewska and G. M. Crooks (1996). Extended long-term culture reveals a highly quiescent and primitive human hematopoietic progenitor population. Blood 88(9): 3306-13.

Hendrikx, P. J., C. M. Martens, A. Hagenbeek, J. F. Keij and J. W. Visser (1996). Homing of fluorescently labeled murine hematopoietic stem cells. Exp Hematol 24(2): 129-40.

Hoffstein, S. T., R. S. Friedman and G. Weissmann (1982). Degranulation, membrane addition, and shape change during chemotactic factor-induced aggregation of human neutrophils. J Cell Biol 95(1): 234-41.

Horn, P. A. and R. Blasczyk (2007). Severe combined immunodeficiency-repopulating cell assay may overestimate long-term repopulation ability. Stem Cells 25(12): 3271-2.

Horn, P. A., B. M. Thomasson, B. L. Wood, R. G. Andrews, J. C. Morris and H. P. Kiem (2003). Distinct hematopoietic stem/progenitor cell populations are responsible for repopulating NOD/SCID mice compared with nonhuman primates. Blood 102(13): 4329-35.

Huang, S., P. Law, K. Francis, B. O. Palsson and A. D. Ho (1999). Symmetry of initial cell divisions among primitive hematopoietic progenitors is independent of ontogenic age and regulatory molecules. Blood 94(8): 2595-604.

Iscove, N. N. and K. Nawa (1997). Hematopoietic stem cells expand during serial transplantation in vivo without apparent exhaustion. Curr Biol 7(10): 805-8.

Jan, Y. N. and L. Y. Jan (2001). Asymmetric cell division in the Drosophila nervous system. Nat Rev Neurosci 2(11): 772-9.

Jordens, I., M. Marsman, C. Kuijl and J. Neefjes (2005). Rab proteins, connecting transport and vesicle fusion. Traffic 6(12): 1070-7.

Kai, T. and A. Spradling (2003). An empty Drosophila stem cell niche reactivates the proliferation of ectopic cells. Proc Natl Acad Sci U S A 100(8): 4633-8.

Kamel-Reid, S. and J. E. Dick (1988). Engraftment of immune-deficient mice with human hematopoietic stem cells. Science 242(4886): 1706-9.

Kamminga, L. M. and G. de Haan (2006). Cellular memory and hematopoietic stem cell aging. Stem Cells 24(5): 1143-9.

Katz, F. E., R. Tindle, D. R. Sutherland and M. F. Greaves (1985). Identification of a membrane glycoprotein associated with haemopoietic progenitor cells. Leuk Res 9(2): 191-8.

Kawabata, K., M. Ujikawa, T. Egawa, H. Kawamoto, K. Tachibana, H. Iizasa, Y. Katsura, T. Kishimoto and T. Nagasawa (1999). A cell-autonomous requirement for CXCR4 in long-term lymphoid and myeloid reconstitution. Proc Natl Acad Sci U S A 96(10): 5663-7.

Keller, H. U., A. Naef and A. Zimmermann (1984). Effects of colchicine, vinblastine and nocodazole on polarity, motility, chemotaxis and cAMP levels of human polymorphonuclear leukocytes. Exp Cell Res 153(1): 173-85.

Kemphues, K. J., J. R. Priess, D. G. Morton and N. S. Cheng (1988). Identification of genes required for cytoplasmic localization in early C. elegans embryos. Cell 52(3): 311-20.

Kiel, M. J., O. H. Yilmaz, T. Iwashita, O. H. Yilmaz, C. Terhorst and S. J. Morrison (2005). SLAM family receptors distinguish hematopoietic stem and progenitor cells and reveal endothelial niches for stem cells. Cell 121(7): 1109-21.

Kierney, P. C. and K. Dorshkind (1987). B lymphocyte precursors and myeloid progenitors survive in diffusion chamber cultures but B cell differentiation requires close association with stromal cells. Blood 70(5): 1418-24.

Kirilly, D. and T. Xie (2007). The Drosophila ovary: an active stem cell community. Cell Res 17(1): 15-25.

Kobayashi, M., J. H. Laver, S. D. Lyman, T. Kato, H. Miyazaki and M. Ogawa (1997). Thrombopoietin, steel factor and the ligand for flt3/flk2 interact to stimulate the proliferation of human hematopoietic progenitors in culture. Int J Hematol 66(4): 423-34.

Kobayashi, T., U. M. Vischer, C. Rosnoblet, C. Lebrand, M. Lindsay, R. G. Parton, E. K. Kruithof and J. Gruenberg (2000). The tetraspanin CD63/lamp3 cycles between endocytic and secretory compartments in human endothelial cells. Mol Biol Cell 11(5): 1829-43.

Kollet, O., A. Spiegel, A. Peled, I. Petit, T. Byk, R. Hershkoviz, E. Guetta, G. Barkai, A. Nagler and T. Lapidot (2001). Rapid and efficient homing of human CD34(+)CD38(-/low)CXCR4(+) stem and progenitor cells to the bone marrow and spleen of NOD/SCID and NOD/SCID/B2m(null) mice. Blood 97(10): 3283-91.

Kondo, M., I. L. Weissman and K. Akashi (1997). Identification of clonogenic common lymphoid progenitors in mouse bone marrow. Cell 91(5): 661-72.

Korbling, M. and P. Anderlini (2001). Peripheral blood stem cell versus bone marrow allotransplantation: does the source of hematopoietic stem cells matter? Blood 98(10): 2900-8.

Krause, D. S., N. D. Theise, M. I. Collector, O. Henegariu, S. Hwang, R. Gardner, S. Neutzel and S. J. Sharkis (2001). Multi-organ, multi-lineage engraftment by a single bone marrow-derived stem cell. Cell 105(3): 369-77.

Kuci, S., J. T. Wessels, H. J. Buhring, K. Schilbach, M. Schumm, G. Seitz, J. Loffler, P. Bader, P. G. Schlegel, D. Niethammer and R. Handgretinger (2003). Identification of a novel class of human adherent CD34- stem cells that give rise to SCID-repopulating cells. Blood 101(3): 869-76.

Lansdorp, P. M. and W. Dragowska (1993). Maintenance of hematopoiesis in serum-free bone marrow cultures involves sequential recruitment of quiescent progenitors. Exp Hematol 21(10): 1321-7.

Lapidot, T., A. Dar and O. Kollet (2005). How do stem cells find their way home? Blood 106(6): 1901-10.

Lapidot, T., F. Pflumio, M. Doedens, B. Murdoch, D. E. Williams and J. E. Dick (1992). Cytokine stimulation of multilineage hematopoiesis from immature human cells engrafted in SCID mice. Science 255(5048): 1137-41.

Larochelle, A., J. Vormoor, H. Hanenberg, J. C. Wang, M. Bhatia, T. Lapidot, T. Moritz, B. Murdoch, X. L. Xiao, I. Kato, D. A. Williams and J. E. Dick (1996). Identification of primitive human hematopoietic cells capable of repopulating NOD/SCID mouse bone marrow: implications for gene therapy. Nat Med 2(12): 1329-37.

Lauffenburger, D. A. and A. F. Horwitz (1996). Cell migration: a physically integrated molecular process. Cell 84(3): 359-69.

Leary, A. G., L. C. Strauss, C. I. Civin and M. Ogawa (1985). Disparate differentiation in hemopoietic colonies derived from human paired progenitors. Blood 66(2): 327-32.

LeBien, T. W. (1998). B-cell lymphopoiesis in mouse and man. Curr Opin Immunol 10(2): 188-95.

Lin, H. (2002). The stem-cell niche theory: lessons from flies. Nat Rev Genet 3(12): 931-40.

Luens, K. M., M. A. Travis, B. P. Chen, B. L. Hill, R. Scollay and L. J. Murray (1998). Thrombopoietin, kit ligand, and flk2/flt3 ligand together induce increased numbers of primitive hematopoietic progenitors from human CD34+Thy-1+Lin- cells with preserved ability to engraft SCID-hu bone. Blood 91(4): 1206-15.

Martinez Arias, A., V. Zecchini and K. Brennan (2002). CSL-independent Notch signalling: a checkpoint in cell fate decisions during development? Curr Opin Genet Dev 12(5): 524-33.

Matsumoto, K., K. Yasui, N. Yamashita, Y. Horie, T. Yamada, Y. Tani, H. Shibata and T. Nakano (2000). In vitro proliferation potential of AC133 positive cells in peripheral blood. Stem Cells 18(3): 196-203.

Mayani, H., W. Dragowska and P. M. Lansdorp (1993). Lineage commitment in human hemopoiesis involves asymmetric cell division of multipotent progenitors and does not appear to be influenced by cytokines. J Cell Physiol 157(3): 579-86.

McGlave, P., C. Verfaillie and J. Miller (1994). Interaction of primitive human myeloid and lymphoid progenitors with the marrow microenvironment. Blood Cells 20(1): 121-6; discussion 126-8.

Mikkola, H. K., C. Gekas, S. H. Orkin and F. Dieterlen-Lievre (2005). Placenta as a site for hematopoietic stem cell development. Exp Hematol 33(9): 1048-54.

Millan, J., M. C. Montoya, D. Sancho, F. Sanchez-Madrid and M. A. Alonso (2002). Lipid rafts mediate biosynthetic transport to the T lymphocyte uropod subdomain and are necessary for uropod integrity and function. Blood 99(3): 978-84.

Miller, J. S., K. A. Alley and P. McGlave (1994). Differentiation of natural killer (NK) cells from human primitive marrow progenitors in a stroma-based long-term culture system: identification of a CD34+7+ NK progenitor. Blood 83(9): 2594-601.

Miller, J. S., V. McCullar, M. Punzel, I. R. Lemischka and K. A. Moore (1999). Single adult human CD34(+)/Lin-/CD38(-) progenitors give rise to natural killer cells, B-lineage cells, dendritic cells, and myeloid cells. Blood 93(1): 96-106.

Milner, L. A. and A. Bigas (1999). Notch as a mediator of cell fate determination in hematopoiesis: evidence and speculation. Blood 93(8): 2431-48.

Miraglia, S., W. Godfrey, A. H. Yin, K. Atkins, R. Warnke, J. T. Holden, R. A. Bray, E. K. Waller and D. W. Buck (1997). A novel five-transmembrane hematopoietic stem cell antigen: isolation, characterization, and molecular cloning. Blood 90(12): 5013-21.

Mizrak, D., M. Brittan and M. R. Alison (2008). CD133: molecule of the moment. J Pathol 214(1): 3-9.

Moore, K. A., H. Ema and I. R. Lemischka (1997). In vitro maintenance of highly purified, transplantable hematopoietic stem cells. Blood 89(12): 4337-47.

Nakamura, Y., K. Ando, J. Chargui, H. Kawada, T. Sato, T. Tsuji, T. Hotta and S. Kato (1999). Ex vivo generation of CD34(+) cells from CD34(-) hematopoietic cells. Blood 94(12): 4053-9.

Nervi, B., D. C. Link and J. F. DiPersio (2006). Cytokines and hematopoietic stem cell mobilization. J Cell Biochem 99(3): 690-705.

Niggli, V. (2003). Microtubule-disruption-induced and chemotactic-peptide-induced migration of human neutrophils: implications for differential sets of signalling pathways. J Cell Sci 116(Pt 5): 813-22.

Nishimura, E. K., S. R. Granter and D. E. Fisher (2005). Mechanisms of hair graying: incomplete melanocyte stem cell maintenance in the niche. Science 307(5710): 720-4.

Nolta, J. A., F. T. Thiemann, J. Arakawa-Hoyt, M. A. Dao, L. W. Barsky, K. A. Moore, I. R. Lemischka and G. M. Crooks (2002). The AFT024 stromal cell line supports long-term ex vivo maintenance of engrafting multipotent human hematopoietic progenitors. Leukemia 16(3): 352-61.

Ohmizono, Y., H. Sakabe, T. Kimura, S. Tanimukai, T. Matsumura, H. Miyazaki, S. D. Lyman and Y. Sonoda (1997). Thrombopoietin augments ex vivo expansion of human cord blood-derived hematopoietic progenitors in combination with stem cell factor and flt3 ligand. Leukemia 11(4): 524-30.

Otegui, M. S., K. J. Verbrugghe and A. R. Skop (2005). Midbodies and phragmoplasts: analogous structures involved in cytokinesis. Trends Cell Biol 15(8): 404-13.

Peled, A., I. Petit, O. Kollet, M. Magid, T. Ponomaryov, T. Byk, A. Nagler, H. Ben-Hur, A. Many, L. Shultz, O. Lider, R. Alon, D. Zipori and T. Lapidot (1999). Dependence of human stem cell engraftment and repopulation of NOD/SCID mice on CXCR4. Science 283(5403): 845-8.

Petit, I., P. Goichberg, A. Spiegel, A. Peled, C. Brodie, R. Seger, A. Nagler, R. Alon and T. Lapidot (2005). Atypical PKC-zeta regulates SDF-1-mediated migration and development of human CD34+ progenitor cells. J Clin Invest 115(1): 168-76.

Petzer, A. L., D. E. Hogge, P. M. Landsdorp, D. S. Reid and C. J. Eaves (1996). Self-renewal of primitive human hematopoietic cells (long-term-culture-initiating cells) in vitro and their expansion in defined medium. Proc Natl Acad Sci U S A 93(4): 1470-4.

Punzel, M. and A. D. Ho (2001). Divisional history and pluripotency of human hematopoietic stem cells. Ann N Y Acad Sci 938: 72-81; discussion 81-2.

Punzel, M., K. A. Moore, I. R. Lemischka and C. M. Verfaillie (1999). The type of stromal feeder used in limiting dilution assays influences frequency and maintenance assessment of human long-term culture initiating cells. Leukemia 13(1): 92-7.

Punzel, M., S. D. Wissink, J. S. Miller, K. A. Moore, I. R. Lemischka and C. M. Verfaillie (1999). The myeloid-lymphoid initiating cell (ML-IC) assay assesses the fate of multipotent human progenitors in vitro. Blood 93(11): 3750-6.

Punzel, M., T. Zhang, D. Liu, V. Eckstein and A. D. Ho (2002). Functional analysis of initial cell divisions defines the subsequent fate of individual human CD34(+)CD38(-) cells. Exp Hematol 30(5): 464-72.

Qian, Z. M., H. Li, H. Sun and K. Ho (2002). Targeted drug delivery via the transferrin receptor-mediated endocytosis pathway. Pharmacol Rev 54(4): 561-87.

Rajendran, L., M. Masilamani, S. Solomon, R. Tikkanen, C. A. Stuermer, H. Plattner and H. Illges (2003). Asymmetric localization of flotillins/reggies in preassembled platforms confers inherent polarity to hematopoietic cells. Proc Natl Acad Sci U S A 100(14): 8241-6.

Rando, T. A. (2006). Stem cells, ageing and the quest for immortality. Nature 441(7097): 1080-6.

Reya, T., S. J. Morrison, M. F. Clarke and I. L. Weissman (2001). Stem cells, cancer, and cancer stem cells. Nature 414(6859): 105-11.

Ringden, O. and K. Le Blanc (2005). Allogeneic hematopoietic stem cell transplantation: state of the art and new perspectives. Apmis 113(11-12): 813-30.

Rocha, V., J. Cornish, E. L. Sievers, A. Filipovich, F. Locatelli, C. Peters, M. Remberger, G. Michel, W. Arcese, S. Dallorso, K. Tiedemann, A. Busca, K. W. Chan, S. Kato, J. Ortega, M. Vowels, A. Zander, G. Souillet, A. Oakill, A. Woolfrey, A. L. Pay, A. Green, F. Garnier, I. Ionescu, P. Wernet, G. Sirchia, P. Rubinstein, S. Chevret and E. Gluckman (2001). Comparison of outcomes of unrelated bone marrow and umbilical cord blood transplants in children with acute leukemia. Blood 97(10): 2962-71.

Ruzankina, Y. and E. J. Brown (2007). Relationships between stem cell exhaustion, tumour suppression and ageing. Br J Cancer 97(9): 1189-93.

Sanchez-Madrid, F. and M. A. del Pozo (1999). Leukocyte polarization in cell migration and immune interactions. Embo J 18(3): 501-11.

Schimmoller, F., I. Simon and S. R. Pfeffer (1998). Rab GTPases, directors of vesicle docking. J Biol Chem 273(35): 22161-4.

Sedwick, C. E. and A. Altman (2002). Ordered just so: lipid rafts and lymphocyte function. Sci STKE 2002(122): RE2.

Shen, Q. and S. Temple (2002). Creating asymmetric cell divisions by skewing endocytosis. Sci STKE 2002(162): PE52.

Shih, C. C., M. C. Hu, J. Hu, J. Medeiros and S. J. Forman (1999). Long-term ex vivo maintenance and expansion of transplantable human hematopoietic stem cells. Blood 94(5): 1623-36.

Shimizu, Y., M. Ogawa, M. Kobayashi, G. Almeida-Porada and E. D. Zanjani (1998). Engraftment of cultured human hematopoietic cells in sheep. Blood 91(10): 3688-92.

Shizuru, J. A., R. S. Negrin and I. L. Weissman (2005). Hematopoietic stem and progenitor cells: clinical and preclinical regeneration of the hematolymphoid system. Annu Rev Med 56: 509-38.

Slezak, S. E. and P. K. Horan (1989). Fluorescent in vivo tracking of hematopoietic cells. Part I. Technical considerations. Blood 74(6): 2172-7.

Somsel Rodman, J. and A. Wandinger-Ness (2000). Rab GTPases coordinate endocytosis. J Cell Sci 113 Pt 2: 183-92.

Spradling, A., D. Drummond-Barbosa and T. Kai (2001). Stem cells find their niche. Nature 414(6859): 98-104.

Suda, J., T. Suda and M. Ogawa (1984). Analysis of differentiation of mouse hemopoietic stem cells in culture by sequential replating of paired progenitors. Blood 64(2): 393-9.

Suda, T., J. Suda and M. Ogawa (1984). Disparate differentiation in mouse hemopoietic colonies derived from paired progenitors. Proc Natl Acad Sci U S A 81(8): 2520-4.

Sutherland, H. J., P. M. Lansdorp, D. H. Henkelman, A. C. Eaves and C. J. Eaves (1990). Functional characterization of individual human hematopoietic stem cells cultured at limiting dilution on supportive marrow stromal layers. Proc Natl Acad Sci U S A 87(9): 3584-8.

Suzuki, A. and S. Ohno (2006). The PAR-aPKC system: lessons in polarity. J Cell Sci 119(Pt 6): 979-87.

Suzuki, T. and S. Chiba (2005). Notch signaling in hematopoietic stem cells. Int J Hematol 82(4): 285-94.

Taupin, P. and F. H. Gage (2002). Adult neurogenesis and neural stem cells of the central nervous system in mammals. J Neurosci Res 69(6): 745-9.

Terstappen, L. W., S. Huang, M. Safford, P. M. Lansdorp and M. R. Loken (1991). Sequential generations of hematopoietic colonies derived from single nonlineage-committed CD34+CD38- progenitor cells. Blood 77(6): 1218-27.

Thiemann, F. T., K. A. Moore, E. M. Smogorzewska, I. R. Lemischka and G. M. Crooks (1998). The murine stromal cell line AFT024 acts specifically on human CD34+CD38- progenitors to maintain primitive function and immunophenotype in vitro. Exp Hematol 26(7): 612-9.

Traycoff, C. M., S. T. Kosak, S. Grigsby and E. F. Srour (1995). Evaluation of ex vivo expansion potential of cord blood and bone marrow hematopoietic progenitor cells using cell tracking and limiting dilution analysis. Blood 85(8): 2059-68.

Viola, A., S. Schroeder, Y. Sakakibara and A. Lanzavecchia (1999). T lymphocyte costimulation mediated by reorganization of membrane microdomains. Science 283(5402): 680-2.

von Levetzow, G., J. Spanholtz, J. Beckmann, J. Fischer, G. Kogler, P. Wernet, M. Punzel and B. Giebel (2006). Nucleofection, an efficient nonviral method to transfer genes into human hematopoietic stem and progenitor cells. Stem Cells Dev 15(2): 278-85.

Wada, H., K. Masuda, R. Satoh, K. Kakugawa, T. Ikawa, Y. Katsura and H. Kawamoto (2008). Adult T-cell progenitors retain myeloid potential. Nature 452(7188): 768-72.

Wagers, A. J. and I. M. Conboy (2005). Cellular and molecular signatures of muscle regeneration: current concepts and controversies in adult myogenesis. Cell 122(5): 659-67.

Wang, J. F., I. W. Park and J. E. Groopman (2000). Stromal cell-derived factor-1alpha stimulates tyrosine phosphorylation of multiple focal adhesion proteins and induces migration of hematopoietic progenitor cells: roles of phosphoinositide-3 kinase and protein kinase C. Blood 95(8): 2505-13.

Watts, J. L., B. Etemad-Moghadam, S. Guo, L. Boyd, B. W. Draper, C. C. Mello, J. R. Priess and K. J. Kemphues (1996). par-6, a gene involved in the establishment of asymmetry in early C. elegans embryos, mediates the asymmetric localization of PAR-3. Development 122(10): 3133-40.

Weissman, I. L. (2000). Translating stem and progenitor cell biology to the clinic: barriers and opportunities. Science 287(5457): 1442-6.

Whetton, A. D., Y. Lu, A. Pierce, L. Carney and E. Spooncer (2003). Lysophospholipids synergistically promote primitive hematopoietic cell chemotaxis via a mechanism involving Vav 1. Blood 102(8): 2798-802.

Whitlock, C. A. and O. N. Witte (1982). Long-term culture of B lymphocytes and their precursors from murine bone marrow. Proc Natl Acad Sci U S A 79(11): 3608-12.

Wodarz, A. (2005). Molecular control of cell polarity and asymmetric cell division in Drosophila neuroblasts. Curr Opin Cell Biol 17(5): 475-81.

Wodarz, A. and W. B. Huttner (2003). Asymmetric cell division during neurogenesis in Drosophila and vertebrates. Mech Dev 120(11): 1297-309.

Wright, D. E., S. H. Cheshier, A. J. Wagers, T. D. Randall, J. L. Christensen and I. L. Weissman (2001). Cyclophosphamide/granulocyte colony-stimulating factor causes selective mobilization of bone marrow hematopoietic stem cells into the blood after M phase of the cell cycle. Blood 97(8): 2278-85.

Xie, T., E. Kawase, D. Kirilly and M. D. Wong (2005). Intimate relationships with their neighbors: tales of stem cells in Drosophila reproductive systems. Dev Dyn 232(3): 775-90.

Xie, T. and A. C. Spradling (2000). A niche maintaining germ line stem cells in the Drosophila ovary. Science 290(5490): 328-30.

Yang, L., L. Wang, H. Geiger, J. A. Cancelas, J. Mo and Y. Zheng (2007). Rho GTPase Cdc42 coordinates hematopoietic stem cell quiescence and niche interaction in the bone marrow. Proc Natl Acad Sci U S A 104(12): 5091-6.

Yin, A. H., S. Miraglia, E. D. Zanjani, G. Almeida-Porada, M. Ogawa, A. G. Leary, J. Olweus, J. Kearney and D. W. Buck (1997). AC133, a novel marker for human hematopoietic stem and progenitor cells. Blood 90(12): 5002-12.

Yu, F., C. T. Kuo and Y. N. Jan (2006). Drosophila neuroblast asymmetric cell division: recent advances and implications for stem cell biology. Neuron 51(1): 13-20.

Zanjani, E. D., G. Almeida-Porada, A. G. Livingston, A. W. Flake and M. Ogawa (1998). Human bone marrow CD34- cells engraft in vivo and undergo multilineage expression that includes giving rise to CD34+ cells. Exp Hematol 26(4): 353-60.

Zhang, J., C. Niu, L. Ye, H. Huang, X. He, W. G. Tong, J. Ross, J. Haug, T. Johnson, J. Q. Feng, S. Harris, L. M. Wiedemann, Y. Mishina and L. Li (2003). Identification of the haematopoietic stem cell niche and control of the niche size. Nature 425(6960): 836-41.

7 Lebenslauf

Name:	Julia Beckmann
Geburtsdatum:	8. Dezember 1976
Geburtsort:	Bergisch Gladbach
Staatsangehörigkeit:	deutsch

Ausbildung:

1983 – 1987	Evangelische Grundschule „Am Broich“, Bergisch Gladbach
1987 – 1996	Dietrich–Bonhoeffer–Gymnasium, Bergisch Gladbach
1996 – 2001	Studium der Biologie an der Philipps–Universität Marburg
11/2000 – 8/2001	Diplomarbeit im Institut für Virologie der Universität zu Köln zum Thema „Charakterisierung der Interaktion der E6–Proteine von Papillomviren mit dem zellulären Koaktivator p300“
9/2001 – 3/2003	Wissenschaftliche Mitarbeiterin im Institut für Hämatologie und Transfusionsmedizin der Rheinischen Friedrich–Wilhelms–Universität Bonn
seit 6/2003	Wissenschaftliche Mitarbeiterin im Institut für Transplantationsdiagnostik und Zelltherapeutika der Heinrich–Heine–Universität Düsseldorf, Arbeitsgruppe Dr. Bernd Giebel

8 Publikationen

8.1 Artikel

Bernd Giebel, Denis Corbeil, Julia Beckmann, Johannes Höhn, Daniel Freund, Kay Giesen, Johannes Fischer, Gesine Kögler and Peter Wernet: Segregation of lipid raft markers including CD133 in polarized human hematopoietic stem and progenitor cells. *Blood* Oct 15; 104: 2332 – 2338, 2004.

Bernd Giebel, Tao Zhang, Julia Beckmann, Jan Spanholtz, Peter Wernet, Anthony D. Ho and Michael Punzel: Primitive human hematopoietic cells give rise to differentially specified daughter cells upon their initial cell division. *Blood* Mar 1; 107: 2146 – 2152, 2006.

Gregor von Levetzow, Jan Spanholtz, Julia Beckmann, Johannes Fischer, Gesine Kögler, Peter Wernet, Michael Punzel and Bernd Giebel: Nucleofection, an efficient nonviral method to transfer genes into human hematopoietic stem and progenitor cells. *Stem Cells Dev* Apr; 15(2): 278-85, 2006.

Andreas Müller–Schiffmann, Julia Beckmann and Gertrud Steger: The E6 Protein of the Cutaneous Human Papillomavirus Type 8 Can Stimulate the Viral Early and Late Promoters by Distinct Mechanisms. *Journal of Virology* Sep; 80: 8718 – 8728, 2006.

Julia Beckmann, Sebastian Scheitza, Johannes Fischer, Peter Wernet, Michael Punzel and Bernd Giebel: Asymmetric cell division within the primitive human hematopoietic stem and progenitor cell compartment: Identification of asymmetrically segregating proteins. *Blood* Jun 15; 109 (12): 5494-501, 2007.

Bernd Giebel und Julia Beckmann: Asymmetric cell divisions of human hematopoietic stem and progenitor cells meet endosomes. *Cell Cycle* Jul; 6(18): 2201-4, 2007.

Julia Beckmann, Lawrence Rajendran and Bernd Giebel: Cell polarity is a prerequisite for migration and homing of primitive human hematopoietic cells. Eingereicht bei *Blood*, Februar 2008.

Lawrence Rajendran, Julia Beckmann, Astrid Magenau, Katharina Gaus, Antonella Viola, Bernd Giebel and Harald Illges: The novel endocytic proteins, flotillins are involved in the polarization of primitive and mature hematopoietic cells. Eingereicht bei *Blood*, März 2008.

8.2 Poster

Bernd Giebel, Julia Beckmann, Gregor von Levetzow, Peter Wernet: Mechanisms of asymmetric cell division of human stem and progenitor cells. Jahrestagung des DFG-Schwerpunktprogramms SPP1109, November 2004, Potsdam.

Bernd Giebel, Julia Beckmann, Jan Spanholtz, Gregor von Levetzow, Gesine Kögler und Peter Wernet: Asymmetric cell division of primitive hematopoietic stem and progenitor cells. Keystone Meeting, Februar 2005, Banff, Kanada.

Julia Beckmann, Peter Wernet und Bernd Giebel: Mechanismen der Polarisierung von hämatopoietischen CD34-positiven Zellen aus Nabelschnurblut. Tag der Nachwuchswissenschaftler 2005, Heinrich-Heine-Universität, Düsseldorf.

Jan Spanholtz, Gregor von Levetzow, Julia Beckmann, Peter Wernet und Bernd Giebel: Die Bedeutung der Zellschicksalsdeterminante NUMB für die Entwicklung humaner hämatopoietischer Stamm- und Vorläuferzellen. Tag der Nachwuchswissenschaftler 2005, Heinrich-Heine-Universität, Düsseldorf.

Julia Beckmann, Sebastian Scheitza, Johannes Fischer, Peter Wernet, Michael Punzel and Bernd Giebel: Asymmetric cell division of primitive human hematopoietic cells. 3rd International Meeting Stem Cell Network North Rhine Westphalia, Mai 2006, Münster.

Jan Spanholtz, Gregor von Levetzow, Ron-Patrick Cadeddu, Julia Beckmann, Sebastian Scheitza, Peter Wernet, Michael Punzel und Bernd Giebel: NUMB, a cell fate determinant known from *Drosophila melanogaster*, inhibits maintenance of primitive human hematopoietic cell fate. 3rd International Meeting Stem Cell Network North Rhine Westphalia, Mai 2006, Münster.

Julia Beckmann, Sebastian Scheitza, Peter Wernet, Johannes Fischer und Bernd Giebel: Asymmetric cell division of primitive human hematopoietic cells. Jahrestagung des DFG-Schwerpunktprogramms SPP1109, September 2006, Dresden.

Jan Spanholtz, Gregor von Levetzow, Ron-Patrick Caddedu, Julia Beckmann, Sebastian Scheitza, Peter Wernet, Michael Punzel und Bernd Giebel: Numb, a cell fate determinant known from *Drosophila melanogaster*, inhibits maintenance of primitive human hematopoietic cell fates. Jahrestagung des DFG-Schwerpunktprogramms SPP1109, September 2006, Dresden.

Ron-Patrick Cadeddu, Julia Beckmann, Gregor von Levetzow, Jan Spanholtz and Bernd Giebel: Mechanisms of Cell Polarity of Human Hematopoietic Stem and Progenitor Cells. GfE-Summerschool, September 2006, Günzburg.

Julia Beckmann, Sebastian Scheitza, Johannes Fischer, Peter Wernet, Michael Punzel and Bernd Giebel: Asymmetric cell division of primitive human hematopoietic cells.1st Congress of the German Society for Stem Cell Research, November 2006, Köln.

Gregor von Levetzow, Jan Spanholtz, Ron-Patrick Cadeddu, Julia Beckmann, Sebastian Scheitza, Peter Wernet, Michael Punzel und Bernd Giebel: NUMB, a cell fate determinant known from *Drosophila melanogaster*, inhibits maintenance of primitive human hematopoietic cell fate. 1st Congress of the German Society for Stem Cell Research, November 2006, Köln.

Julia Beckmann, Sebastian Scheitza, Johannes Fischer, and Bernd Giebel: Asymmetric cell division of primitive human hematopoietic cells. 4th International Meeting Stem Cell Network North Rhine Westphalia, Oktober 2007, Düsseldorf.

Gregor von Levetzow, Jan Spanholtz, Julia Beckmann, Johannes Fischer, Michael Punzel, Bernd Giebel: Numb, a cell fate determinant known from *Drosophila melanogaster*, inhibits maintenance of primitive human hematopoietic cell fates. 4th International Meeting Stem Cell Network North Rhine Westphalia, Oktober 2007, Düsseldorf.

Julia Beckmann, Sebastian Scheitza, Johannes Fischer, and Bernd Giebel: Asymmetric cell division of primitive human hematopoietic cells. EuroStells Meeting, Januar 2008, Barcelona.

9 Danksagung

Für die bereitwillige Übernahme der Betreuung meiner Arbeit bedanke ich mich herzlich bei PD. Dr. M. Uhrberg und bei Prof. J.H. Hegemann.

Vielen Dank an Dr. Bernd Giebel für die unermüdliche Anteilnahme am Fortschritt und die Hilfe bei Problemlösungen während meiner Arbeit, die hohe Diskussionsbereitschaft sowie für die für die Kölner Unterstützung in der „verbotenen“ Stadt und die Kreativität im Umgang mit zahlreichen Redewendungen.

Ich möchte mich besonders bei allen bedanken, die mich während dieser langen Zeit unterstützt und aufgebaut haben. Meine Eltern und meine Schwestern haben mir in organisatorischen und vor allem in seelischen und moralischen Fragen unermüdlich mit Rat und Tat zur Seite gestanden - ihr habt den goldenen Telefonhörer verdient! Danke!

Ein besonderer Dank geht an Jan und Rebecca Spanholtz, die mir in experimentellen Fragen, bei der Durchführung, durch immer aufnahmebereite Ohren, zahlreiche wunderbare Abende (nicht immer) laotischer und sportlicher Art, Abhilfe bei Minusbeträgen auf dem Lachkonto und bei Folgeschäden aufgrund von stark erhöhter TVC-Aktvität sowie mit der Notfallration auf Rädern sehr geholfen haben. Anika, auch dir vielen Dank für die Unterstützung, die Lach- (und Sach-) Geschichten und das Verständnis ohne Worte! Bei Frau Lange bedanke ich mich für den steten Austausch der Erfahrungen, die Organisation von zahlreichen unterhaltsamen Abendprogrammen und viele lustige Momente. Silke und Christian danke ich für die geduldige Anteilnahme und die Motivation sowie für kommode Wochenenden, schöne gemeinsame Reisen, irre Staubkörner und andere total vertwistete Untiefen.

Nicht zuletzt möchte ich den anderen Mitgliedern der AG Giebel danken: Gregor- für stetiges Interesse an meiner Arbeit (…UUUUUUNNNNDDD????), viele interessante Wortschöpfungen und Unterhaltungen über den Tellerrand hinaus, Svenja für die leider zu kurze Verschönerung der Anfangszeit im ITZ und zahlreiche erstaunliche Geschichten, Sebastian - für viele Mitosen, André für seine konstant gute Laune, beruhigende Wirkung und Hilfsbereitschaft, Simon - für so manche passende ironische Bemerkung. Liska und Esther: danke, dass endlich

mal wieder ein paar Mädels in der dritten Etage dabei sind! Auch an Bärbel geht ein Dank für die animierte Steinlaus und weitere gemeinsame Lach-Momente!

Sandra, Jin, Julia, Kathrin, Theodora, Martina und den restlichen Mitgliedern der NWG Uhrberg danke ich für Asyl und offene Ohren, allen GL-Mädels für viel Geduld und Verständnis, wenn die Experimente doch mal wieder länger gedauert haben, Lawrence für viele anregende Diskussionen und den ansteckenden Enthusiasmus. Dr. J. Fischer danke ich für die Akuthilfe am FC-500 und am Sorter, Kerstin und Ute aus der Apherese für Geduld und Erbarmen.

Beim Mittwochabend-Team, besonders Dirk und Kerstin und natürlich Lars bedanke ich mich dafür, dass sie mir den Rücken für den Endspurt gestärkt haben.

Und natürlich ein Riiiiesendankeschön an den Vogel, für deine Anteilnahme an meiner Arbeit, die geduldige Unterstützung von nah und fern, das Ertragen des ewigen „ich komme doch später…“ und von vielen weiteren Einschränkungen der gemeinsamen Zeit, den stetigen Wiederaufbau, deine Kochkünste (HM!), ungewöhnliche Reiseziele und unzählige lustige und erheiternde Momente. Danke für alles!!!

10 Anhang

10.1 Segregation of lipid raft markers including CD133 in polarized human hematopoietic stem and progenitor cells (Giebel et al., 2004)

Segregation of lipid raft markers including CD133 in polarized human hematopoietic stem and progenitor cells

Bernd Giebel, Denis Corbeil, Julia Beckmann, Johannes Höhn, Daniel Freund, Kay Giesen, Johannes Fischer, Gesine Kögler, and Peter Wernet

During ontogenesis and the entire adult life hematopoietic stem and progenitor cells have the capability to migrate. In comparison to the process of peripheral leukocyte migration in inflammatory responses, the molecular and cellular mechanisms governing the migration of these cells remain poorly understood. A common feature of migrating cells is that they need to become polarized before they migrate. Here we have investigated the issue of cell polarity of hematopoietic stem/progenitor cells in detail. We found that human CD34$^+$ hematopoietic cells (1) acquire a polarized cell shape upon cultivation, with the formation of a leading edge at the front pole and a uropod at the rear pole; (2) exhibit an amoeboid movement, which is similar to the one described for migrating peripheral leukocytes; and (3) redistribute several lipid raft markers including cholesterol-binding protein prominin-1 (CD133) in specialized plasma membrane domains. Furthermore, polarization of CD34$^+$ cells is stimulated by early acting cytokines and requires the activity of phosphoinositol-3-kinase as previously reported for peripheral leukocyte polarization. Together, our data reveal a strong correlation between polarization and migration of peripheral leukocytes and hematopoietic stem/progenitor cells and suggest that they are governed by similar mechanisms. (Blood. 2004;104:2332-2338)

Introduction

During ontogenesis the earliest progenitors of the mammalian adult hematopoietic system are initially formed in the intraembryonic aorta-gonad-mesonephros (AGM) and it seems very likely that such AGM-derived hematopoietic stem cells (HSCs) emigrate and colonize the fetal liver, the main site of embryonic hematopoiesis. During neonatal stages, HSCs migrate again; they leave the fetal liver to enter the blood stream and home to the bone marrow (BM), the main side of adult hematopoiesis.[1] More than 30 years of clinical experience as well as several animal models have demonstrated that neonatal and adult HSCs retain their ability to migrate into the BM and the capacity to reconstitute the entire hematopoietic system.[2] It appears that the homing process of transplanted HSCs is based on a naturally occurring process in which adult HSCs and progenitors travel from BM to blood and back to functional niches in BM and maybe into other organs.[3] Remarkably, despite the central role of these phenomena in hematopoietic stem cell biology and their therapeutic relevance, the molecular and cellular mechanisms, which involve chemokines for navigation, and adhesive proteins for interactions, to guide them to their appropriate niche, remain poorly understood.[4-8]

In contrast, more is known about the migration process of peripheral leukocytes in inflammatory responses in which they are attracted to leave the blood stream and enter tissues by crossing the vascular endothelium. As reviewed by Sanchez-Madrid and del Pozo,[9] the first requirement for cells that initiate migration is the acquisition of a polarized morphology that enables them to turn intracellularly generated forces into net cell locomotion. In this context it has been shown that chemokines trigger processes that induce changes in the organization of the cytoskeleton, resulting in an observable switch from a spherical into a polarized cell shape. It is established that this polarization requires the activity of phosphoinositol-3-kinase (PI3K), an enzyme involved in signal transduction events.[10,11] Polarized leukocytes form lamellipodia-like structures at the front side (ie, the leading edge) and contain a pseudopod-like projection at the rear pole called a uropod, a leukocyte-specific structure that plays an important role in cell motility and adhesion.[9] These morphologic changes are accompanied not only by the redistribution of several intracellular but also transmembrane proteins (eg, chemokine receptors become localized at the leading edge while other intercellular cell adhesion molecules, including intercellular adhesion molecules [ICAMs], CD43, and CD44, concentrate at the uropod).[9]

In our current studies we have observed that human CD34$^+$ cells acquire a polarized cell shape resembling the morphologic phenotype of migrating peripheral leukocytes when they are cultured ex vivo. Since the human CD34$^+$ cell fraction is highly enriched for HSCs and hematopoietic progenitor cells (HPCs),[12] we wondered whether there are parallels between the polarization

From the Institut für Transplantationsdiagnostik and Zelltherapeutika, Heinrich-Heine-Universität Düsseldorf, Düsseldorf, Germany; Medical Clinic and Polyclinic I, University Carl Gustav Carus, Dresden, Germany; and Max-Planck-Institute of Molecular Cell Biology and Genetics, Dresden, Germany.

Submitted February 10, 2004; accepted April 10, 2004. Prepublished online as Blood First Edition Paper, July 1, 2004; DOI 10.1182/blood-2004-02-0511.

Supported by grants from the Deutsche Forschungsgemeinschaft (SPP1109 GI 336/1-1, B.G. and P.W.; CO298/2-1, D.C.) as well as from the Forschungskommission of the Heinrich-Heine-Universität-Düsseldorf and the Stem Cell Network Nordrhein-Westfalen.

The online version of the article contains a data supplement.

Reprints: Bernd Giebel, Institut für Transplantationsdiagnostik and Zelltherapeutika, Heinrich Heine Universität Düsseldorf, Moorenstr 5, Geb 14.80, D-40225 Düsseldorf, Germany; e-mail: giebel@itz.uni-duesseldorf.de.

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

and migration processes of HSCs/HPCs and peripheral leukocytes. Here we have investigated this issue by immunochemistry and green fluorescent protein (GFP)–based approaches using a panel of uropod and leading-edge protein and lipid markers as well as the new hematopoietic stem and progenitor cell marker prominin-1/CD133 (human AC133 antigen)[13-15] (for review see Bhatia[16] and Corbeil et al[17]).

Materials and methods

Cell preparation and culture conditions

Umbilical cord blood (CB), BM, and peripheral blood (PB) of granulocyte colony-stimulating factor (G-CSF)–treated stem cell donors were obtained from unrelated donors after informed consent. Approval for BM and PB was obtained from the ethics commission of the Heinrich-Heine University, and approval for CB was obtained from the Paul-Ehrlich Institute. Informed consent was provided according to the Declaration of Helsinki. Mononuclear cells were isolated from individual sources by Ficoll (Biocoll Separating Solution; Biochrom, Berlin, Germany) density gradient centrifugation. Remaining red blood cells were lysed at 4°C in 0.83% ammonium chloride with 0.1% potassium hydrogen carbonate, followed by a phosphate-buffered saline (PBS) washing step. $CD34^+$ cells were isolated by magnetic cell separation using the MidiMacs technique according to the manufacturer's instructions (Miltenyi Biotec, Bergisch Gladbach, Germany), yielding $CD34^+$ cells of 65.5% ± 14.3% purity.

Freshly enriched $CD34^+$ cells were cultured in a humidified atmosphere at 37°C and 5% CO_2 at a density of approximately 1×10^5 cells/mL in serum-free (Stemspan H3000; Stemcell Technologies Inc, Vancouver, BC, Canada) or serum-containing tissue culture medium (Myelocult H5100; Stemcell Technologies Inc) in the absence or presence of early acting cytokines (fetal liver tyrosine kinase 3 ligand [FLT3L], stem cell factor [SCF], thrombopoietin [TPO]; each at 10 ng/mL final concentration; PeproTech Inc, Rocky Hill, NJ). To inhibit the PI3K activity of isolated cells we have added Ly294002 (Calbiochem, Bad Soden, Germany) at a final concentration of 50 μM to serum-free or serum-containing media supplemented with early acting cytokines.

Migration assays

Migratory potential of cultivated cells was analyzed by transmigration assays using 3-μm pore filters (Costar Transwell, 6.5-mm diameter; Corning Incorporated, Corning, NY). CB-derived $CD34^+$ cells were cultured for 2 days in Myelocult H5100 supplemented with early acting cytokines as described under "cell preparation and culture conditions." The Transwell filters were washed with Myelocult H5100 before they were loaded with 100-μL cell suspension of cultivated cells. Afterward they were carefully transferred to another well containing 600 μL Myelocult H5100 supplemented with early acting cytokines and 100 ng/mL stromal cell–derived factor-1α (SDF-1α; R&D Systems Inc, Minneapolis, MN) and cultured overnight in a humidified atmosphere at 37°C and 5% CO_2. To study the influence of PI3K activity on cell migration, the same assays were performed and Ly294002 was added at a final concentration of 100 μM to both to the top cell suspension and to the medium in the bottom chambers. The cultivation of the same amount of cells in 600 μL Myelocult H5100 supplemented with early acting cytokines and 100 ng/mL SDF-1α in the absence or presence of Ly294002 (100 μM) served as controls. Following overnight incubation, filters were carefully removed and the percentage of cells recovered in the bottom compartment was evaluated.

Immunofluorescence and microscopy

$CD34^+$ cells were generally cultured for 2 days in the presence of early acting cytokines in Myelocult H5100 medium before immunostaining. To conserve their polarized morphology, the $CD34^+$ cells were prefixed for 5 minutes at room temperature with paraformaldehyde (Sigma-Aldrich Chemie, Taufkirchen, Germany) at a final concentration of 0.2% in the medium. Cells were then incubated with the AC133-phycoerythrin (PE) antibody (1:10; AC133/1; Miltenyi Biotec) diluted in PBS containing 10% donkey serum (Jackson Immuno Research Laboratories, West Grove, PA) for at least 10 minutes at 4°C and postfixed with 4% paraformaldehyde in PBS for 20 minutes at 4°C. Because PE is not a suitable fluorochrome for immunofluorescence microscopy, we counterstained the AC133-labeled cells with cyanin 3 (Cy3)–conjugated AffiniPure Fab fragment donkey antimouse immunoglobulin G (IgG; 1:50; Jackson Immuno Research Laboratories) diluted in PBS containing 10% donkey serum for at least 10 minutes at 4°C. Remaining mouse epitopes were saturated with unconjugated AffiniPure Fab fragment rabbit antimouse IgG (1:10; Jackson Immuno Research Laboratories). Cells were divided in different aliquots and stained with one of the following primary mouse antibodies: anti-CD34–fluorescein isothiocyanate (FITC; HPCA-2; BD PharMingen, Heidelberg, Germany), anti-CD43 (1G10; BD PharMingen), anti-CD44–FITC (J173; Immunotech, Marseille, France), anti-CD45–FITC (2D1; BD PharMingen), anti-CD50 (TU41; BD PharMingen), anti-CD54–FITC (84H10; Immunotech), anti-GM3 IgM (GMR6; Seikagaku America, East Falmouth, MA), or rat anti-CXCR4 (1D9; BD PharMingen). These antibodies were counterstained using Cy2-conjugated secondary antibodies (goat antimouse IgG, donkey antimouse IgM, and goat antirat IgG + IgM; Jackson Immuno Research Laboratories). Labeled cells were mounted in 75% glycerin containing propylgallat (50 mg/mL) and DAPI (4,6 diamidino-2-phenylindole; 200 ng/mL; Roche, Mannheim, Germany).

Cells were observed with an Axioplan 2 fluorescence microscope (Carl Zeiss, Goettingen, Germany) using a ×20 dry (Figure 2A-B) or a ×100 oil immersion objective (Figures 2Ci-2Dii, 3), respectively. In general, analyses of living cells were performed at 37°C in a humidified 5% CO_2 atmosphere using a DMIRB inverse fluorescence microscope equipped with a CO_2 incubator chamber and a ×20 dry objective (Figures 1,4) (Leica, Bensheim, Germany). All pictures and videos were taken with an Axiocam digital camera and processed using Axiovision 3.1 Software (Carl Zeiss).

To analyze the degree of cell polarization, the living cells were photographed and their structure was evaluated. Cells with a comma-shaped morphology or with a recognizable tip (uropod) were determined as being polarized, whereas round and oval cells were determined as being nonpolarized. The percentage of viable cells was evaluated by trypan blue staining (0.4% solution; Sigma-Aldrich).

Plasmid construction and transfection of $CD34^+$ cells

The eukaryotic expression vector plasmid enhanced GFP (pEGFP)–N1-CD133, containing the entire coding sequence of human CD133 fused in-frame to the N-terminus of GFP, was constructed by selective polymerase chain reaction (PCR) amplification of the corresponding cDNA (GenBank accession no. AF027208) using the oligonucleotides 5′-TTGGAGTTTCTCGAGCTATGGCCCTCGTACT-3′ and 5′-TTCAACATCAGCTCGAGATGTTGTGATGG-3′ as 5′ and 3′ primers, respectively. The resulting PCR fragment was digested with *Xho*I and cloned into the corresponding site of pEGFP-N1 vector (BD Clontech, Heidelberg, Germany). The pEGFP-N1-CD44 plasmid encoding for human CD44 fused in-frame to the N-terminus of GFP was obtained by selective PCR amplification of the corresponding cDNA (GenBank accession no. AY101192) with the oligonucleotides 5′-CGCCTCGAGATCCTCCAGCTCCTTT-3′ and 5′-ATGGTGTAGAATTCGCACCCCAATC-3′ as 5′ and 3′ primers, respectively. The resulting PCR fragment was digested with *Xho*I and *Eco*RI and cloned into the corresponding sites of pEGFP-N1 vector. In both constructs, GFP is fused in-frame to the cytosolic C-terminal domain of the CD marker and their expression is under control of the cytomegalovirus (CMV) promoter.

CB-derived $CD34^+$ cells (1.5×10^5 to 3×10^5 cells) were transfected with 10 μg of plasmid DNA using the new Amaxa Nucleofection technology according to the manufacturer's instructions (Amaxa Biosystems, Cologne, Germany). As effectors we have used the pEGFP-N1-CD133 or pEGFP-N1-CD44 plasmids and, as a control, the plasmid yellow fluorescent protein–N1 (pEYFP-N1) or pEGFP-N1 vector, respectively. Following transfection, cells were immediately transferred into Myelocult H5100 supplemented with early acting cytokines. The

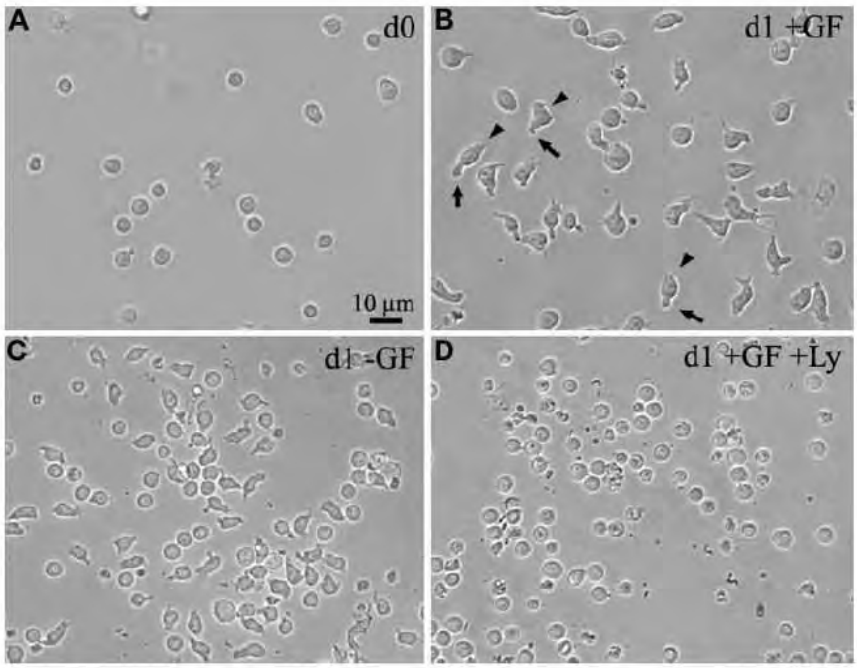


Figure 1. The human CD34+ cells acquire a morphologic polarity upon in vitro cultivation. (A-C) Light micrographs of human CD34+ cells freshly isolated from the umbilical cord blood (A) or cultured for 1 day (d1) in serum-free medium in the presence (B) or absence (C) of early acting cytokines as growth factors (GF). (D) The human CD34+ cells were cultured in serum-free medium in the presence of early acting cytokines and PI3K inhibitor Ly294002 (Ly). All panels are shown at the same magnification. Note, CD34+ cells that were cultured for 1 day in the presence of early acting cytokines (B) increase in size and acquire a polarized cell shape forming a leading edge at the front (arrowheads) and a uropod at the rear pole (arrows). Even in the absence of early acting cytokines, CD34+ cells can acquire a polarized cell shape, although they do not increase in size (C). The cell polarization and the growth process are inhibited by the PI3K inhibitor (D).

efficiency of individual transfections was evaluated by staining the transfected cells 24 hours after the DNA incorporation with an anti-CD34–PE antibody (8G12; BD PharMingen) and analyzed by flow cytometry or fluorescence microscopy (see "Immunofluorescence and microscopy").

Flow cytometry

Flow cytometric analyses were performed on a Cytomics FC 500 flow cytometer equipped with the RXP software (Beckman Coulter, Krefeld, Germany).

Results

Morphologic polarization of human CD34+ cells

Human CD34+ cells freshly isolated from different sources (CB, BM, and PB) are small (ie, 5-6 μm), round, and without any morphologic sign of cell polarity (Figure 1A). Remarkably, a high proportion of these cells increase in size and acquire a polarized cell shape when grown on uncoated plastic dishes in the presence of early acting cytokines (SCF, TPO, FLT3L; 10 ng/mL each) in either serum-supplemented or serum-free tissue culture medium (Table 1; Figure 1B). It should be mentioned that these early acting cytokines have been reported to preserve the multipotency and engraftment potential of human HSCs/HPCs in short-term cultures.[18] Although the number of cells possessing a polarized shape is high and comparable between individual donor samples under serum-containing conditions, it is more variable under serum-free conditions (Table 1). Cells acquiring a polarized morphology form a leading-edge–like structure at one end and a uropod-like structure at the opposite side (Figure 1B, arrowheads and arrows, respectively). Even in the absence of any growth factor, some cells acquire a polarized cell shape in serum-containing or serum-free medium (Table 1), but they neither increase in size (Figure 1C) nor proliferate (data not shown). Although we did not add SDF-1 or any other chemokine, the cultivated cells were highly dynamic and exhibited an amoeboid movement that is very similar to the one described for migrating peripheral leukocytes[9] (see the time-lapse videomicroscopy imaging in supplemented material). Additionally, we have observed that flow cytometrically sorted lin−CD34+CD38low/− cells as well as lin−CD34+CD38+ cells acquire a polarized cell shape upon cultivation in cytokine-containing media (data not shown).

Table 1. Polarization and migration of CD34+ cells depend on PI3K activity

	% polarized cells	n	% living cells	n	% migrated cells	n
Culture condition d0-d1; values d1						
Serum-free + GF	64.90 ± 18.70	7	97.37 ± 1.84	3	NE	0
Serum-free	37.75 ± 19.91	7	90.91 ± 1.91	3	NE	0
Serum-free + GF + Ly294002	3.91 ± 1.47	3	92.55 ± 1.11	3	NE	0
Serum + GF	82.86 ± 5.84	7	96.74 ± 2.02	3	NE	0
Serum	42.84 ± 25.61	3	83.53 ± 16.22	3	NE	0
Serum + GF + Ly294002	9.73 ± 3.20	3	91.05 ± 1.41	3	NE	0
Culture condition d2-d3; values d3						
Serum + GF + SDF-1α	73.10 ± 3.85	3	94.48 ± 1.51	3	15.6 ± 4.20	3
Serum + GF + SDF-1α + Ly294002	14.60 ± 7.12	3	87.39 ± 4.74	3	3.3 ± 0.25	3

Data are for CB-derived CD34+ cells; mean ± SD.
n indicates number of independent experiments; d0, freshly isolated cells; GF, growth factors (SCF, TPO, FLT3L); and NE, not estimated.

Polarization and migration of CD34+ cells depends on PI3K activity

When PI3K activity is inhibited by addition of Ly294002, peripheral leukocytes lose their polarized shape and round up.[10] To investigate if PI3K activity is also required for the polarization of CD34+ cells we have cultured CB-derived CD34+ cells in early acting cytokine-supplemented media, in the presence or absence of Ly294002. While most of the CD34+ cells present in the control group acquired a polarized cell shape after 1 day (Table 1; Figure 1B), treated cells remained round (Table 1; Figure 1D) and viable (Table 1). Furthermore, when adding Ly294002 to polarized CD34+ cells cultured for 2 days in serum and early acting cytokine-containing medium, many of them lose their polarized morphology and round up but remain viable (Table 1). Taken together these data suggest that the PI3K pathway is involved in the polarization process of CD34+ cells.

Because cell polarity is an essential prerequisite for the migration process of several cell types,[9] we have tested whether CD34+ cells that lose their polarized cell shape have a reduced migration capacity. Therefore, we have examined the migration rate of CB-derived CD34+ cells, which were originally cultured for 2 days in serum and early acting cytokine-containing medium, in the presence or absence of the PI3K inhibitor Ly294002 using a transmigration assay. In the absence of Ly294002, 15.6% ± 4.20% of the CD34+ cells migrated through 3-μm pores of Transwell filters, whereas only 3.3% ± 0.25% of the cells treated with this PI3K inhibitor passed through the pores (Table 1).

The hematopoietic stem cell marker CD133 is redistributed to the uropod-like structure of migrating human CD34+ cells

A hallmark of the polarization of migrating peripheral leukocytes is an asymmetric redistribution of certain cell surface proteins into the well-defined architectural structures, the uropod and the leading edge.[9] This prompted us to examine, by immunochemistry, the distribution of hematopoietic stem and progenitor marker CD133 in nonpolarized and polarized (ie, migrating) CD34+ cells. In our hands more than 90% of CD34+ cells isolated from CB, PB, or BM express CD133 (data not shown). Interestingly, we found that CD133, which is distributed over the entire cell surface of freshly isolated CB-derived CD34+ cells (Figure 2A,Ci-ii), is redistributed into the tip of the uropod-like structure in polarized CD34+ cells (Figure 2B,Di-ii). Under the same conditions, the cell surface redistribution of CD133 is not observed in nonpolarized cells (Figure 2B arrowhead). The same phenomenon is observed with CD34+ cells isolated from BM and PB (data not shown).

Segregation of several plasma membrane markers in polarized human CD34+ cells

Given the high similarity observed between the migrating CD34+ cells and peripheral leukocytes we decided to investigate the subcellular distribution of several membrane protein markers, including adhesion molecules and one membrane receptor, known to be enriched either in the uropod or at the leading edge of migrating peripheral leukocytes.[9] Immunochemistry revealed that CD markers previously reported to be concentrated into the uropod of migrating peripheral leukocytes,[9] including CD43, CD44, CD50 (ICAM3), and CD54 (ICAM1), are also enriched in the uropod-like structure of CB-derived CD34+ cells and colocalized with CD133 (Figure 3A-D). Similar data were obtained with PB-derived CD34+ cells (data not shown).

In contrast, we found that the CXCR4 chemokine receptor, which has been reported to be redistributed at the leading edge of B and T lymphocytes upon exposure to chemokines,[19-21] appeared as a gradient with its highest expression in the leading edge of CD34+ cells (Figure 3G). Finally, the segregation of CD

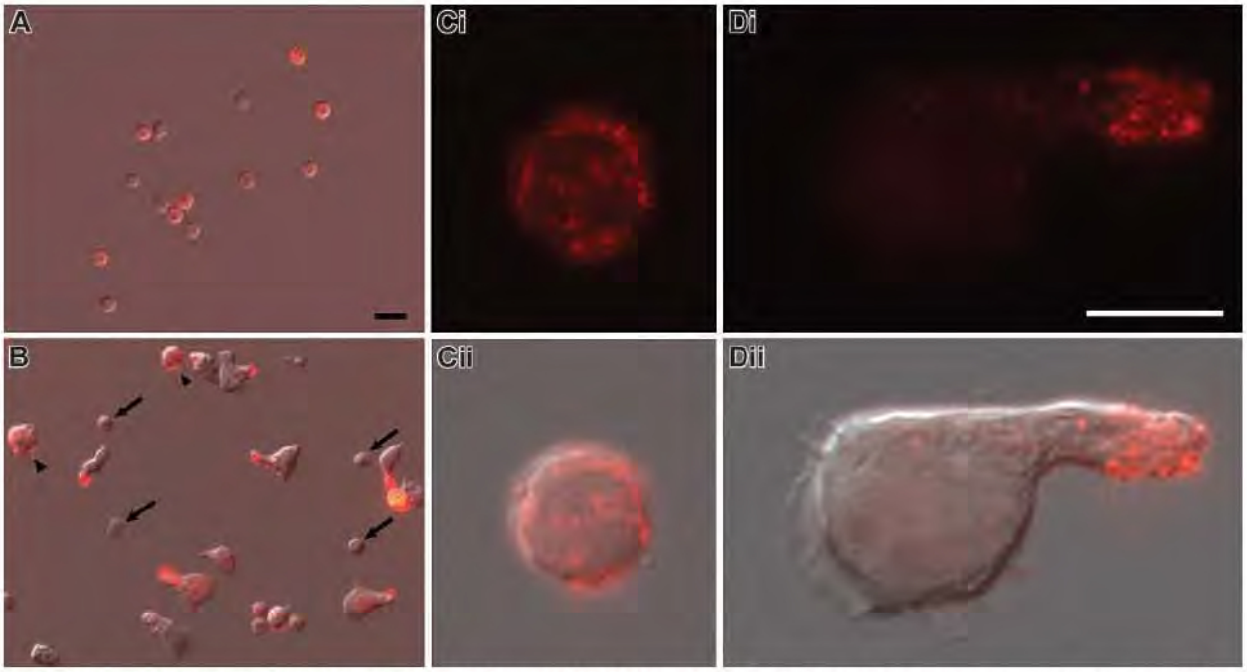


Figure 2. Cell surface redistribution of CD133 into the uropod of polarized CD34+ cells. (A-D) Human CD34+ cells, freshly isolated from umbilical cord blood (A,Ci-ii) or cultured for 2 days in the presence of early acting cytokines (B,Di-ii), were labeled with AC133 antibody (anti-CD133; red) and observed by immunofluorescence (Ci,Di). The overlays with the corresponding differential interference contrast images are shown (A-B,Cii,Dii). Note that the cultured CD133− cells remain small and round (arrows in B), whereas cultured CD133+ cells increase in size and CD133 becomes localized into the uropod of polarized cells (B,Di-ii) but remains distributed all over the surface of nonpolarized CD133+ cells (arrowheads in B). Same magnification was used in panels A and B (scale bar = 10 μm; A) or in panels Ci and Di (scale bar = 5 μm; Di), respectively.

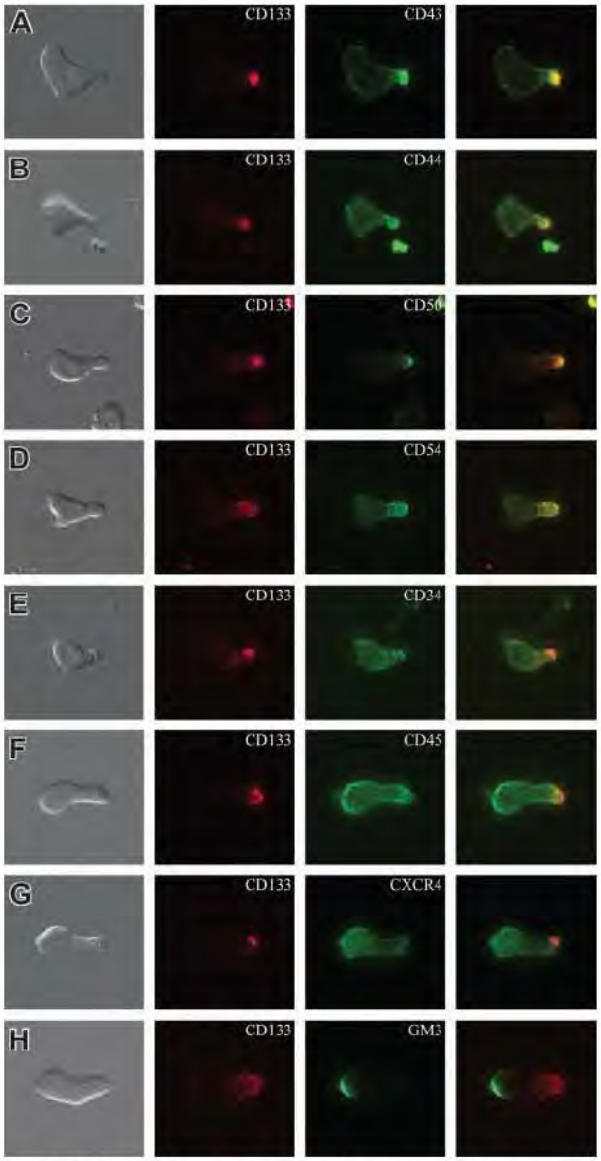


Figure 3. Cell surface distribution of plasma membrane markers in polarized CD34+ cells. (A-H) CD34+ cells isolated from umbilical cord blood and cultured for 2 days in serum-containing medium supplemented with early acting cytokines were subjected to double labeling using AC133 antibody (anti-CD133) and an antibody directed against another cell surface antigen, as indicated, and analyzed by double immunofluorescence. The CD133 immunofluorescence (red) is shown in the second column, the immunofluorescence of various cell surface antigens (green) in the third column, and the corresponding differential interference contrast image as well as the merge are shown in the first and the fourth column, respectively. Note that the chemokine receptor CXCR4 (G) and the ganglioside GM3 (H) are concentrated in the leading edge of the front pole, whereas CD43 (A), CD44 (B), ICAM3/CD50 (C), and ICAM1/CD54 (D) are enriched in the uropod of the polarized cells and colocalized with CD133. All panels are shown at the same magnification.

markers observed here is not a common characteristic shared by all of them since CD34 and CD45 are found equally distributed all over the surface of polarized CD34+ cells (Figure 3E and F, respectively).

Recently, it has been demonstrated that during polarization of T cells the monosi θ aloganglioside GM3, a raft-associated lipid, redistributes to the leading edge of activated leukocytes.[22] To corroborate further the similarity underlying the polarization process between CD34+ cells and activated leukocytes, we analyzed the distribution of GM3 in CD34+ cells by immunochemistry. Remarkably, we found that GM3 is localized at the leading edge of polarized CD34+ cells (Figure 3H).

CD44-GFP and CD133-GFP fusion proteins are localized in the uropod of transfected human CD34+ cells

The morphologic data strongly suggest that certain plasma membrane markers are partitioned into specialized regions or domains of migrating human CD34+ cells. To rule out that the compartmentalization observed is generated by the use of antibodies creating an artificial cluster of a given membrane marker, we monitored the distribution of the protein markers CD44 and CD133 fused to green fluorescent protein (GFP) in living CB-derived CD34+ cells. Under the specific and new nonviral transfection conditions described here (see "Plasmid construction and transfection of CD34 cells" in "Materials and methods"), up to 80% of CB transfected cells expressed the fluorescence protein reporter gene (Figure 4Cii quadrants A2 and A4), which is distributed into the entire cytoplasm (Figure 4Ci). No fluorescence is observed with untransfected cells (Figure 4B quadrants A2 and A4). Interestingly, fluorescence microscopy revealed that CD44-GFP fusion protein, which is expressed in more than 75% of transfected cells (Figure 4Dii quadrants A2 and A4), is associated with the entire plasma membrane being enriched in the uropod of the CB-derived cells (Figure 4Di). Likewise, CD133-GFP is selectively concentrated at the tip of the uropod of polarized CD34+ cells (Figure 4Ei), as previously observed by immunochemistry for the endogenous CD133 (Figures 2-3). It should be mentioned that in comparison to fluorescence protein alone and CD44-GFP, CD133-GFP is detected only in a specific subpopulation of CB-derived cells (ie, CD34+ cells; Figure 4Ei quadrant A2).

Discussion

Here we report 4 major observations. First, HSCs/HPCs acquire a polarized cell shape by a molecular mechanism dependent on the PI3K pathway. Second, polarized HSCs/HPCs exhibit an amoeboid movement, which is similar to the one described for migrating peripheral leukocytes. Third, the polarization of HSC/HPC plasma membrane leads to a redistribution of a particular set of markers from which several of them have been previously reported to be associated with lipid microdomains (lipid rafts). Fourth, the stem cell marker CD133 is selectively concentrated in the uropod of polarized HSCs/HPCs.

It is well established that HSCs/HPCs have the ability to migrate.[1-3] Despite the evidence that SDF-1 triggers migration of HSCs/HPCs,[4-8] the current knowledge of the mechanisms governing stem cell migration remains limited. For other cell types it has been shown that the acquisition of cell polarity is an essential prerequisite for the migration process.[9] Although it has been noticed that some CD34+ cells acquire a polarized cell shape upon adhesion to fibronectin[23] or when migrating under the influence of SDF-1 through a fibronectin-coated Transwell filter or a 3-dimensional meshwork of extracellular matrix components,[5,24] the issue of cell polarity in HSCs/HPCs has not been analyzed in detail.

We were able to document that the CD34+ cells grown in suspension cultures in the presence of early acting cytokines acquire a polarized cell shape with a defined leading edge at the front pole and a uropod at the rear pole. This morphologic phenotype is highly related to the one described for polarized, migrating peripheral leukocytes. Since the polarization of peripheral leukocytes depends on the activity of chemokines,[20,25,26] it is

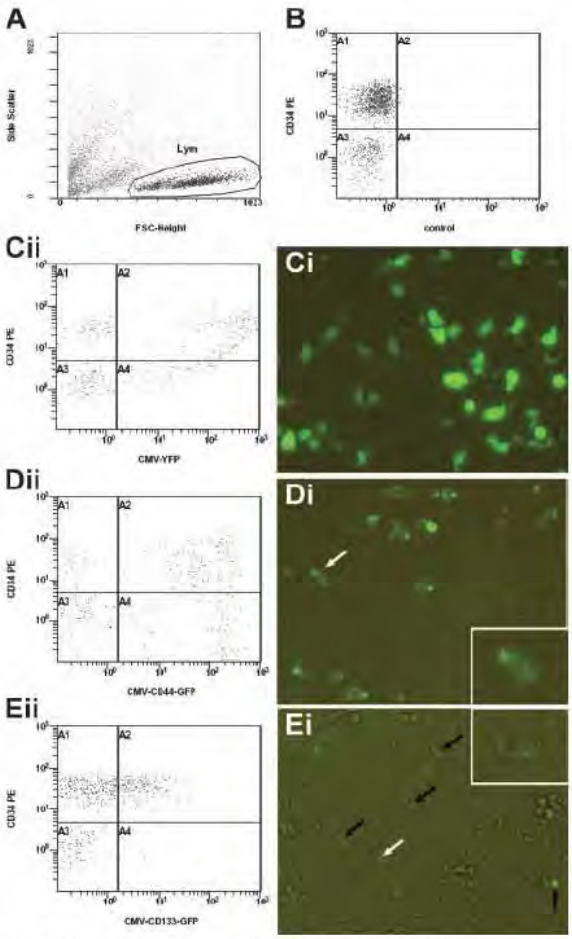


Figure 4. Subcellular localization of CD44-GFP and CD133-GFP fusion proteins in transfected CD34+ cells. Using the new Amaxa nucleofection technology, human CD34+ cells enriched from umbilical cord blood were transfected with the expression plasmid encoding either for YFP (Ci), CD44-GFP (Di-ii) or CD133-GFP (Ei-ii) under the control of the cytomegalovirus promoter. Transfected cells and, as negative control, untransfected cells (B) were cultivated for 1 day in serum-containing medium supplemented with early acting cytokines and were analyzed by flow cytometry (A-B,Cii,Dii,Eii) and fluorescence microscopy (Ci,Di,Ei). (A-B,Cii,Dii,Eii) A representative experiment (n = 5) of the CB-derived cells untransfected (B) or transfected with different expression plasmids (Cii,Dii,Eii) was stained with a PE-conjugated anti-CD34 antibody and analyzed by flow cytometry. The cells analyzed in panels B, Cii, Dii, and Eii were gated according to the morphology depicted on a forward scatter/side scatter plot (A). Note, cells shown in panel Cii are extremely positive for YFP; most of them stick to the right border of the plot. (Ci,Di,Ei). Differential interference contrast image shows fluorescence overlay of YFP (Ci), CD44-GFP (Di), or CD133-GFP (Ei) in living transfected CD34+-enriched cells. The YFP is strongly expressed throughout the cytoplasm of the cells, whereas CD44-GFP and CD133-GFP are concentrated in the uropod of the migrating cells (arrows in Di and Ei). The white arrows indicate the cells shown in the insets (high magnification).

very suggestive that similar factors are not only involved in the migration process of HSCs but also required for the polarization of these cells. However, our data show that chemokines, including SDF-1, do not appear essential for the polarization of CD34+ cells. Moreover, although it is not known yet which factor triggers such polarization, we provide evidence that the activation of the PI3K pathway is crucial for this process, being consistent with results obtained for peripheral leukocytes.[10,11] It is interesting to note that SCF, which was added to most of our cultures, has been reported to activate the PI3K signaling pathway similarly to SDF-1.[10,11,27-29] SCF also has the capability to act as a chemoattractant for human HPCs,[8] arguing that other factors than chemokines can induce polarization and migration of CD34+ cells. Finally, it is important to point out that the serum-free culture medium used in our assays contains insulin, which is another potential activator of the PI3K.[30] Therefore, insulin might be responsible for the polarization of CD34+ cells under serum-free conditions in the absence of cytokines.

As mentioned in "Introduction," the polarization process seems to be a prerequisite for cell migration. Indeed we show that CD34+ cells treated with a PI3K inhibitor lose not only their polarized morphology but also their ability to migrate, suggesting that polarization and migration are closely connected to each other in CD34+ cells, as they are in other cell types. The inhibition of these cellular processes may explain why the homing of CD34+ cells is disturbed when PI3K signaling is perturbed.[31]

In addition to the importance of the PI3K signaling, we have demonstrated that polarization of CD34+ cells is accompanied by the redistribution of certain transmembrane proteins. As in other leukocytes, CD43, CD44, CD50 (ICAM3), and CD54 (ICAM1) become concentrated into the uropod of CD34+ cells, whereas other molecules such as CD34 or CD45 remain distributed all over the plasma membrane.

The phenotypic similarity between polarized progenitor cells and other leukocytes is not restricted to their morphology and the distribution of uropod markers. We show that the chemokine receptor CXCR4, which is known to be enriched in the leading edge of migrating lymphocytes, is distributed in a gradient-like fashion with its maximal concentration in the leading edge of polarized CD34+ cells. In agreement with this finding, van Buul and colleagues[32] have reported recently that a CXCR4-GFP fusion protein redistributes to the leading edge of the KG1a cells, an acute myeloid leukemia (AML)–derived cell line.

There is growing evidence that many membrane proteins are associated with certain lipids (eg, sphingolipid or cholesterol-based lipids). Such lipids are often clustered in special microdomains, in so-called lipid rafts.[33] Depending on the lipids that form a special raft, lipid rafts traffic to certain plasma membrane domains (eg, the apical or basal plasma membrane). In this context they seem to function as platforms that are important to govern the subcellular distribution of associated membrane proteins.[33] Indeed, it has been shown that polarization of peripheral leukocytes depends on the controlled redistribution of such specialized lipid rafts.[22,34] In activated T cells for example, the monosialoganglioside GM3-enriched lipid rafts traffic to the leading edge, while others redistribute to the uropod pole, concentrating a specific set of membrane proteins, such as ICAM3, CD43, and CD44, there.[22,34] The observation that these raft-associated markers are also concentrated at the uropod pole and GM3 at the leading edge of CD34+ cells further reinforces existing parallels in the polarization process of HSCs/HPCs with peripheral leukocytes and suggests that lipid rafts are also important for the polarization of HSCs/HPCs. Therefore, it is tempting to speculate that cues and mechanisms underlying the polarization and migration of precursors and more mature hematopoietic cells appear common to all of them.

Interestingly, we found that the stem cell marker CD133, a cholesterol-binding protein,[35] is also redistributed into the uropod of migrating CD34+ cells. Since CD133 is preferentially associated with plasma membrane protrusions in all cell types where it is expressed,[35-38] and is incorporated into lipid rafts,[35] it seems very likely that special lipid rafts organize the delivery and/or retention of CD133 in the uropod. As CD133 is selectively associated with microvilli and other plasma membrane protrusions within the apical plasma membrane in different epithelia of embryonic and adult tissues,[37,38] it might be possible that epithelial cells and

HSCs/HPCs use a similar mechanism to distribute CD133. In agreement with the postulation that the targeting and retention of proteins into a specialized plasma membrane domain of distinct cell types are mediated by a common intracellular machinery,[39] the uropod of polarized HSCs/HPCs and therefore of other leukocytes would correspond to the apical domain of polarized epithelial cells.

In conclusion, our data show for the first time that the polarization and migration processes of $CD34^+$ cells are highly related to those reported for migrating peripheral leukocytes. The comprehensive knowledge about these mechanisms in peripheral leukocyte biology should increase our understanding about HSC/HPC traffic during normal and malignant hematopoiesis and should help to improve the process of homing and engraftment of stem cells in clinical trials.

Acknowledgment

We thank Michael Punzel for general discussion, comments on the manuscript, and providing us with PB and BM samples.

References

1. Dzierzak E. Hematopoietic stem cells and their precursors: developmental diversity and lineage relationships. Immunol Rev. 2002;187:126-138.
2. Weissman IL. Translating stem and progenitor cell biology to the clinic: barriers and opportunities. Science. 2000;287:1442-1446.
3. Wright DE, Wagers AJ, Gulati AP, Johnson FL, Weissman IL. Physiological migration of hematopoietic stem and progenitor cells. Science. 2001; 294:1933-1936.
4. Peled A, Petit I, Kollet O, et al. Dependence of human stem cell engraftment and repopulation of NOD/SCID mice on CXCR4. Science. 1999;283: 845-848.
5. Peled A, Kollet O, Ponomaryov T, et al. The chemokine SDF-1 activates the integrins LFA-1, VLA-4, and VLA-5 on immature human CD34(+) cells: role in transendothelial/stromal migration and engraftment of NOD/SCID mice. Blood. 2000;95:3289-3296.
6. Aiuti A, Webb IJ, Bleul C, Springer T, Gutierrez-Ramos JC. The chemokine SDF-1 is a chemoattractant for human CD34+ hematopoietic progenitor cells and provides a new mechanism to explain the mobilization of CD34+ progenitors to peripheral blood. J Exp Med. 1997;185:111-120.
7. Mohle R, Bautz F, Rafii S, Moore MA, Brugger W, Kanz L. 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;91:4523-4530.
8. Kim CH, Broxmeyer HE. In vitro behavior of hematopoietic progenitor cells under the influence of chemoattractants: stromal cell-derived factor-1, steel factor, and the bone marrow environment. Blood. 1998;91:100-110.
9. Sanchez-Madrid F, del Pozo MA. Leukocyte polarization in cell migration and immune interactions. EMBO J. 1999;18:501-511.
10. Sotsios Y, Whittaker GC, Westwick J, Ward SG. The CXC chemokine stromal cell-derived factor activates a Gi-coupled phosphoinositide 3-kinase in T lymphocytes. J Immunol. 1999;163:5954-5963.
11. Vicente-Manzanares M, Rey M, Jones DR, et al. Involvement of phosphatidylinositol 3-kinase in stromal cell-derived factor-1 alpha-induced lymphocyte polarization and chemotaxis. J Immunol. 1999;163:4001-4012.
12. Krause DS, Fackler MJ, Civin CI, May WS. CD34: structure, biology, and clinical utility. Blood. 1996; 87:1-13.
13. Fargeas CA, Corbeil D, Huttner WB. AC133 antigen, CD133, prominin-1, prominin-2, etc: prominin family gene products in need of a rational nomenclature. Stem Cells. 2003;21:506-508.
14. Miraglia S, Godfrey W, Yin AH, et al. A novel five-transmembrane hematopoietic stem cell antigen: isolation, characterization, and molecular cloning. Blood. 1997;90:5013-5021.
15. Yin AH, Miraglia S, Zanjani ED, et al. AC133, a novel marker for human hematopoietic stem and progenitor cells. Blood. 1997;90:5002-5012.
16. Bhatia M. AC133 expression in human stem cells. Leukemia. 2001;15:1685-1688.
17. Corbeil D, Roper K, Fargeas CA, Joester A, Huttner WB. Prominin: a story of cholesterol, plasma membrane protrusions and human pathology. Traffic. 2001;2:82-91.
18. Luens KM, Travis MA, Chen BP, Hill BL, Scollay R, Murray LJ. Thrombopoietin, kit ligand, and flk2/flt3 ligand together induce increased numbers of primitive hematopoietic progenitors from human CD34+Thy-1+Lin- cells with preserved ability to engraft SCID-hu bone. Blood. 1998;91: 1206-1215.
19. Nieto M, Frade JM, Sancho D, Mellado M, Martinez AC, Sanchez-Madrid F. Polarization of chemokine receptors to the leading edge during lymphocyte chemotaxis. J Exp Med. 1997;186:153-158.
20. Vicente-Manzanares M, Montoya MC, Mellado M, et al. The chemokine SDF-1alpha triggers a chemotactic response and induces cell polarization in human B lymphocytes. Eur J Immunol. 1998;28: 2197-2207.
21. Pelletier AJ, van der Laan LJ, Hildbrand P, et al. Presentation of chemokine SDF-1 alpha by fibronectin mediates directed migration of T cells. Blood. 2000;96:2682-2690.
22. Gomez-Mouton C, Abad JL, Mira E, et al. Segregation of leading-edge and uropod components into specific lipid rafts during T cell polarization. Proc Natl Acad Sci U S A. 2001;98:9642-9647.
23. Fruehauf S, Srbic K, Seggewiss R, Topaly J, Ho AD. Functional characterization of podia formation in normal and malignant hematopoietic cells. J Leukoc Biol. 2002;71:425-432.
24. Voermans C, Anthony EC, Mul E, van der Schoot E, Hordijk P. SDF-1-induced actin polymerization and migration in human hematopoietic progenitor cells. Exp Hematol. 2001;29:1456-1464.
25. del Pozo MA, Sanchez-Mateos P, Nieto M, Sanchez-Madrid F. Chemokines regulate cellular polarization and adhesion receptor redistribution during lymphocyte interaction with endothelium and extracellular matrix: involvement of cAMP signaling pathway. J Cell Biol. 1995;131:495-508.
26. Nieto M, Navarro F, Perez-Villar JJ, et al. Roles of chemokines and receptor polarization in NK-target cell interactions. J Immunol. 1998;161:3330-3339.
27. Lev S, Givol D, Yarden Y. Interkinase domain of kit contains the binding site for phosphatidylinositol 3' kinase. Proc Natl Acad Sci U S A. 1992;89: 678-682.
28. Timokhina I, Kissel H, Stella G, Besmer P. Kit signaling through PI 3-kinase and Src kinase pathways: an essential role for Rac1 and JNK activation in mast cell proliferation. EMBO J. 1998;17: 6250-6262.
29. Feng LX, Ravindranath N, Dym M. Stem cell factor/c-kit up-regulates cyclin D3 and promotes cell cycle progression via the phosphoinositide 3-kinase/p70 S6 kinase pathway in spermatogonia. J Biol Chem. 2000;275:25572-25576.
30. Cheatham B, Vlahos CJ, Cheatham L, Wang L, Blenis J, Kahn CR. Phosphatidylinositol 3-kinase activation is required for insulin stimulation of pp70 S6 kinase, DNA synthesis, and glucose transporter translocation. Mol Cell Biol. 1994;14: 4902-4911.
31. Wang JF, Park IW, Groopman JE. Stromal cell-derived factor-1alpha stimulates tyrosine phosphorylation of multiple focal adhesion proteins and induces migration of hematopoietic progenitor cells: roles of phosphoinositide-3 kinase and protein kinase C. Blood. 2000;95:2505-2513.
32. van Buul JD, Voermans C, van Gelderen J, Anthony EC, van der Schoot CE, Hordijk PL. Leukocyte-endothelium interaction promotes SDF-1-dependent polarization of CXCR4. J Biol Chem. 2003;278:30302-30310.
33. Simons K, Ikonen E. Functional rafts in cell membranes. Nature. 1997;387:569-572.
34. Millan J, Montoya MC, Sancho D, Sanchez-Madrid F, Alonso MA. Lipid rafts mediate biosynthetic transport to the T lymphocyte uropod subdomain and are necessary for uropod integrity and function. Blood. 2002;99:978-984.
35. Roper K, Corbeil D, Huttner WB. Retention of prominin in microvilli reveals distinct cholesterol-based lipid micro-domains in the apical plasma membrane. Nat Cell Biol. 2000;2:582-592.
36. Corbeil D, Roper K, Hannah MJ, Hellwig A, Huttner WB. Selective localization of the polytopic membrane protein prominin in microvilli of epithelial cells: a combination of apical sorting and retention in plasma membrane protrusions. J Cell Sci. 1999;112(pt 7):1023-1033.
37. Weigmann A, Corbeil D, Hellwig A, Huttner WB. Prominin, a novel microvilli-specific polytopic membrane protein of the apical surface of epithelial cells, is targeted to plasmalemmal protrusions of non-epithelial cells. Proc Natl Acad Sci U S A. 1997;94:12425-12430.
38. Corbeil D, Roper K, Hellwig A, et al. The human AC133 hematopoietic stem cell antigen is also expressed in epithelial cells and targeted to plasma membrane protrusions. J Biol Chem. 2000;275:5512-5520.
39. Keller P, Simons K. Post-Golgi biosynthetic trafficking. J Cell Sci. 1997;110(pt 24):3001-3009.

10.2 Primitive human hematopoietic cells give rise to differentially specified daughter cells upon their initial cell division (Giebel et al., 2006)

Primitive human hematopoietic cells give rise to differentially specified daughter cells upon their initial cell division

Bernd Giebel, Tao Zhang, Julia Beckmann, Jan Spanholtz, Peter Wernet, Anthony D. Ho, and Michael Punzel

It is often predicted that stem cells divide asymmetrically, creating a daughter cell that maintains the stem-cell capacity, and 1 daughter cell committed to differentiation. While asymmetric stem-cell divisions have been proven to occur in model organisms (eg, in *Drosophila*), it remains illusive whether primitive hematopoietic cells in mammals actually can divide asymmetrically. In our experiments we have challenged this question and analyzed the developmental capacity of separated offspring of primitive human hematopoietic cells at a single-cell level. We show for the first time that the vast majority of the most primitive, in vitro–detectable human hematopoietic cells give rise to daughter cells adopting different cell fates; 1 inheriting the developmental capacity of the mother cell, and 1 becoming more specified. In contrast, approximately half of the committed progenitor cells studied gave rise to daughter cells, both of which adopted the cell fate of their mother. Although our data are compatible with the model of asymmetric cell division, other mechanisms of cell fate specification are discussed. In addition, we describe a novel human hematopoietic progenitor cell that has the capacity to form natural killer (NK) cells as well as macrophages, but not cells of other myeloid lineages. (Blood. 2006;107: 2146-2152)

Introduction

Somatic stem cells are defined as undifferentiated cells, which can self-renew over a long period of time and give rise to progenitor cells that are committed to differentiation upon their further development. Since both an uncontrolled expansion as well as loss of stem cells would be fatal for multicellular organisms, the decision of self-renewal versus differentiation needs to be tightly controlled. Therefore, a key question in stem-cell biology is how and which mechanisms govern this decision.

Hematopoietic stem cells (HSCs) are the most investigated mammalian stem cells. More than 30 years of clinical experience as well as experiments with animals demonstrated that neonatal and adult HSCs retain the ability to reconstitute the hematopoietic systems of patients after myeloablative treatment.[1] Therefore, an important feature of HSCs is the capacity to replenish all lineages of mature blood cells. Beside this, they also have the potential to expand in vivo as revealed by sequential transplantation experiments using limiting numbers of mouse HSCs to reconstitute the hematopoietic systems of primary and secondary lethally irradiated hosts.[2,3] However, although HSCs can be maintained in vitro in close contact to adequate stroma cells,[4-8] no evaluated in vitro condition has been reported so far, which supports the expansion of these cells over a period of several weeks. These findings demonstrate that the surrounding environment has a major influence on the cell fate of HSCs and their daughter cells. In this context, it was recently shown that by participating in the formation of special HSC-supporting niches, osteoblasts regulate the size of the HSC pool in vivo.[9-11]

In addition to data supporting a hematopoietic stem-cell niche model, results of further studies suggest that cells of the hematopoietic stem- and progenitor-cell compartments are able to divide asymmetrically. In an initial set of experiments Ogawa's group analyzed the differentiation of murine and human hematopoietic progenitor cells (HPCs). After separation of paired-progenitor cells they cultured these cells individually and observed that in some cases siblings gave rise to colonies that significantly differed from each other (ie, they gave rise to different cell lineages and/or to colonies of different sizes).[12-14] Recently, some evidence for the occurrence of asymmetric cell division of mouse HSCs was provided in a transplantation model.[15,16]

In studies using human HSC-enriched cells of the $CD34^+CD38^-$ fraction we and others have shown that $CD34^+CD38^-$ cells are heterogeneous in respect to their function and their proliferation kinetics (ie, the proliferation rate of more primitive cells is lower than that of committed ones).[17-19] Furthermore, it was observed that approximately 30% of the $CD34^+CD38^-$ cells gave rise to daughter cells with heterogeneous proliferation kinetics, perhaps the result of an asymmetric cell division.[17,19,20]

To increase the evidence for the occurrence of asymmetric cell divisions within the most primitive in vitro–detectable hematopoietic cell compartment we have separated the progenies of HSC candidates by micromanipulation and analyzed their developmental potential in primitive human progenitor assays.

From the Institute for Transplantation Diagnostics and Cellular Therapeutics, Heinrich-Heine-University Düsseldorf, Germany; Department of Medicine V, Ruprecht-Karls University of Heidelberg, Germany; and Cancer Center Union Hospital, Huazhong University of Science and Technology Wuhan, People's Republic of China.

Submitted August 5, 2005; accepted October 15, 2005. Prepublished online as *Blood* First Edition Paper, October 25, 2005; DOI 10.1182/blood-2005-08-3139.

Supported by grants from the Deutsche Forschungsgemeinschaft (SPP1109 GI 336/1-2 to B.G. and P.W.; HO 914/2-1 to A.D.H.), from the Forschungskommission of the HHU-Düsseldorf (B.G., M.P.), and from the Faculty Research Program of the University of Heidelberg (F203532 to M.P.).

B.G. and T.Z. contributed equally to this work.

Reprints: Michael Punzel or Bernd Giebel, Institute for Transplantation Diagnostics and Cell Therapeutics, Heinrich-Heine-University Düsseldorf, D-40225 Düsseldorf, Germany; e-mail: punzel@itz.uni-duesseldorf.de or giebel@itz.uni-duesseldorf.de.

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

Materials and methods

Cell source and preparation

Human umbilical cord blood (CB) was obtained from the umbilical vein after delivery of the placenta from mothers after informed consent according to the Declaration of Helsinki. Approval for CB was obtained from the Paul-Ehrlich Institute. Mononuclear cells were isolated from individual sources by Ficoll (Biocoll Separating Solution; Biochrom AG, Berlin, Germany) density gradient centrifugation. $CD34^+$ cells were isolated by magnetic cell separation using the MidiMacs technique according to the manufacturer's instructions (Miltenyi Biotec, Bergisch Gladbach, Germany).

Immunofluorescence

For the proliferation kinetics, freshly isolated $CD34^+$ cells were stained with PKH2 (Sigma-Aldrich Chemie, Taufkirchen, Germany) as described previously.[19] Before the cells were analyzed by flow cytometry they were stained with AC133-phycoerythrin (PE; Miltenyi Biotec) and anti-CD34–PE/Cytochrome 5 (PCy5) antibodies (BD PharMingen, Heidelberg, Germany). Flow cytometric analyses were performed on a Cytomics FC 500 flow cytometer equipped with the RXP software (Beckman Coulter, Krefeld, Germany).

For functional analysis $CD34^+$-enriched cells were stained with anti-CD34–PE (BD PharMingen) and anti-CD38–allophycocyanin (APC; BD PharMingen) antibodies. Individual $CD34^+CD38^-$ cells were sorted into 96-well plates (NUNC, Roskilde, Denmark) using the Automated Cell Deposition Unit (ACDU) on a FACSvantage-SE flow cytometry system (BD Immunosystems, Heidelberg, Germany) equipped with an Apple G3 Power computer (Palo Alto, CA). To ensure that only a single cell was deposited, the ACDU was set up in a low-event "through-put" (200-500 events/second).

Cell culture (bulk proliferation assays)

Freshly enriched and PKH2-stained $CD34^+$ cells were cultured in a humidified atmosphere at 37°C and 5% CO_2 at a density of approximately 1×10^5 cells/mL in serum containing tissue-culture medium (Myelocult H5100; Stemcell Technologies, Vancouver, BC, Canada) supplemented with 1000 U/mL penicillin and 100 U/mL streptomycin (Invitrogen, Karlsruhe, Germany) in the presence of early-acting (recombinant human fetal liver tyrosine kinase 3 ligand [rhFLT3L], recombinant human stem-cell factor [rhSCF], and recombinant human thrombopoietin [rhTPO], each at 10 ng/mL final concentration; PeproTech, Rocky Hill, NJ) or late-acting cytokines (10 ng/mL recombinant human interleukin-3 [rhIL-3], 500 U/mL rhIL-6, 10 ng/mL recombinant human granulocyte-macrophage colony-stimulating factor [rhGM-CSF], 2.5 ng/mL recombinant human basic fibroblast growth factor [rh-bFGF],10 ng/mL recombinant human insulin-like growth factor-1 [rhILGF; all Cell Systems, St Katharinen, Germany], 2.5 U/mL recombinant human erythropoietin [rhEpo; Roche Diagnostics, Mannheim, Germany], and 50 ng/mL rhSCF [R&D Systems, Minneapolis, MN]), respectively.

Functional assays (single-cell experiments)

Individual sorted $CD34^+CD38^-$ cells were cultured for 10 days in Myelocult (Cell Systems) supplemented with 10 ng/mL rhIL-3, 500 U/mL rhIL-6, 10 ng/mL rhGM-CSF, 2.5 ng/mL rhbFGF, 10 ng/mL rhILGF (all Cell Systems), 2.5 U/mL rhEpo (Roche), 50 ng/mL rhSCF (R&D Systems), 1000 U/mL penicillin, and 100 U/mL streptomycin (Invitrogen) as described before.[19]

Before cell deposition the 96-well plates (Nunc) were precoated with bovine serum albumin (BSA) as described previously,[19,21] and each well was subsequently filled with 150 μL culture medium as described. After single-cell deposition, cells were maintained in a humidified atmosphere at 37°C and 5% CO_2 and fed every 48 hours with fresh medium. The proliferation of each cell was determined by light microscopy every 12 to 24 hours.

Immediately after single cells divided, the corresponding daughter cells were separated using a customized micromanipulation-system (Nikon-Germany, Düsseldorf, Germany) and individually transferred into the myeloid-lymphoid–initiating cell (ML-IC) assay.

ML-IC assay

This readout assay was described extensively in our previous reports.[19,21] Briefly, separated daughter cells were individually deposited into 96-well plates (Nunc) containing a confluent, irradiated AFT024 feeder-cell layer. After 2 weeks of culture in a humidified atmosphere at 37°C and 5% CO_2 in RPMI-medium containing 20% fetal bovine serum supplemented with 10 ng/mL rhFLT3L, 10 ng/mL rhSCF, 10 ng rhIL-7, and 10 ng/mL rhTPO (all Cell Systems), the content of each well was harvested by trypsination and split into 4 equal fractions. As described before, 2 fractions were transferred into duplicates of the lymphoid natural killer initiating cell (NK-IC) readout assay;[21,22] the other 2 fractions were transferred into duplicates of the myeloid LTC-IC assay.[19,21]

Culture conditions in readout assays

Lymphoid differentiation readout (NK-IC assay). Transferred cells were cocultured with the murine cell line AFT024 in Dulbecco modified Eagle medium (DMEM) and Ham F12-medium (Invitrogen) mixed in a 2:1 (vol/vol) relation containing 20% heat-inactivated human AB serum (Cambrex, Taufkirchen, Germany), ascorbic acid (20 mg/mL; Invitrogen), selenium selenite (50 μM; Invitrogen), 2-mercaptoethanol (25 μM), and ethanolamine (50 μM; Invitrogen). The following cytokines were added to these cultures: rhIL-2 (1000 U/mL), rhIL-3 (5 ng/mL), rhIL-7 (20 ng/mL), rhSCF (10 ng/mL), and rhFlt3L (10 ng/mL). At weekly intervals half-media exchanges were performed using 10% instead of 20% human AB serum. Starting at week 2, the only cytokine added to the cultures was rhIL-2. After 5 to 7 weeks of culture, wells containing viable cells were harvested and cells were analyzed flow cytometrically using antibodies recognizing the NK cell–specific antigens CD16 and CD56 as well as CD3.

Confirming previous studies from our group and others the cells arising in the NK-IC assay express the NK cell–specific antigens CD16 and CD56 and are negative for the T-cell marker CD3. As such cells are able to kill cells of the commonly used NK cell target cell line K562, they are considered as functional NK cells.[19,21-24] In agreement with this assumption, we found in our ongoing experiments that the arising cells express additional NK cell–specific antigens like NKp30/NKp44/NKp46 and NKG2A/CD94 as well as different killer immunoglobulin-like receptor (KIR) transcripts (M.P. and M. Uhrberg, manuscript in preparation).

Myeloid differentiation readout (LTC-IC assay). Transferred cells were cocultured with the murine cell line AFT024 in Iscove modified Dulbecco medium (IMDM; Invitrogen) supplemented with 12.5% fetal calf serum (FCS), 12.5% horse serum (Cell Systems), 2 mM L-glutamine (Invitrogen), 1000 U/mL penicillin, 100 U/mL streptomycin (Invitrogen), and 10^{-6} M hydrocortisone. Cultures were maintained for 5 weeks in a humidified atmosphere at 37°C and 5% CO_2 and fed once a week. At week 5 all wells were overlaid with clonogenic methylcellulose medium (Methylcellulose [Fluka, Buchs, Switzerland] in a final concentration of 1.12% containing IMDM and supplemented with 30% FCS, 3 U/mL erythropoietin [Cell Systems], and supernatant of the bladder carcinoma cell line 5637 [10%]). Wells were scored for the occurrence of secondary colony-forming cells (CFCs) after an additional 2 weeks. As the initially deposited cells and their offspring were cultured for more than 8 weeks before reading out the secondary colony-forming cells as LTC-ICs, our myeloid readout reflects more primitive myeloid progenitors than those detected in conventional LTC-IC assays.

Statistics

Experimental results from different experiments were reported as mean ± standard deviation of the mean (SD). Significance analyses were performed with the paired Student *t* test.

Results

Experimental design

We and others have demonstrated that primitive human hematopoietic cells give rise to daughter cells following different proliferation kinetics.[17,19,20] However, the cell fate of primitive hematopoietic cell siblings has not been analyzed at the single-cell level until now. Therefore, we decided to determine the developmental capacity of arising daughter cells individually. Because it is challenging to identify a single human primitive hematopoietic cell and subsequently separate its offspring, we compared the effect of 2 different culture conditions on the proliferation kinetics of primitive hematopoietic cells in bulk experiments first. For these analyses we assessed effects of 2 distinct cytokine cocktails, which we have successfully applied in our previous analysis,[19,24] 1 cocktail containing late-acting cytokines (LACs), and the other consisting of early-acting cytokines (EAC; ie, SCF, FLT3L, and TPO). The latter cocktail has been shown to be particularly effective in maintaining and slightly expanding human HSCs in suspension cultures up to 7 days.[25-27] In order to compare the data presented here with our former results we performed our experiments in Dexter-type cultures.

Proliferation kinetics of primitive hematopoietic cells under different cytokine conditions

Isolated $CD34^+$ cells (89.9% ± 5.9% purity; n = 3) were labeled with the fluorescent dye PKH2 and cultured up to a week in serum containing media supplemented either with EACs or LACs. Starting at day 3 cultured cells from individual aliquots were harvested every other day and simultaneously counterstained with antibodies recognizing the human stem-cell surrogate markers CD34 and CD133.[28,29]

By analyzing the influence of the culture conditions on $CD34^+$ cells we observed that under both conditions cells increase in size and granularity (Figure 1A). Cells cultured in the presence of LACs expand more (45.2-fold ± 17.7-fold at day 5 compared with day 0; n = 3) than in the presence of EACs (14.7-fold ± 5.6-fold at day 5 compared with day 0; n = 3). Under both conditions most cells remain $CD34^+$ until day 5 (Figure 1B) (LACs, 61.4% ± 20.2%; n = 3; EACs, 85.5% ± 10.4%; n = 3), while the percentage of $CD133^+$ cells decreases over time (Figure 1B) (LACs, 14.6% ± 6.7%; n = 3; EACs, 33.0% ± 5.2%; n = 3 at day 5). However, the absolute numbers of $CD133^+$ cells increase under both conditions (LACs, 8.6-fold ± 1.0-fold; n = 3; EACs, 5.4-fold ± 1.0-fold at day 5 compared with day 0; n = 3).

In addition, we show that under both conditions the average of the newly detected $CD34^+CD133^-$ cells is less positive for PKH2 than $CD34^+CD133^+$ cells, suggesting that most arising $CD34^+CD133^-$ cells have had a higher proliferation rate than the $CD34^+CD133^+$ cells (Figure 1C). Regarding their PKH2 staining, the LAC-stimulated $CD34^+CD133^+$ cell fraction is more heterogeneous than the corresponding EAC-stimulated fraction (Figure 1C), suggesting that EACs stimulate proliferation of $CD34^+CD133^+$ cells more homogeneously than LAC conditions. Within the LAC-stimulated fraction we observed more remaining PKH^{bright} $CD34^+CD133^+$ cells than in the EAC-stimulated fraction (at day 5: LACs, 47.7% ± 24.1%; EACs, 39.4% ± 28.3% of the initially cultured PKH^{bright} $CD34^+CD133^+$ cells, n = 3, $P = .05$; Figure 1C). Remarkably, the PKH^{bright} fraction of LAC-stimulated cells contain less $CD133^-$ cells than the EAC-stimulated fraction, resulting in a sharp contrast of PKH^{bright} $CD34^+CD133^+$ versus PKH^+ $CD34^+CD133^-$ cells within the LAC-stimulated fraction (Figure 1C).

According to our previous results primitive hematopoietic cells cultured under LAC conditions get highly enriched in the PKH^{bright} or the so-called slow dividing fraction.[19,24] This is most likely due

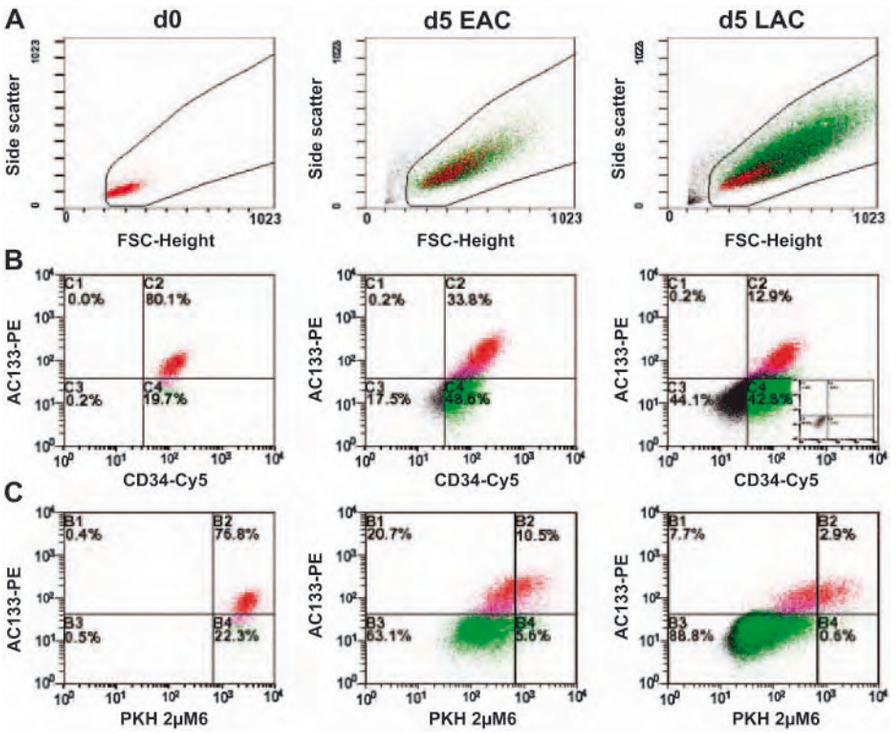


Figure 1. Flow cytometric analysis of bulk experiments. Since the amount of cells depicted in the plots is normalized to the cell number initially used, plots can be compared semiquantitatively. Cells analyzed in panels B and C were gated according to the morphology depicted on the forward scatter/side scatter plots shown in panel A. Plots in panel B represent the distribution of CD34 and CD133 antigens in freshly isolated or in expanded $CD34^+$ cells, respectively. A characteristic isotype control of expanded cells is shown as inlet in the right panels. To identify the individual subpopulations in panels A or C, $CD34^+CD133^+$ cells are labeled in red, $CD34^+CD133^-$ cells in green, and $CD34^-CD133^-$ cells in black. The PKH2 staining, representing the number of cell divisions single cells have performed, is plotted against the CD133 antigen distribution in panel C. $PKH2^{bright}$ cells are plotted within the right quadrants of the diagrams shown in panel C. See "Results" for more details.

to the fact that the most primitive hematopoietic cells do not immediately respond to LACs and therefore remain quiescent for the first few days under LAC conditions. Since the vast majority of the $CD34^+$ cells get activated under EAC conditions and show similar cell division kinetics, the LAC conditions provide an opportunity to discriminate between primitive and more mature $CD34^+$ cells according to just the way they initiate their cell division. Therefore, we concluded that LAC conditions were more suitable for our intended single-cell studies on primitive hematopoietic cells than EAC conditions.

Our conclusion was additionally supported by the finding that the percentage of the stem-cell surrogate marker CD133 within the slow dividing fraction (PKH^{bright} cells) was significantly higher under LAC than under EAC conditions (at day 5: LAC, 61.1% ± 3.1%; EAC, 50.3% ± 3.9%; n = 3, $P = .005$).

Proliferation of individual primitive human hematopoietic cells

Due to the fact that freshly isolated $CD34^+$ cells express similar amounts of CD133 (Figure 1B) and with the necessity to enrich the most primitive hematopoietic cells as much as possible, we decided to perform our single-cell analyses with $CD34^+CD38^-$ cells.

In 4 independent experiments we sorted a total of 176 single $CD34^+CD38^-$ cells per experiment into individual wells of 96-well plates. To determine the deposition frequency we analyzed each well 12 hours after finishing the sorting procedure by bright-field microscopy. We recovered a total of 556 single deposited cells, which corresponds to a deposition frequency of 79.0% ± 4.0%.

In previous experiments under LAC conditions, the most primitive hematopoietic cells remained quiescent for up to 5 days and underwent their first in vitro cell division between day 5 and day 10 after deposition.[19] Therefore, we tracked the division process of each single cell within the first 10 days and grouped them into 3 different categories as shown in Table 1: (1) category I indicates cells that divided before day 5 (59% ± 12%); (2) category II, cells that performed their first cell division between day 5 and day 10 (31% ± 11%); and (3) category III, cells which did not perform any cell division within the first 10 days of culture (10% ± 4%).

Analyses of the developmental potential of individually separated daughter cells

According to our focus on the most primitive human hematopoietic cells we only included cells from category II for the micromanipulation-based daughter cell analyses. Within 24 hours after performing their initial cell division, emerging daughter cells of category II cells were separated by micromanipulation and individually transferred into expansion cultures containing the murine fetal liver–derived stromal cell line AFT024.[21] As shown in Figure 2, the expansion cultures were harvested after 2 weeks and split into 4 separate aliquots, which were transferred into secondary readout systems: 2 into primitive myeloid assays (LTC-IC) and 2 into primitive lymphoid assays (NK-IC). This enabled us to determine whether initially deposited cells gave rise to primitive progeny in any of the functional assays.

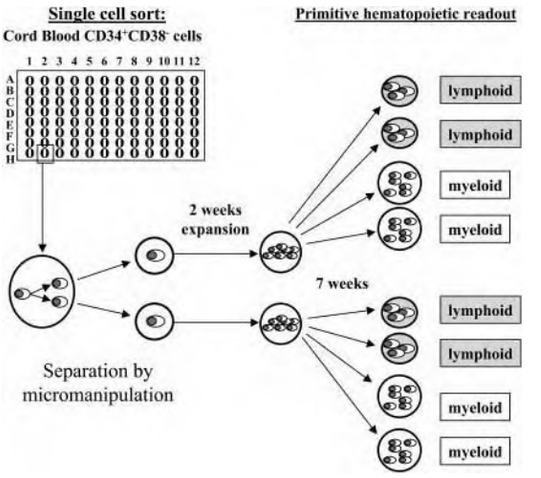


Figure 2. Experimental setup. Individual $CD34^+CD38^-$ cells were deposited by fluorescent cell sorting into 96-well plates (1 cell/well), and observed at half-daily intervals. Initially deposited cells that performed their initial cell division between culture days 5 and 10 (category II) were considered for further analyses. Shortly after the first cell division, the arising daughter cells were separated by micromanipulation and individually transferred into secondary plates containing irradiated AFT024 cells as a stromal feeder. After 2 weeks of expansion the entire progeny of each individual daughter cell was split into 4 aliquots and transferred in equal amounts (in duplicates) into primitive myeloid (LTC-IC) or primitive lymphoid (NK-IC) readout assays, respectively. After an additional 7 weeks the assays were analyzed as described in "Materials and methods." Originally deposited cells as well as singularized daughter cells that had both LTC-IC as well as NK-IC capacity were retrospectively considered ML-ICs.[21]

As shown in Table 2 we formed 3 groups, discriminating initially deposited cells which gave rise to (1) 2 colony forming daughter cells (category IIa; 55 pairs of daughter cells); (2) those in which only 1 daughter cell formed colonies (category IIb; 21 pairs); and (3) the ones in which none of the daughter cells formed any colonies (category IIc; 101 pairs).

Category IIa and IIb cells, 76 cells in total, were further subgrouped regarding to the cell fate adopted by the individual daughter cells (Table 3). We used the following definitions: (1) initially plated cells that only gave rise to primitive myeloid hematopoiesis were defined as LTC-ICs; (2) those that gave rise only to NK cells were defined as NK-ICs; and (3) cells that were able to generate both LTC-ICs as well as NK-ICs were defined as ML-ICs, closely related to the most primitive human hematopoietic compartment.[21]

In several cases colonies were found in the LTC-IC assays that did not fulfill the well-defined morphologic criteria of secondary colony formation. Since they resembled a macrophage-like morphology without clonogenic proliferation we did not categorize them as

Table 1. Categorization of individual $CD34^+CD38^-$ cells according to the occurrence of their initial in vitro cell division under LAC-stimulated culture conditions

	Deposited cells	Category I: first division before day 5	Category II: first division days 5-10	Category III: first division after day 10
Experiment 1	130	93	26	11
Experiment 2	147	65	63	19
Experiment 3	139	78	54	7
Experiment 4	140	90	34	16
Total cell no. (mean % ± SEM)	556	326 (59.0 ± 11.7)	177 (31.5 ± 11.1)	53 (9.5 ± 3.5)

Values in table are total numbers of cells except where indicated.

Table 2. Categorization of individual CD34$^+$CD38$^-$ cells performing their initial in vitro cell division under LAC-stimulated culture conditions between days 5 and 10

	Investigated daughter cell pairs	Category IIa: 2 colony-initiating daughter cells	Category IIb: 1 colony-initiating daughter cell	Category IIc: No colony-initiating daughter cells
Experiment 1	26	3	1	22
Experiment 2	63	16	5	42
Experiment 3	54	13	7	34
Experiment 4	34	23	8	3
Total cell no. (mean % ± SEM)	177	55 (32.2 ± 24.5)	21 (12.1 ± 8.5)	101 (55.8 ± 32.7)

Values in table are total numbers of cells except where indicated.

LTC-ICs but as macrophage colony-forming units (CFUs-M). Remarkably, we found many cases in which individual cells had the NK-IC capacity and gave rise to CFU-M–forming progeny. To discriminate these cells from ML-ICs we called them macrophage/NK cell–initiating cells (M-NK-ICs).

In total, 15 of the original deposited cells had the ML-IC capacity; 2 of them transmitted the ML-IC fate to both daughter cells, and in the remaining cases only 1 of the daughter cells adopted both, the LTC-IC as well as the NK-IC capacity (Table 4). Remarkably, in 6 (40%) cases the non–ML-IC daughter cell gave rise to NK-ICs and formed macrophage-like colonies in the LTC-IC assay, demonstrating that viable offspring of the corresponding daughter cells were transferred into the latter assay. These results suggest that in these cases the first-generation ML-IC daughter cells contained different developmental capacities. Surprisingly, we never found the constellation in which 1 ML-IC daughter cell adopted the myeloid and the other 1 the lymphoid potential.

Only 3 of the 76 originally deposited cells were determined exclusively as LTC-ICs; 2 of them gave rise to daughter cells, both containing the LTC-IC potential (Table 4). Originally deposited cells of the newly defined M-NK-IC group transmitted this cell fate to both daughter cells in 46% of the cases studied, and to only 1 daughter cell in 54% (Table 4). In the latter cases the non–M-NK-IC daughter cell died or gave rise to macrophage-like cells in LTC-IC assays. We never found any daughter cell that had the NK-IC potential only. Seventeen of the originally deposited cells had NK-IC potential only, which was transmitted in 7 cases to both daughter cells; in the remaining 10 cases only 1 of the siblings gave rise to NK cells in our assays (Table 4). Most of the cells (13 of 17) that were retrospectively named CFUs-M transmitted this fate to both daughter cells; in the remaining cases, only 1 daughter cell gave rise to the macrophage-like cells.

In summary, most of the originally deposited cells, retrospectively determined as ML-ICs, gave rise to daughter cells with different cell fates. In contrast, siblings of more-committed mother cells seem to inherit a higher ratio of identical cell fates in our assays (Table 4).

Discussion

Here, we analyzed primitive human hematopoietic cells in bulk cultures and at a single-cell level, and report 3 major findings: (1) we realized that upon cultivation CD34$^+$ cells split up into a CD34$^+$CD133$^+$ and a CD34$^+$CD133$^-$ fraction; (2) using our newly established single-cell separation approach we determined the cell fate of primitive human hematopoietic cells and their first-generation daughter cells individually and present evidence for the existence of progenitor cells containing the capacity to form NK cells and macrophages, but which lack more primitive myeloid capacities (LTC-ICs); and (3) we demonstrate that the most primitive ML-ICs have a high tendency (87%) to transmit their cell fate to only 1 of the arising daughter cells. In contrast, the ratio of more-committed progenitor cells giving rise to progeny adopting identical cell fates to those adopting different cell fates is more balanced.

Although there is increasing evidence that special hematopoietic niches are required to maintain HSCs,[9-11] it is often suggested that hematopoietic stem cells can divide asymmetrically to form another HSC and a more-specified daughter cell.[30] The latter hypothesis is highly supported by the finding that immediate progeny of primitive hematopoietic progenitor cells often adopt different cell fates in myeloid readout systems.[12-14,16,18] However, as mentioned by Takano and colleagues, due to the lack of an appropriate assay, the lymphoid potential of separated cells was not analyzed in these studies.[16] In this study we used the ML-IC assay, a sensitive and efficient assay for the detection of both the myeloid and lymphoid potentials of individual cells,[19,21] and analyzed the myeloid and lymphoid potentials of progenies of primitive human hematopoietic cells at the single-cell level.

Supporting our previous data, we found that under LAC conditions approximately 30% of the deposited CD34$^+$CD38$^-$ cells performed their initial cell division between culture day 5 and 10.[19] We were able to determine retrospectively the cell fate of approximately 45% of the initially deposited cells that performed their first cell division between culture days 5 and 10. In 55% of the cases studied none of the separated daughter cells gave rise to any recognizable colony in our readout systems. Since similar frequencies were obtained in our previous and other studies, in which nonseparated CD34$^+$CD38$^-$ cells were analyzed in ML-IC assays,[19,31] we suggest that loss of cells during the micromanipulation procedure is more or less negligible. The efficiency of our micromanipulation procedure is additionally supported by the fact that we obtain similar ML-IC frequencies of nonseparated and separated CD34$^+$CD38$^-$ cells performing their first cell division under LAC conditions between culture days 5 and 10 (15 [8.5%] cells of 177) or after day 5 (10.3% ± 3.5%),[19] respectively.

In this context it should be mentioned that all but 1 initially deposited cell, which gave rise to only 1 colony-forming daughter cell, were retrospectively determined to be more mature cells (M-NK-ICs, NK-ICs, or CFUs-M), suggesting that the non–colony-forming daughter cell was more committed and terminally differentiated during the long-term culture period.

It is interesting to note that using the ML-IC assay we could identify a novel human progenitor cell that has not been described before. This progenitor, which we called M-NK-IC, has the capability to initiate NK cell development and also gives rise to macrophages but not to secondary clonogenic myeloid colonies in LTC-IC assays. Similar to our findings, murine fetal liver but not adult hematopoietic progenitors have been described as containing

Table 3. Cell fate classification of initially deposited colony-initiating CD34$^+$CD38$^-$ cells

	Category IIa and IIb cells	ML-ICs	LTC-ICs	M-NK-ICs	NK-ICs	CFUs-M
Experiment 1	4	1	0	1	2	0
Experiment 2	21	5	2	5	2	7
Experiment 3	20	4	0	5	8	3
Experiment 4	31	5	1	13	5	7
Total cell no. (mean % ± SEM)	76	15 (21.2 ± 4.0)	3 (3.2 ± 4.5)	24 (28.9 ± 8.7)	17 (28.9 ± 19.2)	17 (17.7 ± 14.0)

Values in table are total numbers of cells except where indicated.

lymphoid and macrophage potential in short-term murine readout systems.[32,33]

According to the prevailing model of hematopoiesis, primitive hematopoietic cells give rise to common myeloid and common lymphoid progenitor cells, which have the developmental capacity to form all myeloid or lymphoid lineages, respectively.[34] Because macrophages belong to the myeloid compartment, the discovery of progenitors containing the M-NK-IC capacity is not compatible with this model. In addition, the recent discovery of primitive hematopoietic cells in mice and humans which contain the lymphoid potential as well as the capacity to form granulocytes and macrophages but not cells of the erythromegakaryocytic lineage, is contrary to this model as well.[35-37] In this context, Adolfsson and colleagues offered a so-called composite model in which primitive hematopoietic cells sequentially lose the capacity to form cells of the megakaryocyte/erythroid and then of the granulocyte/macrophage potential during lymphoid commitment.[35] Our findings together with those of others[32,33,38] support this hypothesis and further suggest that next to the megakaryocyte/erythroid potential the granulocytic developmental capacity is lost during early lymphoid commitment, leaving cells containing the capacity to initiate lymphoid and macrophage development.

In previous studies it was found that siblings of primitive hematopoietic cells often adopt different proliferation kinetics, whereas more-committed progenitor cells give rise to daughter cells dividing in a more uniform fashion. Differences in the proliferation kinetics are often interpreted as a result of an asymmetric cell division.[17,19,20] The theory of asymmetric cell division is further supported by the finding that individual daughter cells of primitive hematopoietic cells frequently adopt different cell fates in myeloid progenitor assays.[12-14,16] However, until now there was no evidence that the most primitive hematopoietic cells containing the lymphoid as well as the myeloid developmental potential give rise to daughter cells adopting different cell fates. Therefore, we demonstrate here for the first time evidence that the most primitive, in vitro–detectable hematopoietic cells, the ML-ICs, give rise to daughter cells adopting different cell fates, which could be the result of an asymmetric cell division. In addition, we provide evidence that offspring of more-committed progenitors that seem to contain similar proliferation kinetics also give rise to cells adopting different cell fates in approximately 50% of the cases studied. These findings demonstrate that proliferation kinetics of arising daughter cells cannot be interpreted as indication for the occurrence of asymmetric cell divisions.

Table 4. Cell fate classification of separated offspring of initially deposited colony-initiating CD34$^+$CD38$^-$ cells

	DC no. 1				
DC no. 2	ML-ICs	LTC-ICs	M-NK-ICs	NK-ICs	CFUs-M
ML-ICs	2	—	—	—	—
LTC-ICs	3	2	—	—	—
M-NK-ICs	6	0	11	0	—
NK-ICs	1	1	0	7	—
CFUs-M	2	0	8	0	13
Dead	1	0	5	10	4
Identical cell fate, no. (%)	2 (13.3)	2 (66.7)	11 (45.8)	7 (41.2)	13 (76.5)
Different cell fate, no. (%)	13 (86.7)	1 (33.3)	13 (54.2)	10 (58.8)	4 (23.5)

The more primitive separated daughter cell is defined as DC no. 1, the other as DC no. 2. Values in table are total numbers of cells except where indicated.

— indicates not applicable.

Surprisingly, none of the deposited cells gave rise to siblings that both adopted only partial capacities of their mother cells. In all cases studied at least 1 of the arising daughter cells took over the developmental capacity of the mother cell, most likely resembling the predicted process of self-renewal. Committed progenitors have a high tendency to expand their cell fate, especially by forming 2 daughter cells containing the same developmental capacity. Cells of the most primitive compartment, the ML-ICs, have a very low tendency to transmit their cell fate to both of the arising daughter cells, which is consistent with the hypothesis that primitive hematopoietic cells have a high tendency to divide asymmetrically.[30]

However, can we really conclude from these data that there are asymmetric cell divisions within the primitive hematopoietic cell compartment? It depends how we define "asymmetric cell division." If we define it from the point of the adopted cell fate, our data would fulfill these criteria. If we define asymmetric cell division as it is used in model organisms such as *Drosophila* or *Caenorhabditis elegans,* in which an asymmetric cell division describes the process in which 2 qualitatively different cells are formed by the different distribution of certain factors which might act as cell fate determinants,[39] our data will not fulfill these criteria. In principle, it could be that even cells that form daughter cells adopting different cell fates divide in a symmetric way, giving rise to 2 equally specified daughter cells. Since the daughter cells in our as well as in other experiments had the ability to stay in close contact with each other before they were separated, they could theoretically have influenced each other's developmental capacity after mitosis. Developmental processes like that are well described. For example, the Notch-mediated process of lateral inhibition selects a single cell within a group of equivalent cells to adopt another cell fate than the remaining cells of that group.[40,41] As Notch is required to maintain primitive hematopoietic cells in an undifferentiated state,[42] it might be possible that 1 of the daughter cells activates the Notch signaling pathway in a process of lateral inhibition in its sister cell, resulting in the maintenance of the primitive cell fate in only 1 of the 2 cells.

Thus, we summarize that our data and data presented before are not sufficient to conclude that primitive hematopoietic cells can indeed divide asymmetrically. To unequivocally demonstrate that hematopoietic cells can divide asymmetrically, markers need to be defined which clearly segregate unequally within mitotic cells. We have learned from model organisms that all cells which divide asymmetrically are polarized during cell division and localize certain molecules to distinct regions of the cells, which then get

transmitted in an unequal way.[39] As we recently could show that several surface molecules, especially CD133, become distributed in a localized fashion in cultivated primitive hematopoietic cells,[43] it will be interesting to analyze the distribution of these markers in dividing primitive hematopoietic cells. As we realized that the more primitive $CD34^+$ cells of the slow-dividing fraction specifically express CD133, it might be possible that upon cell division CD133 segregates into one of the arising daughter cells, perhaps confirming the concept of asymmetric cell division within the primitive hematopoietic cell compartment.

Acknowledgments

The authors like to thank Rainer Saffrich and Martin Peschel (Nikon-Germany) for their excellent support in establishing the micromanipulation system, as well as Volker Eckstein, Johannes Fischer, and Katrin Miesala for their technical support. Umbilical cord blood samples were kindly provided by the Department of Gynecology and Obstetrics of the University of Heidelberg and by Gesine Kögler of the Heinrich-Heine University, Düsseldorf.

References

1. Korbling M, Anderlini P. Peripheral blood stem cell versus bone marrow allotransplantation: does the source of hematopoietic stem cells matter? Blood. 2001;98:2900-2908.
2. Iscove NN, Nawa K. Hematopoietic stem cells expand during serial transplantation in vivo without apparent exhaustion. Curr Biol. 1997;7:805-808.
3. Krause DS, Theise ND, Collector MI, et al. Multi-organ, multi-lineage engraftment by a single bone marrow-derived stem cell. Cell. 2001;105:369-377.
4. Moore KA, Ema H, Lemischka IR. In vitro maintenance of highly purified, transplantable hematopoietic stem cells. Blood. 1997;89:4337-4347.
5. Nolta JA, Thiemann FT, Arakawa-Hoyt J, et al. The AFT024 stromal cell line supports long-term ex vivo maintenance of engrafting multipotent human hematopoietic progenitors. Leukemia. 2002;16:352-361.
6. Punzel M, Moore KA, Lemischka IR, Verfaillie CM. The type of stromal feeder used in limiting dilution assays influences frequency and maintenance assessment of human long-term culture initiating cells. Leukemia. 1999;13:92-97.
7. Thiemann FT, Moore KA, Smogorzewska EM, Lemischka IR, Crooks GM. The murine stromal cell line AFT024 acts specifically on human CD34+CD38- progenitors to maintain primitive function and immunophenotype in vitro. Exp Hematol. 1998;26:612-619.
8. Shih CC, Hu MC, Hu J, Medeiros J, Forman SJ. Long-term ex vivo maintenance and expansion of transplantable human hematopoietic stem cells. Blood. 1999;94:1623-1636.
9. Arai F, Hirao A, Ohmura M, et al. Tie2/Angiopoietin-1 signaling regulates hematopoietic stem cell quiescence in the bone marrow niche. Cell. 2004; 118:149-161.
10. Calvi LM, Adams GB, Weibrecht KW, et al. Osteoblastic cells regulate the haematopoietic stem cell niche. Nature. 2003;425:841-846.
11. Zhang J, Niu C, Ye L, et al. Identification of the haematopoietic stem cell niche and control of the niche size. Nature. 2003;425:836-841.
12. Suda T, Suda J, Ogawa M. Disparate differentiation in mouse hemopoietic colonies derived from paired progenitors. Proc Natl Acad Sci U S A. 1984;81:2520-2524.
13. Suda J, Suda T, Ogawa M. Analysis of differentiation of mouse hemopoietic stem cells in culture by sequential replating of paired progenitors. Blood. 1984;64:393-399.
14. Leary AG, Strauss LC, Civin CI, Ogawa M. Disparate differentiation in hemopoietic colonies derived from human paired progenitors. Blood. 1985;66:327-332.
15. Ema H, Takano H, Sudo K, Nakauchi H. In vitro self-renewal division of hematopoietic stem cells. J Exp Med. 2000;192:1281-1288.
16. Takano H, Ema H, Sudo K, Nakauchi H. Asymmetric division and lineage commitment at the level of hematopoietic stem cells: inference from differentiation in daughter cell and granddaughter cell pairs. J Exp Med. 2004;199:295-302.
17. Brummendorf TH, Dragowska W, Zijlmans J, Thornbury G, Lansdorp PM. Asymmetric cell divisions sustain long-term hematopoiesis from single-sorted human fetal liver cells. J Exp Med. 1998;188:1117-1124.
18. Mayani H, Dragowska W, Lansdorp PM. Lineage commitment in human hemopoiesis involves asymmetric cell division of multipotent progenitors and does not appear to be influenced by cytokines. J Cell Physiol. 1993;157:579-586.
19. Punzel M, Zhang T, Liu D, Eckstein V, Ho AD. Functional analysis of initial cell divisions defines the subsequent fate of individual human CD34(+)CD38(-) cells. Exp Hematol. 2002;30: 464-472.
20. Huang S, Law P, Francis K, Palsson BO, Ho AD. Symmetry of initial cell divisions among primitive hematopoietic progenitors is independent of ontogenic age and regulatory molecules. Blood. 1999; 94:2595-2604.
21. Punzel M, Wissink SD, Miller JS, Moore KA, Lemischka IR, Verfaillie CM. The myeloid-lymphoid initiating cell (ML-IC) assay assesses the fate of multipotent human progenitors in vitro. Blood. 1999;93:3750-3756.
22. Miller JS, McCullar V, Punzel M, Lemischka IR, Moore KA. Single adult human CD34(+)/Lin-/CD38(-) progenitors give rise to natural killer cells, B-lineage cells, dendritic cells, and myeloid cells. Blood. 1999;93:96-106.
23. Miller JS, McCullar V. Human natural killer cells with polyclonal lectin and immunoglobulinlike receptors develop from single hematopoietic stem cells with preferential expression of NKG2A and KIR2DL2/L3/S2. Blood. 2001;98:705-713.
24. Punzel M, Liu D, Zhang T, Eckstein V, Miesala K, Ho AD. The symmetry of initial divisions of human hematopoietic progenitors is altered only by the cellular microenvironment. Exp Hematol. 2003; 31:339-347.
25. Kobayashi M, Laver JH, Lyman SD, Kato T, Miyazaki H, Ogawa M. Thrombopoietin, steel factor and the ligand for flt3/flk2 interact to stimulate the proliferation of human hematopoietic progenitors in culture. Int J Hematol. 1997;66:423-434.
26. Luens KM, Travis MA, Chen BP, Hill BL, Scollay R, Murray LJ. Thrombopoietin, kit ligand, and flk2/flt3 ligand together induce increased numbers of primitive hematopoietic progenitors from human CD34+Thy-1+Lin- cells with preserved ability to engraft SCID-hu bone. Blood. 1998;91: 1206-1215.
27. Ohmizono Y, Sakabe H, Kimura T, et al. Thrombopoietin augments ex vivo expansion of human cord blood-derived hematopoietic progenitors in combination with stem cell factor and flt3 ligand. Leukemia. 1997;11:524-530.
28. Krause DS, Fackler MJ, Civin CI, May WS. CD34: structure, biology, and clinical utility. Blood. 1996; 87:1-13.
29. Yin AH, Miraglia S, Zanjani ED, et al. AC133, a novel marker for human hematopoietic stem and progenitor cells. Blood. 1997;90:5002-5012.
30. Ho AD. Kinetics and symmetry of divisions of hematopoietic stem cells. Exp Hematol. 2005;33: 1-8.
31. Theunissen K, Verfaillie CM. A multifactorial analysis of umbilical cord blood, adult bone marrow and mobilized peripheral blood progenitors using the improved ML-IC assay. Exp Hematol. 2005; 33:165-172.
32. Lacaud G, Carlsson L, Keller G. Identification of a fetal hematopoietic precursor with B cell, T cell, and macrophage potential. Immunity. 1998;9: 827-838.
33. Mebius RE, Miyamoto T, Christensen J, et al. The fetal liver counterpart of adult common lymphoid progenitors gives rise to all lymphoid lineages, CD45+CD4+CD3- cells, as well as macrophages. J Immunol. 2001;166:6593-6601.
34. Reya T, Morrison SJ, Clarke MF, Weissman IL. Stem cells, cancer, and cancer stem cells. Nature. 2001;414:105-111.
35. Adolfsson J, Mansson R, Buza-Vidas N, et al. Identification of Flt3+ lympho-myeloid stem cells lacking erythro-megakaryocytic potential a revised road map for adult blood lineage commitment. Cell. 2005;121:295-306.
36. Haddad R, Guardiola P, Izac B, et al. Molecular characterization of early human T/NK and B-lymphoid progenitor cells in umbilical cord blood. Blood. 2004;104:3918-3926.
37. Lu M, Kawamoto H, Katsube Y, Ikawa T, Katsura Y. The common myelolymphoid progenitor: a key intermediate stage in hemopoiesis generating T and B cells. J Immunol. 2002;169:3519-3525.
38. Cumano A, Paige CJ, Iscove NN, Brady G. Bipotential precursors of B cells and macrophages in murine fetal liver. Nature. 1992;356:612-615.
39. Betschinger J, Knoblich JA. Dare to be different: asymmetric cell division in Drosophila, C. elegans and vertebrates. Curr Biol. 2004;14: R674–685.
40. Giebel B. The notch signaling pathway is required to specify muscle progenitor cells in Drosophila. Mech Dev. 1999;86:137-145.
41. Martinez Arias A, Zecchini V, Brennan K. CSL-independent Notch signalling: a checkpoint in cell fate decisions during development? Curr Opin Genet Dev. 2002;12:524-533.
42. Ohishi K, Varnum-Finney B, Bernstein ID. The notch pathway: modulation of cell fate decisions in hematopoiesis. Int J Hematol. 2002;75:449-459.
43. Giebel B, Corbeil D, Beckmann J, et al. Segregation of lipid raft markers including CD133 in polarized human hematopoietic stem and progenitor cells. Blood. 2004;104:2332-2338.

10.3 Nucleofection, an Efficient Nonviral Method to Transfer Genes into Human Hematopoietic Stem and Progenitor Cells (von Levetzow et al., 2006)

STEM CELLS AND DEVELOPMENT 15:278–285 (2006)

Technical Report

Nucleofection, an Efficient Nonviral Method to Transfer Genes into Human Hematopoietic Stem and Progenitor Cells

GREGOR VON LEVETZOW,[1,2] JAN SPANHOLTZ,[1,2] JULIA BECKMANN,[1] JOHANNES FISCHER,[1] GESINE KÖGLER,[1] PETER WERNET,[1] MICHAEL PUNZEL,[1] and BERND GIEBEL[1]

ABSTRACT

The targeted manipulation of the genetic program of single cells as well as of complete organisms has strongly enhanced our understanding of cellular and developmental processes and should also help to increase our knowledge of primary human stem cells, e.g., hematopoietic stem cells (HSCs), within the next few years. An essential requirement for such genetic approaches is the existence of a reliable and efficient method to introduce genetic elements into living cells. Retro- and lentiviral techniques are efficient in transducing primary human HSCs, but remain labor and time consuming and require special safety conditions, which do not exist in many laboratories. In our study, we have optimized the nucleofection technology, a modified electroporation strategy, to introduce plasmid DNA into freshly isolated human HSC-enriched $CD34^+$ cells. Using enhanced green fluorescent protein (eGFP)-encoding plasmids, we obtained transfection efficiencies of approximately 80% and a mean survival rate of 50%. Performing functional assays using GFU-GEMM and long-term culture initiating cells (LTC-IC), we demonstrate that apart from a reduction in the survival rate the nucleofection method itself does not recognizably change the short- or long-term cell fate of primitive hematopoietic cells. Therefore, we conclude, the nucleofection method is a reliable and efficient method to manipulate primitive hematopoietic cells genetically.

INTRODUCTION

FOR MORE THAN 30 YEARS, hematopoietic stem cells (HSCs) have been successfully used in a various number of clinical applications (1). However, the current understanding about the molecular and cellular mechanisms governing the biology of these cells remains limited. In a variety of different organisms (e.g., *Drosophila* and *Caenorhabditis elegans*) or cellular systems, the understanding of different cellular processes was fundamentally improved by genetic approaches (2). In this regard over- or ectopic-expression studies, as well as inhibition of expression by RNA interference, revealed important results, similar to naturally occurring mutations. Therefore, an effective method to manipulate human HSCs or hematopoietic progenitor cells (HPCs) genetically should help to increase the current understanding of the basic mechanisms governing the biology of human HSCs and HPCs. Additionally, an effective transfection method might help to improve HSC/HPC applications in clinical trials.

In principle, there are different strategies to manipulate human HSCs/HPCs genetically, i.e., techniques based on viral or nonviral gene transfer. Although viral strategies are highly efficient to transfer genes into primary cells (up to 90%) (3), they are time consuming and require special safety precautions to minimize the risk of

[1]Institute for Transplantation Diagnostics and Cell Therapeutics, Heinrich-Heine-University Düsseldorf, D-40225 Düsseldorf, Germany.

[2]Both authors contributed equally to this work.

exposure to biohazards (4), most nonviral techniques, e.g. electroporation, require a prestimulation of the corresponding cells and result in a maximal, regularly nonpermanent transfection efficiency of 30% (5,6).

Recently, a new and highly efficient nonviral method called nucleofection technology was described. This modified electroporation strategy, which delivers the transfected nucleic acids directly into the nuclei of most cell types investigated so far, permits transient transfection of primary cells without any prestimulation (7–10). Indeed, we and others have successfully applied this technique to transfect freshly isolated human $CD34^+$ cells and have obtained transfection efficiency rates of up to 80% when using green fluorescent protein (GFP)-encoding plasmids (11,12). Because the survival rate of transfected cells dropped significantly with decreasing cell numbers used in a single nucleofection reaction, we now have optimized the technology to transfect as little as 2×10^5 freshly isolated $CD34^+$ cells.

For many experiments, especially those involving immature cell types, it is required that the cell fate of treated cells is not significantly altered by the transfection method itself. Therefore, we have also assessed whether the optimized nucleofection technology has any influence on the developmental potential of human HSCs/HPCs.

MATERIALS AND METHODS

Cell preparation

Umbilical cord blood (CB) samples were obtained from donors after informed consent according to the Declaration of Helsinki. Mononuclear cells were isolated from individual samples by Ficoll (Biocoll Separating Solution, Biochrom AG, Berlin Germany) density gradient centrifugation. Remaining red blood cells were lysed at 4°C in 0.83% ammonium chloride with 0.1% potassium hydrogen carbonate, followed by a phosphate-buffered saline (PBS) washing step. $CD34^+$ cells were isolated by magnetic cell separation using the MidiMacs technique according to the manufacturer's instructions (Miltenyi Biotec, Bergisch Gladbach, Germany), yielding $CD34^+$ cells of 80.2 ± 8.6 % purity ($n = 19$).

Transfection of $CD34^+$ cells

The pEGFP-N1 vector (BD Clontech, Heidelberg, Germany) was amplified in *Escherichia coli* strain DH12S (Invitrogen GmbH, Karlsruhe, Germany) and purified using an Endofree-Plasmid-Maxi-preparation kit (Qiagen,Hilden, Germany) according to the manufacturer's instructions.

$CD34^+$-enriched cells were divided into aliquots of equal size ($2–5 \times 10^5$ cells per aliquot) and were either not transfected, transfected without DNA (mock), or transfected with 5 μg of the vector pEGFP-N1 as described in the text. By rinsing the transfection cuvettes two times with 1 ml of I20 [Iscove's modified Dulbecco's medium (Invitrogen GmbH) supplemented with 20% fetal calf serum (FCS; Biochrom, Berlin, Germany)] transfected cells were transferred into a 15-ml plastic tube and washed in a total volume of 10 ml of I20. Pelleted cells were incubated for 15 min in a humidified atmosphere at 37°C and 5% CO_2, then they were resuspended in 1 ml of Myleocult H5100 (Stem Cell Technologies Inc, Vancouver, Canada) supplemented with early-acting cytokines (fetal liver tyrosine kinase-3 ligand [FLT3L], stem cell factor [SCF], thrombopoietin [TPO], each at 10 ng/ml final concentration [all PeproTech, Inc., Rocky Hill, NJ]). Cells were cultured at a density of $\approx 1 \times 10^5$ cells/ml, either in the presence or absence of the general caspase inhibitor Z-VAD-FMK (BD Biosciences, Heidelberg, Germany) in a humidified atmosphere at 37°C and 5% CO_2.

Flow cytometry and cell sorting

After 2 days, cultivated mock- and GFP-transfected cells, as well as nontransfected cells, were stained with an anti-CD34-PeCy5 antibody (clone 581; BD Pharmingen, Heidelberg, Germany). $CD34^+$ cells of the controls and successfully transfected $CD34^+GFP^+$ cells were highly purified using a Coulter EPICS Elite ESP fluorescence cell-sorting system equipped with the Expo32 software (Beckman Coulter, Krefeld, Germany). For functional assays, defined numbers of appropriate cells were immediately sorted into corresponding media. Flow cytometric analyses were performed on a Cytomics FC 500 flowcytometer equipped with the RXP software (Beckman Coulter).

Functional assays

Primitive hematopoietic progenitors were assessed as long-term culture initiating cells (LTC-IC) as described before (13). Briefly, 5,000 cells were sorted into LTBMC medium consisting of Iscove's modified Dulbecco medium (IMDM; Invitrogen GmbH) containing 12.5% horse serum, 12.5% FCS for human myeloid LTC (both Stem Cell Technologies Inc), 2 mM L-glutamine, 100 U/ml penicillin, and 100 U/ml streptomycin (all Invitrogen GmbH). The cells were transfered into 96-well tissue culture plates (Costar, Corning Incorporated, New York) containing irradiated stroma cells of the murine fetal liver cell line AFT024 (14) in limiting dilutions (LDA; 22 replicates per concentration: 150, 50, 15, 5 cells/well). Half-medium exchanges were performed once a week. After 5 weeks of culture in a humidified atmosphere at 37°C and 5% CO_2, all medium was replaced by secondary clonogenic methylcellulose medium consisting of 1,12% methylcellulose (Sigma-Aldrich Chemie GmbH, Steinheim, Germany) in IMDM (Invitro-

gen GmbH) containing 30% FCS for human myeloid CFC (Stem Cell Technologies, Inc.), 2 U/ml erythropoietin (EPO; NeoRecormon; Roche Diagnostics GmbH, Mannheim, Germany), and 10% supernatant of the human bladder carcinoma cell line 5637 (13,15). Cells were cultured for another 2 weeks before individual wells were scored for the presence or absence of secondary colony-forming units (CFU). Using Poisson statistics and the weighted mean method, the frequencies of LTC-IC were calculated (15,16).

To perform CFU-GEMM assays, 250 cells were sorted into MethoCult GF H4434 containing recombinant human SCF, interleukin-3 (IL-3), granulocyte/macrophage colony-stimulating factor (GM-CSF), and EPO (Stem Cell Technologies Inc). The cells were incubated in a humidified atmosphere at 37°C and 5% CO_2. Hematopoietic colonies were scored after 10–14 days as described before (13,15).

Stromal feeders for LTC-IC assays

The murine fetal liver cell line AFT024 was used as a stromal feeder layer to support hematopoietic growth in LTC-IC assays. The cell line was maintained at 33°C in Dulbecco's modified Eagle's medium (DMEM; Invitrogen GmbH) containing 20% FCS (Biochrom AG) and 160 μM 2-mercaptoethanol (Invitrogen GmbH). The 96-well plates (Costar, Corning Incorporated) used for the LTC-IC assays were coated with 0.1% gelatin (Stem Cell Technologies, Inc.) and seeded with AFT024 cells. Cells were grown to confluence and irradiated with 2,000˘rad. One day after irradiation, all medium was replaced by LTBMC medium.

Immunofluorescence and microscopy

Analyses of living cells were performed at 37°C in a humidified, 5% CO_2 atmosphere using a DMIRB inverse fluorescence microscope (Leica, Bensheim, Germany). Pictures were taken with an Axiocam digital camera and processed using Axiovision Software (Carl Zeiss, Goettingen, Germany).

Statistics

Experimental results from different experiments were reported as mean ± standard deviation of the mean (SD). Significance analyses were performed with the paired Student's *t*-test.

RESULTS

Optimization of the nucleofection technology to transfect primitive hematopoietic cells

As described recently, we have successfully applied the nucleofection technology to transfect freshly isolated $CD34^+$ cells with different GFP-encoding plasmids (12). When following the manufacturer's instructions (especially using the nucleofection program U08) and using more than 5×10^5 $CD34^+$-enriched cells, we obtained satisfying transfection and survival rates. However, when using less than 5×10^5 $CD34^+$-enriched cells in a single transfection reaction, the survival rate decreased dramatically, and transfection buffer-derived floe-like structures arose within the concomitant cell culture. To overcome these difficulties when transfecting as little as 2×10^5 $CD34^+$-enriched cells, we tested different nucleofection programs and obtained the best results using the nucleofection program U01. However, even a large proportion of the transfected cells were still viable at day 1 post transfection (p.t.), most of them died within two days p.t. (data not shown). In accordance with the original protocol, we initially cultured transfected $CD34^+$ cells in the presence of the transfection buffer. When the transfection buffer was removed immediately after the transfection procedure, the survival rate of cultivated, transfected cells was obviously increased (data not shown).

Electroporation processes create minipores in cell membranes that selectively enable the passage of small ions, resulting in a colloidal osmotic swelling that kills cells (18,19). Because the postpulse pelleting procedure, i.e., the immediate precipitation and incubation of pelleted, transfected cells for 15 min at 37°C, was reported to suppress this swelling in human primary hematopoietic stem cells (19,20), we adapted this procedure to our protocol. Using our improved protocol and 5 μg of Endofree-preparted plasmid DNA (pEGFP-N1), we now obtain survival rates at day 2 p.t. of 52.9% ± 18.9% (n = 19) with a mean transfection efficiency of 79.8% ± 14.1% (n = 19). Upon usage of higher amounts of plasmid DNA (10 μg), the viability of transfected cells dropped down (data not shown).

Effects of apoptosis inhibitor Z-VAD-FMK on cell survival

Recently, it was shown that the electrotransfection-induced DNA uptake resulted in the induction of large-scale apoptosis in $CD34^+$ cells isolated from peripheral blood, and that this apoptotic effect was clearly reduced when caspase inhibitors, e.g., Z-VAD-FMK, were added to the postpulse culture media (20). To analyze whether inhibition of caspases also increase the survival rate of nucleofected $CD34^+$ cells, we compared the survival rate of nontransfected, mock-transfected, and GFP-transfected $CD34^+$ cells that were cultured in the presence or absence of Z-VAD-FMK (20 μM or 120 μM) for 2 days (n = 6). As depicted in Table 1, we did not find any significant impact of Z-VAD-FMK on the survival rate of GFP transfected $CD34^+$ cells; a slight increase was observed when added at a final concentration of 120 μM (Table 1).

TABLE 1. MEAN SURVIVAL RATE OF CULTURED NONTRANSFECTED AND TRANSFECTED CELLS 2 DAYS POST TRANSFECTION

	Untreated	*20 μM Z-VAD-FMK*	*120 μM Z-VAD-FMK*
Control	84.5% ± 4.8%	76.3% ± 12.4%	78.9% ± 11.0%
		$p = 0.061$	$p = 0.171$
Mock	76.7% ± 10.5%	76.3% ± 10.7%	76.1% ± 6.2%
		$p = 0.246$	$p = 0.045$
GFP	49.5% ± 19.0%	52.8% ± 16.3%	60.4% ± 7.6%
		$p = 0.130$	$p = 0.044$

Content of $CD34^+$ cells, 78.8% ± 11.6%; GFP transfection rate, 85.1% ± 5.0%; $n = 6$; p Values are given in relation to corresponding untreated cell fractions.

Effects of the nucleofection procedure on the cell growth of $CD34^+$ cells

As we have described recently, upon cultivation $CD34^+$ cells increase in size and acquire a dynamic, polarized cell morphology, forming a leading edge at the front and an uropod at the rear pole (12). To analyze whether the nucleofection procedure has any morphological effect on $CD34^+$ cells, we compared GFP-transfected, mock-transfected, and nontransfected cells. We realized that most cultivated mock- or GFP-transfected $CD34^+$ cells acquire a polarized cell shape similar to that of nontransfected cells (Fig. 1). However, whereas nontransfected $CD34^+$ cells reach their final size at culture day 2, transfected $CD34^+$ cells seem to be delayed in their cell growth. This difference vanishes largely until day 3 posttransfection (Fig. 2A). It should be mentioned that we observed an additional reduction in cell growth upon treating transfected cells with Z-VAD-FMK (data not shown).

To analyze whether the transfection procedure has any obvious influence on the expression of stem cell-associated surface marker, we compared the content of CD34 and CD133 expression of nontransfected as well as of GFP-transfected cells over time. Under the con-

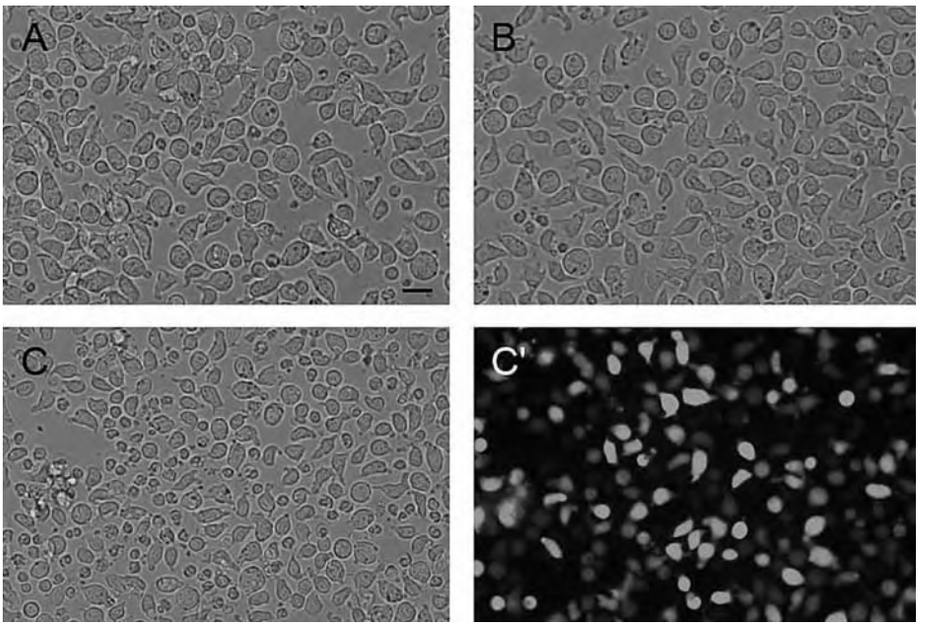


FIG. 1. Upon cultivation, nontransfected as well as transfected $CD34^+$ cells acquire a polarized cell shape. (**A**) Nontransfected $CD34^+$ cells. **(B)** Mock-transfected $CD34^+$ cells. **(C,C′)** GFP-transfected $CD34^+$ cells. All depicted cells were derived from the same $CD34^+$ preparation. Apart from the nucleofection reaction, cells were treated the same. Photos were taken at day 2 post transfection. GFP-transfected cells are clearly smaller than non- or mock-transfected cells (compare **C** to **A** or **B**, respectively). Note that most cells, even the GFP-transfected cells, acquired a polarized cell shape.

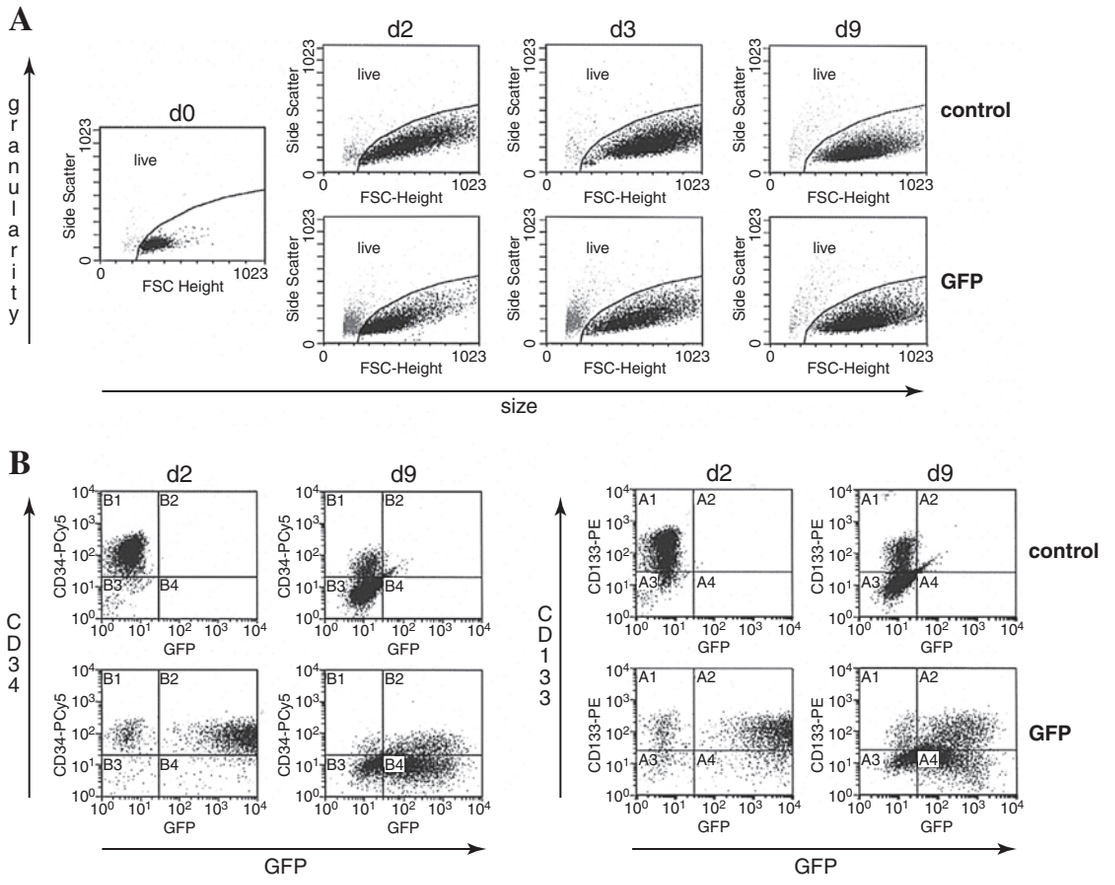


FIG. 2. Flow cytometric analysis of nontransfected and GFP-transfected cells. **(A)** Comparison of the size (FSC-height) and granularity (side scatter) of freshly isolated $CD34^+$ (day 0) and GFP-transfected cells (GFP) versus nontransfected cells $CD34^+$ cells at day 2, day 3, or day 9 past transfection. Upon cultivation, cultured cells increase in size and granularity. While cultivated control cells reach their final size after 2 days, the growth of GFP-transfected cells is delayed approximately 1 day. The "live" gate includes the living and excludes the dead cells. **(B)** Expression of CD34 and CD133 in GFP-transfected or nontransfected control cells that were cultured for 2 or 9 days in the presence of early-acting cytokines. Note that GFP-transfected cells express comparable levels of CD34 and CD133 expression than non-transfected cells. Only cells within the live gate (shown in **A**) are plotted.

ditions used, we observed similar dynamics of CD133 and CD34 expression in cultivated nontransfected or GFP-transfected $CD34^+$ cells, respectively (Fig. 2B). Starting from culture day 3, CD133 and CD34 expression declines over time leaving approximately 20% $CD133^+CD34^+$ cells at day 9 p.t. (Fig. 2B). In this context, it is worth mentioning that although the strength of GFP expression decreases more than 20-fold, approximately 50% of the cultured offspring of originally GFP-transfected cells still express GFP after 9 days (Fig. 2B).

Colony-forming potential of GFP-nucleofected $CD34^+$ cells

To identify potential effects of the nucleofection procedure on the colony-forming potential of $CD34^+$ cells, we performed CFU-GEMM assays of either transfected or nontransfected $CD34^+$ cells. To allow transfected cells to recover from the transfection procedure and to express the *trans*-gene, they were cultured for 2 days in the presence of early-acting cytokines. Then, viable GFP^+ $CD34^+$ cells or $CD34^+$ cells of the controls were purified by cell sorting and transferred in discrete numbers into the CFU-GEMM assay.

According to our data, GFP transfected $CD34^+$ cells form fewer colonies than non- and mock-transfected control cells do. However, we did not find any significant alteration in the ratio of erythroid to myeloid to mixed colonies in our assays (Table 2). The addition of the caspase inhibitor Z-VAD-FMK to the cultures of the 2-day lasting recovery phase did not reveal any positive effect on the frequency of colony formation of transfected or nontransfected cells (data not shown).

Table 2. Mean Colony Formation of Nontransfected and Transfected Cells and Rate of White to Red to Mixed Colonies of Colonies Formed

	Survival rate	*Colonies per 250 CD34$^+$ cells*	*Rate of red colonies*	*Rate of white colonies*	*Rate of mixed colonies*
Control	82.3% ± 6.0%	119.6% ± 64.7%	18.2% ± 11.3%	62.8% ± 14.2%	19.0% ± 11.6%
Mock	74.2% ± 9.7%	108.6 ± 56.0	15.3% ± 12.1%	64.0% ± 16.9%	20.8% ± 12.8%
	$p = 0.007$	$p = 0.331$	$p = 0.206$	$p = 0.440$	$p = 0.386$
GFP	50.5% ± 17.5%	86.3 ± 76.2	16.0% ± 10.5%	60.3% ± 15.8%	23.7% ± 20.8%
	$p < 0.001$	$p = 0.097$	$p = 0.288$	$p = 0.245$	$p = 0.177$

Content of CD34$^+$ cells, 78.8% ± 10.0%; GFP transfection rate, 79.6% ± 15.6%, $n = 10$, p values are given in relation to corresponding control cell fractions.

LTC-IC content of GFP-nucleofected CD34$^+$ cells

To test if more primitive hematopoietic cells within the CD34$^+$ cell fraction can be transfected without losing their primitive cell fate, we performed LTC-IC assays of successfully transfected CD34$^+$ cells in comparison to CD34$^+$ cells of the controls. Again, to allow the cells to recover from the transfection procedure, we cultured them for 2 days in the presence of early-acting cytokines, before, either in the presence or in the absence of Z-VAD-FMK.

According to our results, we detected a slight reduction in the LTC-IC frequency of transfected versus nontransfected cells (Table 3). A beneficial effect of the caspase inhibitor was not observed (data not shown).

DISCUSSION

In addition to their clinical relevance, primary human HSCs provide an attractive and challenging system to study certain biological processes, e.g., the process regulating the decision whether a stem cell is maintained as a stem cell or becomes committed to differentiate. To analyze such processes genetically, it is extremely helpful to have a reliable and highly efficient method to genetically manipulate these cells without altering their cell fate just by the method itself. As we and others have published recently, the nucleofection technology is very efficient method to transfect human HSC-enriched CD34$^+$ cells transiently (11,12). Herein we report the optimization of the nucleofection protocol to transfect transiently as little as 2×10^5 freshly isolated, human HSC-enriched CD34$^+$ cells and the effects of the transfection procedure on the short-term as well as on the long-term cell fate of these cells.

Using our optimized conditions and an enhanced (e) GFP-encoding plasmid, we obtained transfection efficiencies of 80.2% ± 8.6% ($n = 19$) and survival rates of 52.9% ± 18.9% at day 2 post transfection. This rate is higher than that reported of the manufacturers, in which precultured human CD34$^+$ cells were transfected with an efficiency of ~70% and a mean survival rate of less than 40% at day 2 post transfection (11). Optimized classical electroporation procedures of human CD34$^+$ cells result in transfection efficiencies of around 30% and survival rates up to 77%, but require a precultivation of transfected cells (5,6), which frequently alters the cell fate of primitive hematopoietic cells (21,22). In several studies, freshly isolated CD34$^+$ cells were electroporated with only low transfection efficiencies (6.9% and 12%) (5,23). Comparable to classical electroporation procedures, transfection rates obtained with liposome-based technologies remain far below the efficiency obtained with the nucleofection technology (24).

Compared to nontransfected control cells (82.3% ± 6.0%; $n = 10$), the cell survival rate of mock-transfected cells is only slightly decreased (74.2% ± 9.7%; $n = 10$) whereas many of the GFP-transfected cells die within the first 48 h post transfection (survival rate, 50.0% ± 17.5%; $n = 10$). These results clearly demonstrate that the lethality observed in our experiments is more related to the presence of the DNA or to the strong GFP expression than to the nucleofection procedure itself. In-

Table 3. Mean LTC-IC Rate of Nontransfected and Transfected Cells

	Survival rate	*LTC-IC in surviving cells*
Control	79.7% ± 7.5%	5.1% ± 1.5%
Mock	74.9% ± 14.3%	4.9% ± 0.5%
	$p = 0.183$	$p = 0.353$
GFP	53.0% ± 23.8%	3.4% ± 1.3%
	$p = 0.028$	$p = 0.059$

Content of CD34$^+$ cells, 79.1% ± 3.3%; GFP transfection rate, 66.7% ± 18.7%, $n = 4$, p Values are given in relation to corresponding control cell fractions.

deed the occurrence of DNA-induced cell death in electroporation experiments and the induction of apoptosis by high levels of GFP expression have been reported before (19,20,25–27).

Consistent with these observations, we realized a reduced colony-forming frequency of isolated GFP^+CD34^+ cells in CFU-GEMM and LTC-IC assays compared to that of isolated $CD34^+$ cells of the mock-transfected or nontransfected controls (Tables 2 and 3). Because the ratio of erythroid to myeloid to mixed colonies formed by GFP^+CD34^+ cells was largely the same as that of the $CD34^+$ control cells, we assume that the DNA and/or GFP-induced cell death is rather unspecific than specific for any of the $CD34^+$ cell subtypes. In summary, these results suggest that DNA transfer into freshly isolated human $CD34^+$ cells by the nucleofection procedure reduces the overall survival rate but has no major impact on the cell fate of surviving cells. Therefore, the nucleofection method is indeed a highly efficient and reliable method to manipulate primitive hematopoietic cells genetically.

Although we observed that approximately 50% of the offspring of the initially transfected cells still express low levels of GFP after 9 days, it was not our aim to analyze whether these cells were stably transfected or whether GFP-encoding plasmids remained transiently in those cells. As mentioned before, culture conditions have a major impact on the fate of HSCs and HPCs; under most stroma-free culture conditions, the majority of primitive cells become committed within the first few days of culture (21,22).

Thus, the nucleofection method is a useful technique to manipulate and dissect the genetic programs that regulate the maintenance or early commitment of HSCs or HPCs. As shown here, by performing CFU-GEMM or LTC-IC assays, it can easily be analyzed whether expression of certain genes modifies the colony-forming frequency or the ratio of erythroid to myeloid to mixed colonies in progenitor assays. As the morphology of cultivated transfected $CD34^+$ cells is not recognizably altered in comparison to cultivated control cells, this method is also very applicable to study the subcellular distribution of introduced GFP-fusion proteins (12). According to the high transfection efficiency, the method might also be helpful for in vivo applications of transfected HSCs/HPCs in scientific or clinical approaches.

ACKNOWLEDGMENTS

The authors thank Dr. Kay Giesen and Dr. Oliver Gresch for initial experimental support and Daniel Moik for a critical reading of the manuscript. This work was funded by grants from the Deutsche Forschungsgemeinschaft (SPP1109 GI 336/1–2 to B.G. and P.W.) as well as from the Forschungskommission of the HHU-Düsseldorf.

REFERENCES

1. Weissman IL. (2000). Translating stem and progenitor cell biology to the clinic: barriers and opportunities. Science 287:1442–1446.
2. Anderson KV and PW Ingham. (2003). The transformation of the model organism: a decade of developmental genetics. Nature Genet 33(Suppl):285–293.
3. Leurs C, M Jansen, KE Pollok, M Heinkelein, M Schmidt, M Wissler, D Lindemann, C Von Kalle, A Rethwilm, DA Williams and H Hanenberg. (2003). Comparison of three retroviral vector systems for transduction of nonobese diabetic/severe combined immunodeficiency mice repopulating human $CD34^+$ cord blood cells. Hum Gene Ther 14:509–519.
4. Baum C, J Dullmann, Z Li, B Fehse, J Meyer, DA Williams and C von Kalle. (2003). Side effects of retroviral gene transfer into hematopoietic stem cells. Blood 101:2099–2114.
5. Van Tendeloo VF, R Willems, P Ponsaerts, M Lenjou, G Nijs, M Vanhove, P Muylaert, P Van Cauwelaert, C Van Broeckhoven, DR Van Bockstaele and ZN Berneman. (2000). High-level transgene expression in primary human T lymphocytes and adult bone marrow $CD34^+$ cells via electroporation-mediated gene delivery. Gene Ther 7:1431–1437.
6. Wu MH, SL Smith and ME Dolan. (2001). High efficiency electroporation of human umbilical cord blood $CD34^+$ hematopoietic precursor cells. Stem Cells 19:492–499.
7. Hamm A, N Krott, I Breibach, R Blindt and AK Bosserhoff. (2002). Efficient transfection method for primary cells. Tissue Eng 8:235–245.
8. Harriague J and G Bismuth. (2002). Imaging antigen-induced PI3K activation in T cells. Nature Immunol 3:1090–1096.
9. Trompeter HI, S Weinhold, C Thiel, P Wernet and M Uhrberg. (2003). Rapid and highly efficient gene transfer into natural killer cells by nucleofection. J Immunol Methods 274:245–256.
10. Lakshmipathy U, B Pelacho, K Sudo, JL Linehan, E Coucouvanis, DS Kaufman and CM Verfaillie. (2004). Efficient transfection of embryonic and adult stem cells. Stem Cells 22:531–543.
11. Gresch O, FB Engel, D Nesic, TT Tran, HM England, ES Hickman, I Korner, L Gan, S Chen, S Castro-Obregon, R Hammermann, J Wolf, H Muller-Hartmann, M Nix, G Siebenkotten, G Kraus and K Lun. (2004). New non-viral method for gene transfer into primary cells. Methods 33:151–163.
12. Giebel B, D Corbeil, J Beckmann, J Hohn, D Freund, K Giesen, J Fischer, G Kogler and P Wernet. (2004). Segregation of lipid raft markers including CD133 in polarized human hematopoietic stem and progenitor cells. Blood 104:2332–2338.
13. Punzel M, SD Wissink, JS Miller, KA Moore, IR Lemischka and CM Verfaillie. (1999). The myeloid-lymphoid initiating cell (ML-IC) assay assesses the fate of multipotent human progenitors in vitro. Blood 93:3750–3756.
14. Moore KA, H Ema and IR Lemischka. (1997). In vitro maintenance of highly purified, transplantable hematopoietic stem cells. Blood 89:4337–4347.

15. Verfaillie C and P McGlave. (1991). Leukemia inhibitory factor/human interleukin for DA cells: a growth factor that stimulates the in vitro development of multipotential human hematopoietic progenitors. Blood 77:263–270.
16. Porter EH and RJ Berry. (1963). The efficient design of transplantable tumour assays. Br J Cancer 17:583–595.
17. Taswell C. (1981). Limiting dilution assays for the determination of immunocompetent cell frequencies. I. Data analysis. J Immunol 126:1614–1619.
18. Watanabe M, Y Shirayoshi, U Koshimizu, S Hashimoto, S Yonehara, Y Eguchi, Y Tsujimoto and N Nakatsuji. (1997). Gene transfection of mouse primordial germ cells in vitro and analysis of their survival and growth control. Exp Cell Res 230:76–83.
19. Li LH, P Ross and SW Hui. (1999). Improving electrotransfection efficiency by post-pulse centrifugation. Gene Ther 6:364–372.
20. Li LH, P McCarthy and SW Hui. (2001). High-efficiency electrotransfection of human primary hematopoietic stem cells. Faseb J 15:586–588.
21. Sorrentino BP. (2004). Clinical strategies for expansion of haematopoietic stem cells. Nature Rev Immunol 4:878–888.
22. Sauvageau G, NN Iscove and RK Humphries. (2004). In vitro and in vivo expansion of hematopoietic stem cells. Oncogene 23:7223–7232.
23. Weissinger F, P Reimer, T Waessa, S Buchhofer, T Schertlin, V Kunzmann and M Wilhelm. (2003). Gene transfer in purified human hematopoietic peripheral-blood stem cells by means of electroporation without prestimulation. J Lab Clin Med 141:138–149.
24. Floch V, G Le Bolc'h, MP Audrezet, JJ Yaouanc, JC Clement, H des Abbayes, B Mercier, JF Abgrall and C Ferec. (1997). Cationic phosphonolipids as non viral vectors for DNA transfection in hematopoietic cell lines and $CD34^+$ cells. Blood Cells Mol Dis 23:69–87.
25. Stacey KJ, IL Ross and DA Hume. (1993). Electroporation and DNA-dependent cell death in murine macrophages. Immunol Cell Biol 71(Pt 2):75–85.
26. Shimokawa T, K Okumura and C Ra. (2000). DNA induces apoptosis in electroporated human promonocytic cell line U937. Biochem Biophys Res Commun 270:94–99.
27. Liu HS, MS Jan, CK Chou, PH Chen and NJ Ke. (1999). Is green fluorescent protein toxic to the living cells? Biochem Biophys Res Commun 260:712–717.

Address reprint requests to:
Dr. Bernd Giebel
Institute for Transplantation Diagnostics and Cell Therapeutics
Heinrich-Heine-University Düsseldorf
Moorenstrasse 5, Geb. 14.80
40225 Düsseldorf, Germany

E-mail: Giebel@itz.uni-duesseldorf.de

Received November 3, 2005; accepted February 2, 2006.

10.4 Asymmetric cell division within the human hematopoietic stem and progenitor cell compartment: Identification of asymmetrically segregating proteins (Beckmann et al., 2007)

Asymmetric cell division within the human hematopoietic stem and progenitor cell compartment: identification of asymmetrically segregating proteins

Julia Beckmann,[1] Sebastian Scheitza,[1] Peter Wernet,[1] Johannes C. Fischer,[1] and Bernd Giebel[1]

[1]Institute for Transplantation Diagnostics and Cellular Therapeutics, Heinrich-Heine-University Düsseldorf, Germany

The findings that many primitive human hematopoietic cells give rise to daughter cells that adopt different cell fates and/or show different proliferation kinetics suggest that hematopoietic stem cells (HSCs) and hematopoietic progenitor cells (HPCs) can divide asymmetrically. However, definitive experimental demonstration is lacking due to the current absence of asymmetrically segregating marker molecules within the primitive hematopoietic cell compartment. Thus, it remains an open question as to whether HSCs/HPCs have the capability to divide asymmetrically, or whether the differences that have been observed are established by extrinsic mechanisms that act on postmitotic progenitors. Here, we have identified 4 proteins (CD53, CD62L/L-selectin, CD63/lamp-3, and CD71/transferrin receptor) that segregate differentially in about 20% of primitive human hematopoietic cells that divide in stroma-free cultures. Therefore, this indicates for the first time that HSCs/HPCs have the capability to divide asymmetrically. Remarkably, these proteins, in combination with the surrogate stem-cell marker CD133, help to discriminate the more primitive human cultivated HSCs/HPCs. Since 3 of these proteins, the transferrin receptor and the tetraspanins CD53 and CD63, are endosomal-associated proteins, they may provide a link between the endosomal compartment and the process of asymmetric cell division within the HSC/HPC compartment. (Blood. 2007;109:5494-5501)

Introduction

Somatic stem cells are undifferentiated cells that can self-renew over a long period of time in vivo and give rise to progenitor cells that are committed to differentiate. Since both uncontrolled expansion as well as loss of stem cells would be fatal for multicellular organisms, the decision of self-renewal versus differentiation needs to be tightly controlled. Therefore, key questions in stem cell biology are how and which mechanisms govern these decisions. Although mammalian hematopoietic stem cells (HSCs) are the most intensively investigated somatic stem cells, the nature of the factors controlling self-renewal and differentiation remain largely unknown.

There is good evidence that HSCs can expand in vivo[1] and be maintained in vitro in close contact to adequate stroma cells.[2-5] These observations point toward the existence of specialized HSC niches, which was already hypothesized as early as 1978.[6] Indeed, it was recently shown that osteoblasts are key elements of HSC niches in the endosteum of bone marrow (BM) and sinusoidal endothelial cells of vascular HSC niches found in the spleen and BM.[7-9] In addition to the data supporting the HSC niche model that likely provides cell extrinsic cue, evidence suggest that cells of the HSC and hematopoietic progenitor cell (HPC) compartment contain capabilities to divide asymmetrically. Ogawa and colleagues showed that after separation of paired murine and human HPCs that were cultured in stroma-free suspension conditions, siblings gave rise to colonies with significantly different characteristics.[10-12] More recently, HSC-enriched cell populations were found to be highly heterogeneous in respect to their function and their proliferation kinetics (ie, the proliferation rate of more primitive cells is slower than that of committed ones).[13-15] Furthermore, it was observed that approximately 30% of primitive hematopoietic cells ($CD34^+CD38^-$ cells) give rise to daughter cells with heterogeneous proliferation kinetics and functions.[14-16]

Recently, we showed that human myeloid-lymphoid initiating cells (ML-ICs), a subfraction of the $CD34^+CD38^-$ cells, when cultured under stroma-free conditions give rise to daughters that adopt different cell fates, with 1 cell inheriting the developmental capacity of the mother cell, and 1 cell becoming more specified.[17] Similarly, in mice, up to 62% of primitive hematopoietic cells ($lin^-CD34^{low/-}c\text{-}kit^+Sca\text{-}1^+$) give rise to daughter cells with different myeloid developmental potentials.[18,19] Although all these observations are in accordance with the model of asymmetric cell division in which primitive hematopoietic cells contain the potential to give birth to 2 intrinsically different daughter cells, it cannot be concluded that the observed differences are indeed the result of an asymmetric cell division. In principle, these differences could have been established by postmitotic, extrinsic decision processes.[17,20,21]

In organisms like *Drosophila melanogaster* and *Caenorhabditis elegans*, in which asymmetric cell divisions have been proven to occur, cells that divide asymmetrically are polarized during cell division and localize specific molecules to distinct regions of the cell, which are then transmitted unequally into the daughters.[22] Therefore, to demonstrate that primitive hematopoietic cells can indeed divide asymmetrically, molecules that clearly segregate asymmetrically during mitoses of these cells need to be identified. Here, we describe the identification of proteins containing extracellular epitopes, which segregate differentially to daughters during mitosis of approximately 20% of the human $CD34^+CD133^+$

Submitted November 2, 2006; accepted February 25, 2007. Prepublished online as *Blood* First Edition Paper, March 1, 2007; DOI 10.1182/blood-2006-11-055921.

The online version of this article contains a data supplement.

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

hematopoietic cells and confirm the occurrence of asymmetric cell division within the human HSC and HPC compartment.

Materials and methods

Cell source and preparation

Human umbilical cord blood (CB), BM, and peripheral blood (PB) of granulocyte colony-stimulating factor (G-CSF)–treated stem cell donors were obtained from unrelated donors after informed consent was obtained in accordance with the Declaration of Helsinki. The use of human cord blood was approved by the ethics committee of Heinrich-Heine University. Mononuclear cells (MNCs) were isolated from individual sources by Ficoll (Biocoll Separating Solution; Biochrom AG, Berlin, Germany) density gradient centrifugation as described previously.[23] $CD34^+$ cells were isolated by magnetic cell separation using the MidiMacs technique according to the manufacturer's instructions (Miltenyi Biotec, Bergisch Gladbach, Germany).

If not stained immediately, freshly purified MNCs were cultured in a humidified atmosphere at 37°C and 5% CO_2 at a density of approximately 1×10^6 cells/mL and freshly enriched $CD34^+$ cells at a density of approximately 1×10^5 cells/mL in I20 (Iscove modified Dulbecco medium [IMDM; Invitrogen, Karlsruhe, Germany] supplemented with 20% fetal calf serum [FCS; Biochrom AG], 1000 U/mL penicillin, and 100 U/mL streptomycin [Invitrogen]) in the presence of early-acting cytokines (fetal liver tyrosine kinase 3 ligand [FLT3L], stem cell factor [SCF], and thrombopoietin [TPO], each at 10 ng/mL final concentration [all from PeproTech, Rocky Hill, NJ]).

Flow cytometry

For the proliferation kinetics, freshly isolated $CD34^+$ cells were stained for 4 minutes with 2 μM PKH2 (Sigma-Aldrich Chemie, Taufkirchen, Germany). After 1 washing step, stained cells were cultured in I20 in the presence of early-acting cytokines. For flow cytometric analyses, cells were stained with AC133-phycoerythrin (PE; Miltenyi Biotec) and anti-CD34–PE/cytochrome 5 (PCy5) antibodies (BD PharMingen, Heidelberg, Germany).

For the screening procedure, MNCs were stained with different combinations of 3 different antibodies as described in paragraph 3 of Results. A list of the antibodies used is given in Table S1, available on the *Blood* website; see the Supplemental Table link at the top of the online article).

Flow cytometric analyses were performed on a Cytomics FC 500 flow cytometer equipped with the RXP software (Beckman Coulter, Krefeld, Germany).

For the functional assays, the cells were highly purified using a Coulter EPICS Elite ESP fluorescence cell sorting system equipped with the Expo32 software (Beckman Coulter).

Immunofluorescence and microscopy

For subcellular localization studies of polarized and dividing primitive hematopoietic cells, $CD34^+$ cells were cultured for 3 or 4 days in the presence of early-acting cytokines in I20 medium before immunostaining. To conserve their morphology, the $CD34^+$ cells were prefixed for 5 minutes at room temperature with paraformaldehyde (Sigma-Aldrich Chemie) at a final concentration of 0.2% in the medium. As the AC133 epitope of CD133 is sensitive to formaldehyde and paraformaldehyde fixation, the cells for the extracellular staining procedure were then incubated with the AC133-PE antibody (1:10; AC133/1; Miltenyi Biotec) diluted in PBS containing 10% donkey serum (Jackson ImmunoResearch Laboratories, West Grove, PA). They were stained for at least 10 minutes at 4°C, and then postfixed with 4% paraformaldehyde in PBS for 20 minutes at 4°C. Because PE is not a suitable fluorochrome for immunofluorescence microscopy, we counterstained the AC133-labeled cells with Cy3-conjugated AffiniPure Fab fragment donkey anti-mouse IgG (1:20; Jackson ImmunoResearch Laboratories) diluted in PBS containing 10% donkey serum for at least 10 minutes at 4°C. Remaining mouse epitopes were saturated with unconjugated AffiniPure Fab fragment rabbit anti-mouse IgG (1:10; Jackson ImmunoResearch Laboratories). Cells were divided in different aliquots and stained with 1 of the following primary mouse antibodies: anti-CD43–FITC (1G10; BD PharMingen), anti-CD44–FITC (J173; Coulter Immunotech, Krefeld, Germany), anti-CD50–FITC (TU41; BD PharMingen), anti-CD54–FITC (84H10; Coulter Immunotech), anti-CD53–FITC (HI29; BD PharMingen), anti-CD62L (FREG56; Coulter Immunotech), anti-CD63–FITC (H5C6; BD PharMingen), or anti-CD71 (YDJ1.2.2; Coulter Immunotech). These antibodies were counterstained using Cy2-conjugated secondary antibodies (1:100; goat anti-mouse IgG; Jackson ImmunoResearch Laboratories).

For the intracellular staining procedure, cultured and prefixed $CD34^+$ cells were fixed with 4% paraformaldehyde in PBS for 20 minutes at 4°C and permeabilized using 0.1% Triton X100 (Sigma-Aldrich Chemie) in PBS. After blocking in 10% donkey serum (Jackson ImmunoResearch Laboratories), cells were incubated with the anti-CD63 antibody (1:50; H5C5; BD PharMingen) for at least 30 minutes at room temperature and counterstained with Cy3-conjugated AffiniPure Fab fragment donkey anti-mouse IgG (1:100; Jackson ImmunoResearch Laboratories) diluted in PBS containing 10% donkey serum for another 30 minutes at room temperature. After saturating remaining mouse epitopes with unconjugated AffiniPure Fab fragment rabbit anti-mouse IgG (1:10; Jackson ImmunoResearch Laboratories), cells were stained with the anti-CD71 antibody (1:50; YDJ1.2.2; Coulter Immunotech) and counterstained with Cy2-conjugated secondary antibodies (1:400 goat anti-mouse IgG; Jackson ImmunoResearch Laboratories).

Labeled cells were mounted in 75% glycerin containing propylgallat (50 mg/mL) and DAPI (200 ng/mL; Roche, Mannheim, Germany) and observed with an Axioplan 2 fluorescence microscope (Carl Zeiss, Goettingen, Germany) using a Zeiss Plan-Neofluar 100× objective lens (1.3 NA O). Pictures were taken with an Axiocam digital camera and processed using Axiovision 4.5 Software (Carl Zeiss).

Functional assays

For the long-term culture-initiating cell (LTC-IC) assays, approximately 6000 sorted cells were cocultured in a limiting dilution with the irradiated murine fetal liver stroma cell line AFT024 in IMDM (Invitrogen) supplemented with 12.5% FCS, 12.5% horse serum (Cell Systems, St. Katharinen, Germany), 2 mM L-glutamine (Invitrogen), 1000 U/mL penicillin, 100 U/mL streptomycin (Invitrogen), and 10^{-6} M hydrocortisone as extensively described previously.[14,24] Briefly, cultures were maintained for 5 weeks in a humidified atmosphere at 37°C and 5% CO_2 and fed once a week. At week 5, all wells were overlaid with clonogenic methylcellulose medium (Methylcel MC; Fluka, Sigma-Aldrich Chemie) in a final concentration of 1.2% containing IMDM, supplemented with 30% FCS for colony-forming cells (CFCs), 5 U/mL erythropoietin (Cell Systems), and supernatant of the bladder carcinoma cell line 5637 (10%). Wells were scored for the occurrence of secondary CFCs after an additional 10 days.

Statistics

Experimental results from different experiments were reported as standard deviation of the mean. Significance analyses were performed with the paired Student *t* test. LTC-IC frequencies were calculated as the reciprocal of the concentration of test cells that gives 37% negative cultures using Poisson statistics and the weighted mean method.

Results

Proliferation kinetics of cultured $CD34^+$ cells

Recently, we have shown that human $CD34^+$ cells grown for 5 days in the presence of early- or late-acting cytokines in suspension cultures segregate into $CD34^+CD133^+$ and $CD34^+CD133^{low/-}$ subfractions. Furthermore, we realized that early-acting cytokines

stimulate proliferation of $CD34^+$ cells more homogeneously than late-acting cytokines.[17] Since we aimed to study the subcellular distribution of antigens in dividing human CB–derived primitive hematopoietic cells and had the experimental requirement to obtain sufficient mitotic cells numbers, we first estimated the proliferation kinetics of $CD34^+$ cells labeled with the fluorescence dye PKH2 and subsequently cultured in the presence of early-acting cytokines. Cells were harvested at different time points, counted, and the expression of PKH2, CD133, and CD34 was analyzed by flow cytometry. According to the PKH2 staining (Figure 1) and in agreement with our previous studies,[23] only a few $CD34^+$ cells undergo cell division within the first 48 hours of cultivation. The proportions of both $CD133^+$ cells (Figure 1) and $CD34^+$ cells (data not shown) were not altered during this period. Analysis of the PKH2 staining at later time points revealed that most of the $CD34^+$ cells in vitro undergo their first cell division between day 2 and day 3 in culture, and subsequent divisions during the next few days (Figure 1). In agreement with our previous report,[17] the content of $CD34^+$ cells does not change during the first 5 days of culture (data not shown). However, with the onset of cell division, the proportion of $CD133^+$ cells decreases between day 2 and 5 in culture (Figure 1). This corresponds to the maximum expansion period of $CD34^+$ cells between days 3 and 4 seen in 3 different CB-derived $CD34^+$ cell samples (expansion rate, 2.3 ± 0.4 times).

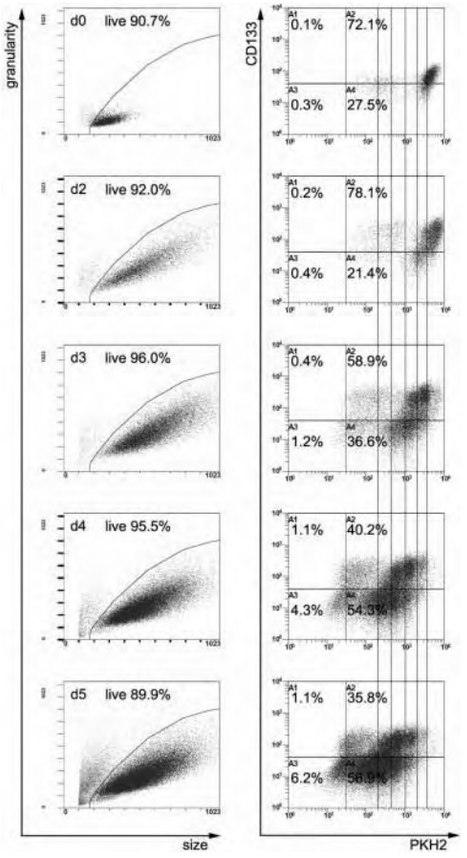


Figure 1. Flow cytometric analyses of PKH2-stained $CD34^+$-enriched cells. PKH2-stained $CD34^+$-enriched cells, either noncultivated (d0) or cultivated in the presence of early-acting cytokines for 2, 3, 4, or 5 days (d2, d3, d4, d5), were measured after labeling with anti-CD34 and anti-CD133 antibodies. The size and the granularity of all cells is plotted in the panels of the first column, and the PKH2 and anti-CD133 staining of the cells located in the live gates (shown in the first column) in the panels of the second column. Quadrants are adjusted according to corresponding isotype controls. Upon cultivation, $CD34^+$ cell increase in size and slightly upregulate CD133 on their cell surface (compare d0 with d2 plots). Starting at day 3, the content of $CD133^+$ cells and the intensity of the PKH2 staining diminishes over time. Since PKH2 is a plasma membrane intercalating dye, its staining gets diluted with each cell division; the PKH2 intensity therefore reflects the number of cell divisions a given cell has performed during cultivation. The perpendicular lines should help to cluster cells regarding the number of cell divisions they had performed. The amount of cells depicted in all plots is normalized to the cell numbers of day 0; therefore, plots can be compared semiquantitatively. Note the small population with the weaker PKH2 staining follows the same kinetics as the large brightly stained population, demonstrating the reliability of the PKH2 experiment.

Because upon stimulation with late-acting cytokines CD133 expression was associated with the more primitive, slowly dividing $CD34^+$ cell fraction,[17] we assumed and verified (fourth paragraph of Results section) that cultivated $CD34^+CD133^+$ cells are more primitive than $CD34^+CD133^{low/-}$ cells, and decided to preferentially analyze $CD34^+CD133^+$ cells in the course of this project. Since the content of $CD34^+CD133^+$ cells was significant higher at culture day 3 compared with days 4 and 5 (day 3, $57.5\% \pm 1.9\%$; day 4, $44.7\% \pm 1.6\%$; day 5, $33.0\% \pm 5.2\%$; $P_{day3/day4} < .003$; $P_{day3/day5} < .02$; $n = 3$), we mainly used day-3 CB-derived $CD34^+$ cells for our further analyses.

Distribution of uropod markers in dividing $CD34^+$ cells

We have recently shown that several surface molecules, and especially CD133, become distributed in a localized fashion in cultivated primitive hematopoietic cells.[23] Now, we have studied their distribution in dividing $CD34^+$ cells. To minimize extrinsic effects on the distribution of these molecules, we generally cultured the cells under stroma-free, nonadherent culture conditions before staining. We analyzed in total 2409 mitotic $CD34^+$ cells from 14 different CB samples that were cultured for 3 or 4 days, respectively. A total of 1379 mitotic cells represented late mitotic stages (telophase), in which the 2 cellular poles can clearly be discriminated (Figure 2). Of the latter, 899 (65.2%) were found to be positive for CD133, a ratio consistent with the content of $CD133^+$ cells within the fraction of $CD34^+$ cells at day 3 of culture. In 892 of these mitotic cells, CD133 was distributed in a symmetrical fashion, displaying its highest concentration at the cleavage furrow or at the midbody, respectively (Figure 2). Only 7 (0.8%) late mitotic cells were found in which the anti-CD133 staining was not symmetrically distributed to the 2 prospective daughter cells; however, according to the appearance of these cells, the nonsymmetric distribution was nonspecific rather than specific.

Furthermore, we analyzed the distribution of other uropod markers in late mitotic $CD34^+$ cells (CD43, CD44, ICAM-1, and ICAM-3). All of these proteins were highly enriched at the cleavage furrow or midbody of the stained cells studied (Table 1; Figure 2). In none of the latter cells we did find any evidence for an asymmetric distribution of these proteins. Together with the results presented in paragraph 6 of the Results section, we assume that under the conditions used here, CD133 and the other uropod markers become highly enriched at the cleavage furrow and at the midbody of all dividing $CD34^+CD133^+$ cells, and thus do not identify putative asymmetric cell divisions.

Phenotypical characterization of the $CD34^+CD133^+$ versus the $CD34^+CD133^{low/-}$ cell fraction

Although our data argues against the possibility that CD133 segregates asymmetrically in dividing $CD34^+CD133^+$ cells, the kinetics of the cultured $CD34^+$ cells are principally compatible with a model in which asymmetric cell divisions give rise to more primitive cells that will maintain the $CD34^+CD133^+$ phenotype, and to committed cells that will reduce their CD133 surface

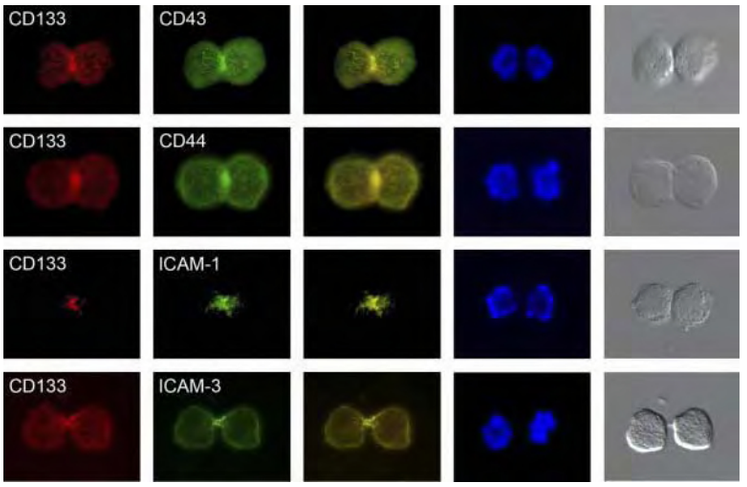


Figure 2. Cell-surface distribution of uropod markers in late mitotic CB-derived CD34$^+$CD133$^+$ cells. Before double labeling with anti-CD133 (red staining) and either anti-CD43, anti-CD44, anti–ICAM-1, or anti–ICAM-3 antibodies (green staining), cells were cultured for 3 days in serum-containing medium supplemented with early-acting cytokines. Note that CD133 as well as the other uropod markers are highly enriched at the cleavage furrow or midbody of dividing CD34$^+$CD133$^+$ cells. Column 1 shows anti-CD133 staining; column 2, anti-CD43, anti-CD44, anti–ICAM-1, and anti–ICAM-3 staining of cultivated CD34$^+$ cells; column 3, merge of columns 1 and 2; column 4, DAPI staining; and column 5, light microscopy images of the stained cells.

expression postmitotically. At this point we hypothesized that if CD34$^+$CD133$^+$ cells indeed can divide asymmetrically to give rise to CD34$^+$CD133$^+$ and CD34$^+$CD133$^{low/-}$ cells, any protein that is expressed differentially among these populations might be a candidate for a protein that segregates differentially in asymmetrically dividing CD34$^+$CD133$^+$ cells.

Thus, to challenge this hypothesis, we next screened for proteins that are expressed differentially on both populations. For the screening procedure, we cultured CB-derived MNCs in the presence of early-acting cytokines for 3 days. Then, cells were stained with anti-CD133–PE and anti-CD34–PCy5 antibodies as well as with 1 of 58 different FITC-conjugated antibodies that recognize different surface antigens. The expression levels of the corresponding antigens were measured by flow cytometry. Using a gating strategy on CD34$^+$ cells, the expression of the different antigens on CD34$^+$CD133$^+$ cells as well as on CD34$^+$CD133$^{low/-}$ cells was analyzed and compared with each other (Figure 3). According to our results, 39 of these antigens were judged to be expressed on cultivated CD34$^+$ cells, with 19 of them uncovering differences between the CD34$^+$CD133$^+$ and CD34$^+$CD133$^{low/-}$ subpopulations (data not shown).

Following this initial screening procedure, we chose a panel of 11 different antibodies and studied the expression of the corresponding antigens on 2 additional CB-derived MNC fractions. While CD47 and CD59 were expressed homogeneously on CD34$^+$ cells, CD13, CD31, CD53, CD62L, CD63, CD71, CD74, and CD105 were expressed in different amounts on CD34$^+$CD133$^+$ cells compared with CD34$^+$CD133$^{low/-}$ cells in all samples. The expression levels of CD164 on these subpopulations were not consistent among the different samples (data not shown). It should be mentioned that in comparative analyses, we have studied the expression of these proteins on MNCs of 2 BM-derived and 2 PB-derived MNC fractions as well and obtained comparable results (data not shown).

Table 1. Analyses of the uropod marker distribution in mitotic CD34$^+$ cells

	n	No. stained telophases	Content of CD133$^+$ telophases, %
CD43	3	95	74.7
CD44	3	195	87.2
ICAM-1	3	124	95.2
ICAM-3	4	135	80.7

n indicates number of CB samples analyzed. The distribution of different antigens was studied in late mitotic cells counterstained with CD133. In all of the late mitotic cells analyzed, antigens were distributed in a symmetric fashion.

According to our results, the expression levels of CD53, CD62L, CD63, and CD71 displayed the highest contrast between CD34$^+$CD133$^+$ and CD34$^+$CD133$^{low/-}$ cells of the MNC fractions (Figure 3). To exclude any influence of CD34$^-$ MNCs on the expression levels of these antigens, we analyzed the expression of CD53, CD62L, CD63, and CD71 on purified CB-derived CD34$^+$ cells that were cultured for 3 days in the presence of early-acting cytokines and obtained comparable results (Figure S1).

Functional characterization of the newly identified CD34$^+$ subpopulations

To functionally characterize the newly identified CD34$^+$ subfractions, we have purified CD133$^+$ and CD133$^{low/-}$ fractions in combination

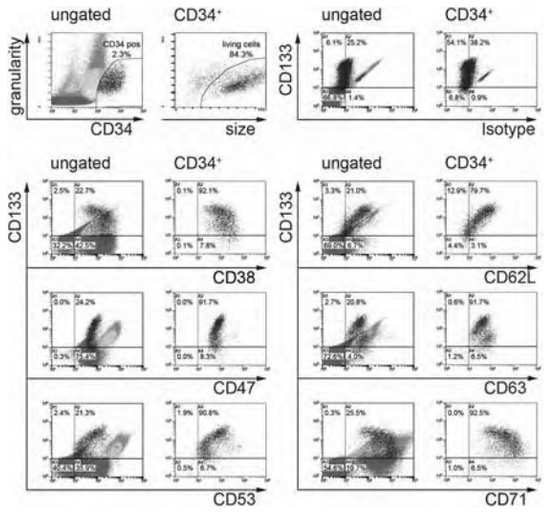


Figure 3. Flow cytometric analyses of CB-derived MNCs that have been cultivated for 3 days in the presence of early-acting cytokines. Plots represent MNCs that are ungated or gated on CD34$^+$ cells. The CD34$^+$ gate used is shown in the first plot of the first row. The size of these cells plotted against their granularity is shown in the second plot of row 1. The remaining plots represent the intensity of CD133 staining against the intensity of the isotype control, an anti-CD38, anti-CD47, anti-CD53, anti-CD62L, anti-CD63, or anti-CD71 staining, respectively. Quadrants are adjusted according to isotype controls of CD34 negative cells. Note that CD34$^+$CD133$^+$ cells contain different levels of CD53, CD63, CD62L, and CD71 than CD34$^+$CD133$^{low/-}$ cells.

with CD38, CD53, CD62L, CD63, or CD71 by fluorescent cell sorting (Figure S1B) and analyzed their primitive myeloid developmental capacity, measured as LTC-ICs. $CD34^+$ cells of the same samples were purified in parallel and analyzed as controls. According to our results, the LTC-IC frequency within the purified $CD34^+CD133^+$ cell fractions was significantly higher than in their $CD34^+CD133^{low/-}$ counterparts or in the total $CD34^+$ cell fractions (Table 2). Compared with the subfraction of cultivated $CD133^+CD38^{low/-}$ and $CD133^+CD71^{low}$ cells, cells of the $CD133^+CD53^+$, $CD133^+CD62L^+$, and $CD133^+CD63^{low}$ subfractions can be recognized as distinct cell populations (Figures 3, S1). Therefore, CD53, CD62L, and CD63 provide markers, which can be used in combination with CD133 to objectively identify potentially more primitive cells within the fraction of cultivated $CD34^+$ cells.

Subcellular distribution of identified antigens in polarized $CD34^+CD133^+$ cells

To subcellularly localize the identified antigens on polarized $CD34^+$ cells, we stained $CD34^+$ cells cultured for 3 days with antibodies against these markers and an anti-CD133 antibody. CD53 and CD63 were highly concentrated in vesicular-like structures at the base of the uropod. CD71 was distributed over the cell surface, including the leading edge of $CD34^+$ cells; in addition, it was highly concentrated in vesicular-like structures at the base of the uropod. CD62L was highly concentrated at the tip of the uropod, where it seemed to colocalize with CD133; in addition, faint staining was sometimes found at the base of the uropod (Figure 4). It should be mentioned that compared with the staining with the other antibodies, the anti-CD62L antibody staining is generally very weak on fixed cells.

CD53, CD62L, CD63, and CD71 segregation during mitosis of $CD34^+CD133^+$ cells

Hence, the proteins identified in our screen are candidates that might segregate differently during mitosis. Therefore, we have analyzed the distribution of CD53, CD62L, CD63, and CD71 on $CD34^+CD133^+$ cells of late mitotic stages (telophase). On approximately 20% of the mitotic cells studied, $CD53^+$ (19.6%), $CD63^+$ (20.5%), and $CD71^+$ (22.1%) vesicular structures were found preferentially in 1 of the prospective daughter cells, suggesting that these structures segregate differentially (Table 3, Figure 5). We also found late telophase $CD34^+CD133^+$ cells that displayed an asymmetric distribution of the CD62L antigen (15.2%). However, due to the weak anti-CD62L antibody staining of fixed cells, the number of asymmetrically dividing $CD133^+CD62L^+$ cells might be underestimated.

Intracellular distribution of CD63 and CD71 in late mitotic $CD34^+$ cells

Since CD53, CD63, and CD71 are expressed on vesicular-like structures and have been reported to be associated with the endosomal traffic,[25-28] the vesicular-like structures might correspond to budding endosomes. To exclude the possibility the asymmetric distribution of these structures is connected to a very dynamic process, which might frequently switch among the daughter cells, we decided to study the intracellular distribution of the identified antigens. Due to an incompatibility between the anti-CD53 antibody and the intracellular staining procedure, we double-stained cultured $CD34^+$ cells with anti-CD63 and anti-CD71 antibodies. In all cases studied, the anti-CD63 and the anti-CD71 staining colocalized (Figure 5B). In support of the data presented in the previous paragraph, we found an asymmetric distribution of the anti-CD63– and anti-CD71–stained structures in approximately 20% of the mitotic cells (Table 3, Figure 5B).

Discussion

Challenging the hypothesis of asymmetric cell division within the primitive hematopoietic cell compartment, we demonstrate here for the first time that primitive human hematopoietic cells indeed contain capabilities to divide asymmetrically. Furthermore, we report the identification of cell surface proteins that, in combination with CD133, can be used to define more primitive hematopoietic cells within the $CD34^+$ cell fraction. In addition, these proteins led to the discovery of a potentially new subcellular plasma-membrane domain on migrating $CD34^+$ cells in which endosomes seem to be formed to bud into the interior of the cell.

Asymmetric cell division of primitive hematopoietic cells

It has been suggested that HSCs can divide asymmetrically to give rise to 1 cell maintaining stem cell fate and to a daughter that is committed to differentiate. In agreement with this hypothesis, the findings of several groups, including our own, have demonstrated that primitive hematopoietic cells give rise to daughter cells adopting different cell fates or realizing different proliferation kinetics, respectively.[10-15,17] Following a definition in which a cell division is defined as asymmetric or symmetric according to the cell fates of its daughter cells,[29] these results would clearly demonstrate that primitive hematopoietic cells divide asymmetrically. However, if a more restrictive definition is used, in which asymmetrically dividing cells

Table 2. Functional analyses of sorted $CD34^+$ sub-fractions in LTC-IC assays

LTC-IC frequency	n	CD34′, %	$CD133^+$, %	$CD133^{low/-}$, %	*P* $CD133^+$ to $CD133^{low/-}$	*P* $CD133^+$ to $CD34^+$
CD38	3	4.2 ± 3.5	$CD38^{low}$, 13.4 ± 1.8	$CD38^+$, 0.3 ± 0.4	< .01	< .02
CD53	5	3.4 ± 2.7	$CD53^+$, 7.9 ± 4.2	$CD53^{low/-}$, 1.1 ± 1.0	< .03	< .03
CD62L	5	3.4 ± 2.7	$CD62L^+$, 8.9 ± 3.9	$CD62L^{low/-}$, 0.3 ± 0.1	< .01	< .01
CD63	5	5.3 ± 2.8	$CD63^{low/-}$, 11.4 ± 3.0	$CD63^+$, 0.3 ± 0.2	< .01	< .01
CD71	5	3.4 ± 2.7	$CD71^{low}$, 12.7 ± 10.0	$CD71^+$, 1.0 ± 0.8	< .06	< .05

n indicates number of experiments; $CD34^+$, LTC-IC frequency of $CD34^+$ cells purified at culture day 3 (note: identical CB- samples for CD53, CD62L, and CD71 analyses were used); $CD133^+$, LTC-IC frequency of purified, cultured $CD34^+$ $CD133^+$ cell fractions which are $CD38^{low}$, $CD53^+$, $CD62L^+$, $CD63^{low/-}$, or $CD71^{low}$, respectively; $CD133^{low}$, LTC-IC frequency of purified, cultured $CD34^+$ $CD133^{low/-}$ cell fractions which are $CD38^+$, $CD53^{low/-}$, $CD62L^{low/-}$, $CD63^+$, or $CD71^+$, respectively; *P* $CD133^+$ to $CD133^{low/-}$, *P* values of significance analyses of the LTC-IC frequency of $CD34^+CD133^+$ cell fractions compared with that of corresponding $CD34^+CD133^{low/-}$ cell fractions; *P* $CD133^+$ to $CD34^+$, *P* values of significance analyses of the LTC-IC frequency of $CD34^+CD133^+$ cell fractions compared with that of corresponding $CD34^+$ cell fractions.

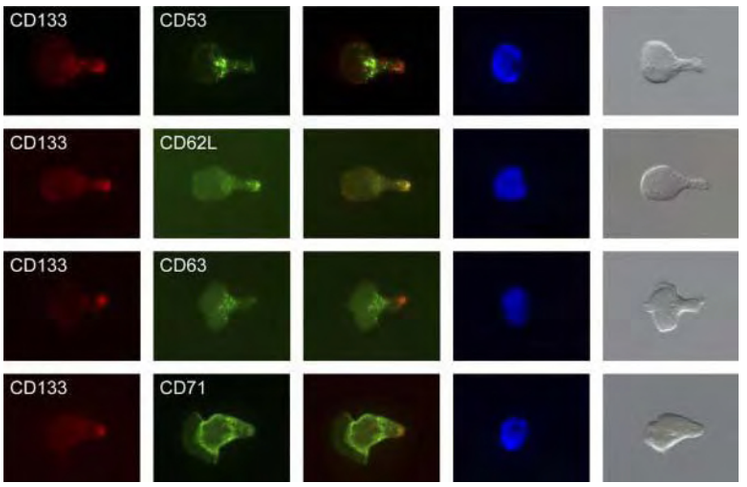


Figure 4. Localization of CD53, CD62L, CD63, and CD71 in polarized CD34$^+$CD133$^+$ cells. CD53, CD63, and CD71 are localized in vesicular-like structures at the base of the uropod. In addition, CD71 is expressed all over the cell surface, including the leading edge (left side of the cell shown in column 2). CD62L is highly localized at the tip of the uropod and colocalizes with CD133. Column 1 shows anti-CD133 staining; column 2, anti-CD53, anti-CD62L, anti-CD63, and anti-CD71 staining of CD34$^+$ cells; column 3, merge of columns 1 and 2; column 4, DAPI staining; and column 5, light microscopy images of the stained cells.

are defined as cells that by the different segregation of certain molecules become qualitatively different, these results are not sufficient to demonstrate asymmetric cell divisions within the primitive human hematopoietic cell compartment. The observed differences in the cell fate or the proliferation kinetics could theoretically be a result of extrinsic mechanisms that act postmitotically and alter the developmental capacities of initially identical daughter cells. A well-analyzed process in which cells with identical developmental capacities become different is the process of lateral inhibition, sometimes also referred as mutual inhibition or lateral specification. In this process, cells with identical developmental capacities mutually influence each others cell fate to become different.[20,21] Since this process is mediated by the Notch signaling pathway, which plays important roles during early and late hematopoiesis,[30] its action is one of several other predictable, postmitotically acting mechanisms which theoretically could account for the observed differences.

However, by the identification of marker proteins that segregate differently in approximately 20% of the mitotic, CB-derived CD34$^+$CD133$^+$ cells, it now becomes evident that primitive hematopoietic cells can indeed give rise to qualitatively different daughter cells and thus divide asymmetrically. Since we raised the cells in stroma-free suspension cultures, and the cells grew nonadherently, we suppose that the asymmetric distribution of the proteins in mitotic cells is assigned intrinsically rather than induced by extrinsic signals that might directly affect the subcellular localization of the identified proteins. This assumption is further enforced by the findings that (1) the ratio of late mitotic cells with asymmetric protein distributions fits into the same range with which primitive human hematopoietic cells deposited as single cells in suspension cultures generate daughter cells realizing different cell fates and different proliferation kinetics, and (2) that the latter rate was not influenced by the different cytokine conditions used to raise these cells in vitro.[14,16]

Although we cannot exclude that daughter cells inheriting different levels of the identified marker proteins can compensate for these differences and adopt identical developmental potentials, this congruency suggests that there is a high correlation between the marker distribution and the acquired cell fates of the daughter cells, which is supported by the fact that CD133 and the expression of the marker proteins we describe here correlates very well with the primitive state of CD34$^+$ cells. More primitive CD34$^+$CD133$^+$ cells express higher levels of CD53 and CD62L as well as lower levels of CD63 and CD71 than the more mature CD34$^+$CD133$^{low/-}$ cells. In this context, it should be noted that, in combination with CD133 and CD62L, CD53 or CD63 more objectively defined subpopulations of CD34$^+$ cells can be recognized and purified than with the CD38 antigen, which is commonly used to discriminate more primitive human HPCs (CD34$^+$CD38$^{low/-}$) from more mature ones (CD34$^+$CD38$^+$).[31,32] According to our findings, we postulate that asymmetrically dividing CD34$^+$CD133$^+$ cells obtaining more CD53 or CD62L or less CD63 are more primitive than their sister cells. As CD71 is distributed along the whole plasma membrane, we suggest that the daughter cells inheriting more of the CD71-stained vesicular-like structures will express higher levels of CD71 than their sister cells. Since CD34$^+$CD133$^+$CD71^{low} cells are more primitive than CD34$^+$CD133^{low}CD71$^+$ cells, we assume that the daughter cells inheriting more of the CD71$^+$ vesicular-like structures are more mature than their sister cells.

Table 3. Distribution of CD53, CD62L, CD63, and CD71 in late mitotic CD34$^+$ cells

Antigen	n	Total no. telophases	Total no. asym telophases	Content of asym telophases, %	Average rate of asym telophases per CB, %
CD53/CD133	3	97	19	19.6	18.9 ± 9.4
CD62L/CD133	4	112	17	15.2	17.8 ± 13.7
CD63/CD133	5	146	30	20.5	23.6 ± 6.5
CD71/CD133	4	131	29	22.1	29.9 ± 16.3
CD63/CD71	4	221	40	18.1	20.3 ± 6.5

The distribution of different antigens was studied in late mitotic cells counterstained with CD133 or in cells that were intracellularly stained with CD63 and CD71.
n indicates number of CB samples analyzed; asym, asymmetric.
*Data shows telophases.

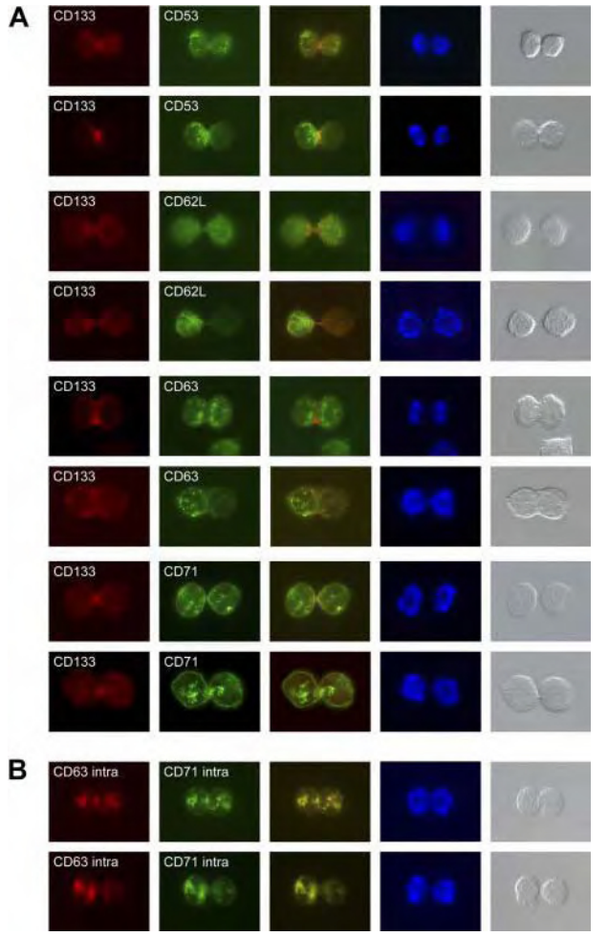


Figure 5. Localization of CD53, CD62L, CD63 and CD71 in dividing CD34$^+$CD133$^+$ cells. (A) Cell-surface distribution of CD53, CD62L, CD63, and CD71 on late mitotic CD34$^+$CD133$^+$ cells. For each of these markers, 1 mitotic cell is shown containing a symmetric distribution of the given antigen (top row), and 1 containing an asymmetric distribution (bottom row; green staining). Cells are counterstained with an anti-CD133 antibody (red staining) and DAPI (blue staining). Light microscopy images of the stained cells are presented in the fifth column. (B) Intracellular distribution of CD63 (red staining) and CD71 (green staining). The overlay of the 2 is given in the third column.

This view is supported by the result of our intracellular staining procedure, in which CD63$^+$ vesicular-like structures, which are supposed to label more mature cells, cosegregate with the CD71-stained structures. In addition, and consistent with our functional data, it was reported several years ago that CD34$^+$CD71^{low} cells are more primitive than CD34$^+$CD71$^+$ cells.[33,34] Similarly, previous evidence showed that CD62L expressing CD34$^+$ cells are more primitive than CD62L$^-$ CD34$^+$ cells.[35,36] To our knowledge, neither CD53 nor CD63 have been associated with the fate of CD34$^+$ cells. Therefore, we have qualified these as new markers to discriminate primitive cultured CD34$^+$ cells from more mature cells.

Tetraspanins

Both CD53 and CD63 encode members of the evolutionary conserved tetraspanin family, a large superfamily of small cell-surface membrane proteins characterized by 4 transmembrane and 2 extracellular domains. At present, 28 members of this family have been documented in humans, many of them expressed on multiple cell types. They seem to organize novel types of cell-surface membrane microdomains, the tetraspanin-enriched microdomains (TEMs), novel signaling platforms which are distinct from lipid rafts.[37,38] However, little is known about their function, though tetraspanins seem to take part in regulating the activation, motility, and antigen presentation of different leukocytes.[37,38] Interestingly, it has been shown that, in addition to the cell surface, many tetraspanins, including CD53 and CD63, are localized on the internal vesicles of multivesicular endosomes and on exosomes.[26] Due to the interaction between CD63 and subunits of AP-2 and AP-3 complexes, it is linked to clathrin-dependent endocytotic pathways and seems to play a role in the recycling of plasma membrane components and their transport to appropriate intracellular compartments.[25,27,39-42]

Since the transferrin receptor (CD71) also cycles between the plasma membrane and the endosomal compartment and can be secreted on exosomes,[28,39] 3 of the 4 identified proteins that frequently segregate asymmetrically in dividing CD34$^+$CD133$^+$ cells are linked to the endosomal/exosomal compartment. This, together with the fact that anti-CD53, anti-CD63, and anti-CD71 staining is highly enriched in vesicular-like structures in polarized as well as in mitotic CD34$^+$CD133$^+$ cells, suggest that the vesicular-like structures correspond to budding endosomes. This assumption is further supported by our finding that in living CD34$^+$ cells, antibodies against CD63 and CD71 become internalized within the region that is highly enriched for these vesicular-like structures (data not shown). Since these presumptive budding endosomes are highly enriched in a region at the base of the uropod, they define a membrane domain that to our knowledge has not been previously described in polarized CD34$^+$ cells. Furthermore, these results reveal a link between the endosomal compartment and mechanisms governing the asymmetric cell division of primitive human hematopoietic cells. It will, in the future, be interesting to determine the similarities between this asymmetric cell division in human HPCs and the role of the endosomal compartment and mechanisms governing asymmetric cell divisions that have been described in *Drosophila*.[43]

Acknowledgments

The authors thank Dr Michael Punzel, Dr Andreas Wodarz, and Dr Verdon Taylor for general discussion and critiques on the manuscript; and Svenja Jünger, Jan Spanholtz, and Gregor von Levetzow for general experimental support. Umbilical cord blood samples were kindly provided by Gesine Kögler of our institute (Institute for Transplantation Diagnostics and Cellular Therapeutics).

This work was supported by grants from the Deutsche Forschungsgemeinschaft (SPP1109 GI 336/1-4 to B.G. and P.W.) and from the Forschungskommission of the HHU-Duesseldorf (B.G.).

Authorship

Contribution: J.B. helped to design the study, performed experiments and assisted in the writing; S.S. performed experiments; P.W. provided intellectual input by discussing the data; J.C.F.

provided essential experimental support; and B.G. designed the study, performed experiments, and wrote the manuscript.

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

Correspondence: Bernd Giebel, Institute for Transplantation Diagnostics and Cell Therapeutics, Heinrich-Heine-University Duesseldorf, D-40225 Duesseldorf, Germany; e-mail: giebel@itz.uni-duesseldorf.de.

References

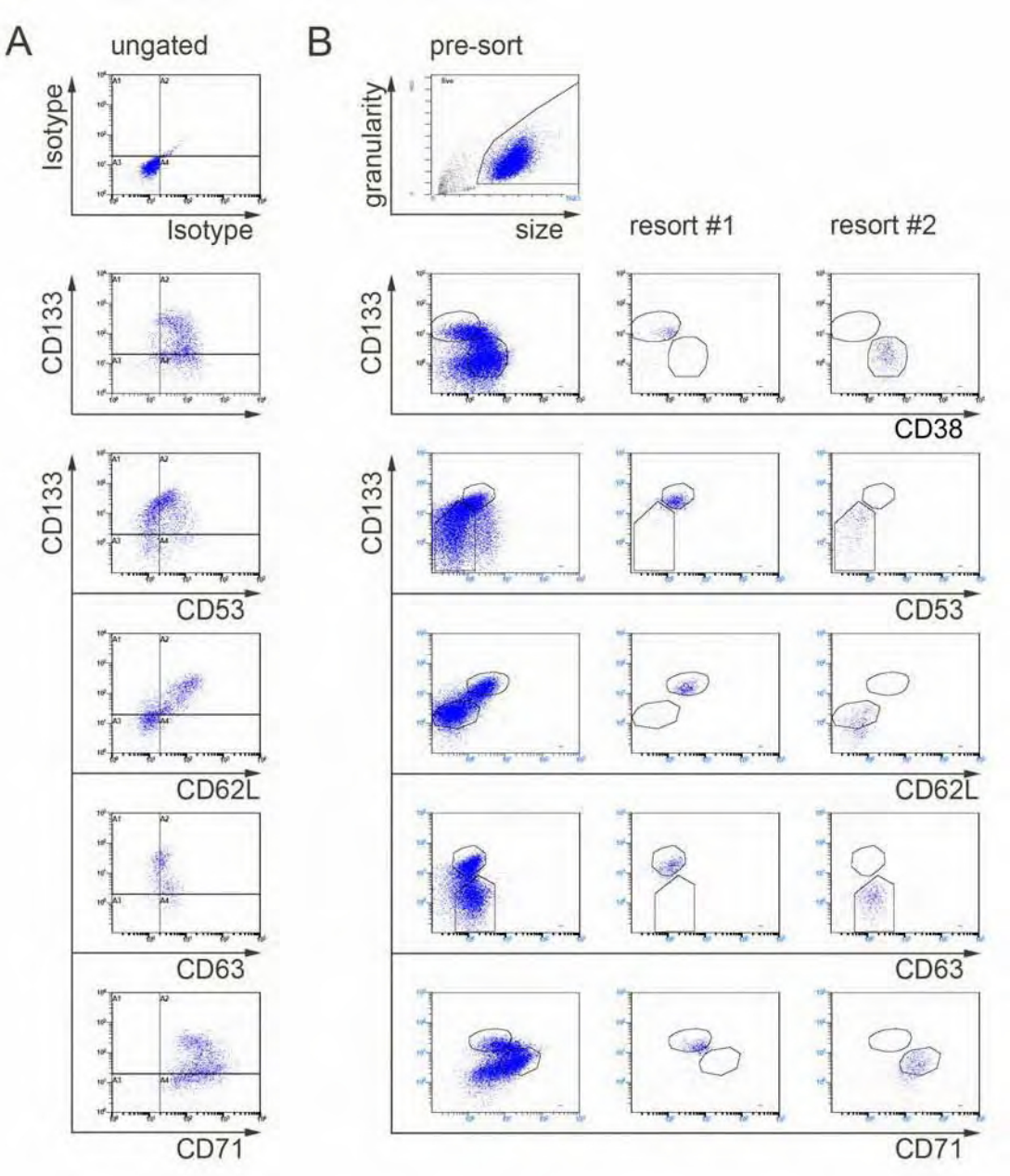
1. Iscove NN, Nawa K. Hematopoietic stem cells expand during serial transplantation in vivo without apparent exhaustion. Curr Biol. 1997;7:805-808.
2. Moore KA, Ema H, Lemischka IR. In vitro maintenance of highly purified, transplantable hematopoietic stem cells. Blood. 1997;89:4337-4347.
3. Nolta JA, Thiemann FT, Arakawa-Hoyt J, et al. The AFT024 stromal cell line supports long-term ex vivo maintenance of engrafting multipotent human hematopoietic progenitors. Leukemia. 2002;16:352-361.
4. Punzel M, Moore KA, Lemischka IR, Verfaillie CM. The type of stromal feeder used in limiting dilution assays influences frequency and maintenance assessment of human long-term culture initiating cells. Leukemia. 1999;13:92-97.
5. Thiemann FT, Moore KA, Smogorzewska EM, Lemischka IR, Crooks GM. The murine stromal cell line AFT024 acts specifically on human CD34+CD38- progenitors to maintain primitive function and immunophenotype in vitro. Exp Hematol. 1998;26:612-619.
6. Schofield R. The relationship between the spleen colony-forming cell and the haemopoietic stem cell. Blood Cells. 1978;4:7-25.
7. Calvi LM, Adams GB, Weibrecht KW, et al. Osteoblastic cells regulate the haematopoietic stem cell niche. Nature. 2003;425:841-846.
8. Kiel MJ, Yilmaz OH, Iwashita T, Terhorst C, Morrison SJ. SLAM family receptors distinguish hematopoietic stem and progenitor cells and reveal endothelial niches for stem cells. Cell. 2005;121:1109-1121.
9. Zhang J, Niu C, Ye L, et al. Identification of the haematopoietic stem cell niche and control of the niche size. Nature. 2003;425:836-841.
10. Suda T, Suda J, Ogawa M. Disparate differentiation in mouse hemopoietic colonies derived from paired progenitors. Proc Natl Acad Sci U S A. 1984;81:2520-2524.
11. Suda J, Suda T, Ogawa M. Analysis of differentiation of mouse hemopoietic stem cells in culture by sequential replating of paired progenitors. Blood. 1984;64:393-399.
12. Leary AG, Strauss LC, Civin CI, Ogawa M. Disparate differentiation in hemopoietic colonies derived from human paired progenitors. Blood. 1985;66:327-332.
13. Mayani H, Dragowska W, Lansdorp PM. Lineage commitment in human hemopoiesis involves asymmetric cell division of multipotent progenitors and does not appear to be influenced by cytokines. J Cell Physiol. 1993;157:579-586.
14. Punzel M, Zhang T, Liu D, Eckstein V, Ho AD. Functional analysis of initial cell divisions defines the subsequent fate of individual human CD34(+)CD38(-) cells. Exp Hematol. 2002;30:464-472.
15. Brummendorf TH, Dragowska W, Zijlmans J, Thornbury G, Lansdorp PM. Asymmetric cell divisions sustain long-term hematopoiesis from single-sorted human fetal liver cells. J Exp Med. 1998;188:1117-1124.
16. Huang S, Law P, Francis K, Palsson BO, Ho AD. Symmetry of initial cell divisions among primitive hematopoietic progenitors is independent of ontogenic age and regulatory molecules. Blood. 1999;94:2595-2604.
17. Giebel B, Zhang T, Beckmann J, Spanholtz J, Wernet P, Ho AD, Punzel M. Primitive human hematopoietic cells give rise to differentially specified daughter cells upon their initial cell division. Blood. 2006;107:2146-2152.
18. Ema H, Takano H, Sudo K, Nakauchi H. In vitro self-renewal division of hematopoietic stem cells. J Exp Med. 2000;192:1281-1288.
19. Takano H, Ema H, Sudo K, Nakauchi H. Asymmetric division and lineage commitment at the level of hematopoietic stem cells: inference from differentiation in daughter cell and granddaughter cell pairs. J Exp Med. 2004;199:295-302.
20. Giebel B. The notch signaling pathway is required to specify muscle progenitor cells in Drosophila. Mech Dev. 1999;86:137-145.
21. Martinez Arias A, Zecchini V, Brennan K. CSL-independent Notch signalling: a checkpoint in cell fate decisions during development? Curr Opin Genet Dev. 2002;12:524-533.
22. Betschinger J, Knoblich JA. Dare to be different: asymmetric cell division in Drosophila, C. elegans and vertebrates. Curr Biol. 2004;14:R674-R685.
23. Giebel B, Corbeil D, Beckmann J, et al. Segregation of lipid raft markers including CD133 in polarized human hematopoietic stem and progenitor cells. Blood. 2004;104:2332-2338.
24. Punzel M, Wissink SD, Miller JS, Moore KA, Lemischka IR, Verfaillie CM. The myeloid-lymphoid initiating cell (ML-IC) assay assesses the fate of multipotent human progenitors in vitro. Blood. 1999;93:3750-3756.
25. Arribas M, Cutler DF. Weibel-Palade body membrane proteins exhibit differential trafficking after exocytosis in endothelial cells. Traffic. 2000;1:783-793.
26. Escola JM, Kleijmeer MJ, Stoorvogel W, Griffith JM, Yoshie O, Geuze HJ. Selective enrichment of tetraspan proteins on the internal vesicles of multivesicular endosomes and on exosomes secreted by human B-lymphocytes. J Biol Chem. 1998;273:20121-20127.
27. Kobayashi T, Vischer UM, Rosnoblet C, et al. The tetraspanin CD63/lamp3 cycles between endocytic and secretory compartments in human endothelial cells. Mol Biol Cell. 2000;11:1829-1843.
28. Qian ZM, Li H, Sun H, Ho K. Targeted drug delivery via the transferrin receptor-mediated endocytosis pathway. Pharmacol Rev. 2002;54:561-587.
29. Morrison SJ, Kimble J. Asymmetric and symmetric stem-cell divisions in development and cancer. Nature. 2006;441:1068-1074.
30. Suzuki T, Chiba S. Notch signaling in hematopoietic stem cells. Int J Hematol. 2005;82:285-294.
31. Hao QL, Shah AJ, Thiemann FT, Smogorzewska EM, Crooks GM. A functional comparison of CD34+ CD38- cells in cord blood and bone marrow. Blood. 1995;86:3745-3753.
32. Bhatia M, Wang JC, Kapp U, Bonnet D, Dick JE. Purification of primitive human hematopoietic cells capable of repopulating immune-deficient mice. Proc Natl Acad Sci U S A. 1997;94:5320-5325.
33. Lansdorp PM, Dragowska W. Long-term erythropoiesis from constant numbers of CD34+ cells in serum-free cultures initiated with highly purified progenitor cells from human bone marrow. J Exp Med. 1992;175:1501-1509.
34. Mayani H, Dragowska W, Lansdorp PM. Cytokine-induced selective expansion and maturation of erythroid versus myeloid progenitors from purified cord blood precursor cells. Blood. 1993;81:3252-3258.
35. Mohle R, Murea S, Kirsch M, Haas R. Differential expression of L-selectin, VLA-4, and LFA-1 on CD34+ progenitor cells from bone marrow and peripheral blood during G-CSF-enhanced recovery. Exp Hematol. 1995;23:1535-1542.
36. Koenig JM, Baron S, Luo D, Benson NA, Deisseroth AB. L-selectin expression enhances clonogenesis of CD34+ cord blood progenitors. Pediatr Res. 1999;45:867-870.
37. Hemler ME. Tetraspanin functions and associated microdomains. Nat Rev Mol Cell Biol. 2005;6:801-811.
38. Tarrant JM, Robb L, van Spriel AB, Wright MD. Tetraspanins: molecular organisers of the leukocyte surface. Trends Immunol. 2003;24:610-617.
39. de Gassart A, Geminard C, Hoekstra D, Vidal M. Exosome secretion: the art of reutilizing nonrecycled proteins? Traffic. 2004;5:896-903.
40. Duffield A, Kamsteeg EJ, Brown AN, Pagel P, Caplan MJ. The tetraspanin CD63 enhances the internalization of the H, K-ATPase beta-subunit. Proc Natl Acad Sci U S A. 2003;100:15560-15565.
41. Mantegazza AR, Barrio MM, Moutel S, et al. CD63 tetraspanin slows down cell migration and translocates to the endosomal-lysosomal-MIICs route after extracellular stimuli in human immature dendritic cells. Blood. 2004;104:1183-1190.
42. Janvier K, Bonifacino JS. Role of the endocytic machinery in the sorting of lysosome-associated membrane proteins. Mol Biol Cell. 2005;16:4231-4242.
43. Shen Q, Temple S. Creating asymmetric cell divisions by skewing endocytosis. Sci STKE. 2002;2002:PE52.

Supplement Table 1: Antibody clones used for flow cytometry and immunostaining

antigen	fluorochrome	antibody clone	company
BCRP	Fitc	5D3	Chemicon
BDCA-1	Fitc	AD5-8E7	Miltenyi
BDCA-2	Fitc	AC144	Miltenyi
BDCA-3	Fitc	AD5-14H12	Miltenyi
CD1a	Fitc	HI149	Pharmingen
CD1c	Fitc	AD5-8E7	Milteny
CD7	Fitc	8H8.1	Immunotech
CD13	Fitc	SJ1D1	Immunotech
CD14	Fitc	RMO52	Immunotech
CD15	Fitc	80H5	Immunotech
CD18	Fitc	7E4	Immunotech
CD25	Fitc	2A3	Pharmingen
CD28	Fitc	CD28.2	Immunotech
CD29	Fitc	K20	Immunotech
CD31	Fitc	WM59	Pharmingen
CD32	Fitc	FLI8-26	Pharmingen
CD33	Fitc	D3HL60-251	Immunotech
CD40	Fitc	5C3	Pharmingen
CD45	Fitc	HI30	Pharmingen
CD45RA	Fitc	ALB11	Immunotech
CD45RO	Fitc	UCHL1	Immunotech
CD47	Fitc	B6H12	Pharmingen
CD48	Fitc	J4-57	Immunotech
CD49d	Fitc	HP2/1	Immunotech
CD49e	Fitc	SAM1	Immunotech
CD53	Fitc	HI29	Pharmingen
CD58	Fitc	AICD58	Immunotech
CD59	Fitc	p282(H19)	Pharmingen
CD62E	Fitc	1.2B6	Serotec
CD62L	Fitc	FREG56	Immunotech
CD62P	Fitc	AK-4	Pharmingen
CD63	Fitc	H5C6	Pharmingen
CD64	Fitc	22	Immunotech
CD69	Fitc	FN50	Pharmingen
CD71	Fitc	YDJ1.2.2	Immunotech
CD74	Fitc	M-B741	Pharmingen

CD80	Fitc	MAB104	Immunotech
CD83	Fitc	HB15a	Immunotech
CD90w	Fitc	F15.42-1-5	Immunotech
CD97	Fitc	VIM3b	Pharmingen
CD99	Fitc	TÜ12	Pharmingen
CD105	Fitc	SN6/N1-3A1	Ancell
CD106	Fitc	BBIG-V3(IE10)	R&D
CD134	Fitc	ACT35	Pharmingen
CD164	Fitc	N6B6	Pharmingen
CD200	Fitc	OX-104	Serotec
Cytokeratin	Fitc	J1B3	Immunotech
GM-CSF Receptor	Fitc	M5D12	Pharmingen
HEA	Fitc	HEA125	Miltenyi
HLA-A2	Fitc	BB7.2	Pharmingen
HLA-ABC	Fitc	B9.12.1	Immunotech
MDR	Fitc	17F9	Pharmingen
vwF	Fitc	AHP062F	Serotec
Isotype	Fitc	MOPC-21	Pharmingen
CD38	Fitc	T16	Immunotech
CD43	Fitc	1G10	Pharmingen
CD44	Fitc	J.173	Immunotech
CD50/ICAM-3	Fitc	TÜ41	Pharmingen
CD54/ICAM-1	Fitc	84H10	Immunotech
CD133	Pe	AC133	Miltenyi
Isotype	Pe	MOPC-21	Pharmingen
CD34	PCy5	581	Pharmingen
CD63	non-conjugated	H5C6	Pharmingen

Supplemented Figure 1: Flow cytometrical analyses and sort-strategy of CB-derived CD34$^+$ enriched cells which have been cultivated for 3 days in the presence of early acting cytokines. The level of anti-CD133 staining is plotted against the anti-CD38, anti-CD53, anti-CD62L, anti-CD63 or anti-CD71 staining, respectively. Cells represented in (A) were analyzed in comparison to an isotype control. (B) Cells of the same cell fractions within the *live* gate (first plot) were sorted according to the gates shown in plot 2-5 of column 1. Column 2 and 3 represent ungated analyses of the sorted cell fractions.



10.5 Asymmetric cell divisions of human hematopoietic stem and progenitor cells meet endosomes (Giebel und Beckmann, 2007)

Extra views:

Asymmetric cell divisions of human hematopoietic stem and progenitor cells meet endosomes

Short title: Asymmetric cell division of human HSC/HPC and endosomes

Bernd Giebel and Julia Beckmann

From the Institute for Transplantation Diagnostics and Cellular Therapeutics, Heinrich-Heine-University Duesseldorf, Duesseldorf, Germany

Correspondence:

Bernd Giebel, Ph.D. Institute for Transplantation Diagnostics and Cellular Therapeutics, Heinrich-Heine-University Duesseldorf, Moorenstr. 5, Geb. 14.80 D-40225 Duesseldorf, Germany
Tel.: +49 211 8119443
FAX: +49 211 8117643
e-mail: giebel@itz.uni-duesseldorf.de

Keywords: Tetraspanins, tetraspanins, endosome, endocytosis, hematopoiesis, stem cell

List of abbreviations:

BM	bone marrow
CB	umbilical cord blood
Dpp	Decapentaplegic
ESCRT	endosomal sorting complex required for transport
HPC	hematopoietic progenitor cell
HSC	hematopoietic stem cell
LAMP-3	lysosome associated membrane protein 3
MVBs	multi vesicular bodies
SARA	Smad-anchor-for-receptor-activation
SOP	sensory organ precursor
Tkv	Thickveins

Abstract

Hematopoietic stem cells (HSC) are undifferentiated cells, which self-renew over a long period of time and give rise to committed hematopoietic progenitor cells (HPC) containing the capability to replenish the whole blood system. Since both uncontrolled expansion as well as loss of HSC would be fatal, the decision of self-renewal *versus* differentiation needs to be tightly controlled. There is good evidence that both HSC niches as well as asymmetric cell divisions are involved in controlling whether HSC self-renew or become committed to differentiate. In this context, we recently identified four proteins which frequently segregate asymmetrically in dividing HSC/HPC. Remarkably, three of these proteins, the tetraspanins CD53 and CD63, and the transferrin receptor are endosome-associated proteins. Here, we highlight these observations in conjunction with recent findings in model organisms which show that components of the endosomal machinery are involved in cell-fate specification processes.

Introduction

Somatic stem cells are undifferentiated cells that are required to maintain homeostasis in a variety of tissues throughout life. To achieve tissue regeneration these cells need to contain livelong lasting self-renewal and differentiation capacities. While uncontrolled expansion of stem cells is often connected to the establishment of cancer,[1-4] loss of stem cells, which occurs during aging, can result in degenerative diseases.[5-8] Thus, a key question in stem cell biology is, how and which mechanisms govern the decision whether stem cells or their sibling cells self-renew or differentiate, a question we and others address at the example of human primitive hematopoietic cells, a fraction of cells containing both, hematopoietic stem cells (HSC) and hematopoietic progenitor cells (HPC).[9-13]

The hematopoietic stem cell niche

Considering that HSC can expand *in vivo*,[14] many attempts have been undertaken to expand repopulating HSC *in vitro*. And although it was found that HSC can be maintained in close contact to certain stroma cells,[15-19] stroma-free culture conditions regularly fail to even support long-term *ex vivo* maintenance of primitive hematopoietic cells.[20-22] Therefore, the surrounding environment and thus a special combination of extrinsic factors are required to keep HSC in a primitive state. These observations are compatible with the model of the HSC niche that was already hypothesized by Raymond Schofield in 1978.[23] According to this model stem cells are associated with other cells that determine their behaviour, i.e. they prevent the stem cells' maturation and thus assure their continued proliferation as stem cells, while their progeny, unless they can occupy similar stem cell niches, become committed to differentiate.[23] Indeed evidence for the occurrence of HSC niches has recently been provided.[24] Osteoblasts in the endosteum of the bone marrow (BM) and sinusoidal endothelial cells in the spleen and BM have been described to participate in the formation of bone marrow or vascular HSC-niches, respectively.[25-27] It seems that the receptor tyrosine kinase Tie2, expressed on HSC, and its cognate ligand Angiopoietin-1 produced by osteoblasts play a crucial role in these niches.[28] Furthermore, osteoblasts express the Notch ligand Jagged-1, which can activate the Notch signalling pathway. This, together with the fact that in co-cultures the long-term supportive effect of osteoblasts is strongly reduced by addition of a γ-secretase inhibitor capable of blocking Notch activation, suggests that another important function of HSC niches is the activation of the Notch signalling pathway,[25] which has also

been associated with the maintenance of murine and human HSC in a variety of different studies.[29-38]

Asymmetric cell division

The significance of asymmetric cell divisions for the development of multi-cellular organisms is widely recognized.[39] Apart from HSC-niches, asymmetric cell divisions have also been postulated to occur within the primitive hematopoietic system. In this context, several groups demonstrated that 20-30% of primitive hematopoietic cells produce daughter cells that differ from each other in respect of their proliferation kinetics and/or their adopted cell fates.[11,40-48] Whether these results are sufficient to demonstrate the occurrence of asymmetric cell divisions within the primitive hematopoietic cell compartment, depends on the definition of the term asymmetric cell division. If asymmetric cell division is defined according to the cell fates the arising daughter cells finally adopt, these data would fulfil the criteria for asymmetric cell divisions. However, by using a more stringent definition, in which cell divisions are defined as asymmetrically, when they give rise to qualitatively different daughter cells, e.g. by the different inheritance of certain intrinsic molecules - some of which might act as cell fate determinants -, these data would not be sufficient to demonstrate the occurrence of asymmetric cell division within the primitive hematopoietic compartment.[11,49]

Thus, to elucidate whether human HSC/HPC also fulfil the criteria for the more stringent definition of an asymmetric cell division, we decided to search for asymmetrically segregating molecules.

Identification of asymmetrically segregating molecules

Most of the primitive human hematopoietic cells can be identified by the expression of the stem cell surrogate markers CD34 and CD133.[50-52] Analyzing the proliferation kinetics of umbilical cord blood derived $CD34^+$ cells by flow-cytometry we observed that initially approximately 90% of the $CD34^+$ cells co-express similar levels of CD133. However, upon cultivation in the presence of early acting cytokines and with the onset of cell divisions between culture day 2 and 3, the relative homogeneously appearing population of $CD34^+CD133^+$ cells gives rise to an additional, more mature population of $CD34^+CD133^{low/-}$ cells, whose content increases over time.[11,12] Under the prediction that the content of CD133 cells can exclusively be altered by asymmetric cell divisions and that each asymmetrically dividing $CD34^+CD133^+$ cell gives rise to one daughter cell maintaining the $CD34^+CD133^+$ phenotype and to one $CD34^+CD133^{low/-}$ daughter cell, which including its descendants

remains $CD133^{low/-}$, and that starting from culture day 2 all cells divide once within 24 hours, the observed kinetics fit to a model in which 20-30% of the $CD34^+CD133^+$ cells divide asymmetrically. Although by using stroma-free culture conditions we failed to detect CD133 as an asymmetrically segregating molecule, the simplicity of the model seemed so persuasive to us that we screened for other cell surface antigens that follow similar kinetics on cultivated $CD34^+$ cells than CD133.[12] After the identification of different antigens that comply with this requirement, we analysed the sub-cellular localisation of some of these antigens on dividing $CD34^+CD133^+$ cells in detail and could identify four proteins, the tetraspanins CD53 and CD63, the transferrin receptor (CD71) and L-selectin (CD62L) which obviously segregate differently in approximately 20% of the dividing $CD34^+CD133^+$ cells.[12] Therefore, HSC/HPC also fulfil the criteria for the more stringent definition of asymmetrically dividing cells, and thus can be regarded as *true* asymmetrically dividing cells.[12] Since the obtained ratio fits into the same range with which primitive human hematopoietic cells give rise to daughter cells realizing different cell fates and/or different proliferation kinetics,[40,43,45,46] it is tempting to speculate that these differences are indeed established by *true* asymmetric cell divisions.

Remarkably, three of the four identified proteins, the transferrin receptor (CD71) and the tetraspanins CD53 and CD63, are associated with endosomal traffic.[53-56] Therefore, our data provide a linkage between the process of asymmetric cell division and the endosomal compartment, maybe indicating that components of the endosomal compartment are importantly involved in cell fate specification processes of primitive hematopoietic cells.[12]

Cell fate specification and the endosomal compartment

A link between the endosomal compartment and mechanisms governing asymmetric cell divisions has been discovered in *Drosophila*.[57] There, so called sensory organ precursor cells (SOPs) sequentially divide asymmetrically to stereotypically generate an external sensory organ consisting out of four different cells.[58] A well analysed feature of this development is that each of the cell divisions creates one cell in which the cell fate determining Notch signalling pathway becomes activated and one in which it remains inactive. Notch itself as well as its ligands are expressed on both siblings; the difference concerning the signalling activity is established by a protein called Numb.[59,60] Numb is an endosomal protein, which segregates mainly into one of the arising daughter cells, the pIIb cells, in which it inhibits the transduction of the Notch signalling by promoting the endocytosis and degradation of Notch itself and/or of Sanpodo, an essential component required for the activation of the Notch signalling pathway.[59-64] Two homologues of *numb*, *numb* and *numblike*, are found in

mammals and like in *Drosophila*, mammalian Numb has been proposed to physically interact with Notch1, inhibit its function and participate in asymmetric cell divisions as it has most clearly been demonstrated in the embryonic nervous system.[65-70] In addition, asymmetric distributions of Numb have also been reported in mitotic retinal precursors, isolated cortical progenitors, muscle satellite cells and T-cells.[71-74]

Remarkably, *Drosophila* as well as mammalian forms of Numb bind to α-Adaptin, an essential component of the AP-2 complex, which links Numb to the clathrin-dependent endocytotic pathway.[62,63,75] Since α-Adaptin co-segregates with Numb during SOP development, Berdnik et al. (2002) could demonstrate that even essential components of the endosomal machinery segregate asymmetrically into corresponding cells.[63] In this context, it should be mentioned that CD63, which we have identified as an asymmetrically segregating protein in approximately 20% of the primitive hematopoietic cells, is considered to be a late endosomal/lysosomal marker also called lysosome associated membrane protein 3 (Lamp-3).[54,76,77] Like Numb it binds to components of AP-2 and thus also links CD63 to clathrin-dependent endocytosis.[49,78]

In a continuation to these observations, Bökel et al. (2006) found a correlation between the establishment of cell-fates and the mitotic segregation of certain endosomes: In the developing *Drosophila* wing, a gradient of the TGF-β homologue Decapentaplegic (Dpp) controls the fate of the wing epithelial cells in a concentration-dependent manner. Upon binding to its receptor Thickveins (Tkv), Dpp induces the phosphorylation of the R-Smad transcription factor Mad and recruits a common Smad to become an active transcription factor that then translocates into the nucleus and induces the expression of target genes. The endosomal protein Smad-anchor-for-receptor-activation (Sara) has been found to be required for the recruitment of R-Smads to type I TGF-β receptors.[79] In mammals as well as in *Drosophila*, Sara accumulates in early endosomes, which in the *Drosophila* wing epithelial cells also contain Dpp and its receptor Tkv.[80,81] During mitosis, i.e. during anaphase, Sara-positive endosomes become localized at the midzone of the central spindle and redistribute during cytokinesis to the central spindle ends, from where they equally segregate into the arising daughter cells.[80] However, in Sara mutant cells the distribution of these endosomes is disturbed and they often segregate asymmetrically into the daughter cells. Since these endosomes still contain Tkv and phosphorylated Mad, arising daughter cells frequently inherit different levels of these proteins. In correlation with that cell fate alterations of such daughter cells were observed in pupal wings.[80]

Recently, Andrews and Ahringer (2007) provide evidence for another connection between the mitotic segregation of endocytotic vesicles and the cell fate.[82] During the first mitotic cell division of *Caenorhabditis elegans*, the zygote divides asymmetrically to create a large anterior AB-cell, which predominantly gives rise to ectodermal cell types, and a smaller posterior P1 cell, which mainly produces mesoderm, endoderm and germ line cells.[83] Shortly before the onset of this first cell division and depending on the activity of cell polarity mediating proteins (Par proteins) subsets of endosomes expressing the early endosomal marker EEA-1 become localized primarily at the anterior pole of the zygotes and segregate asymmetrically mainly into the anterior located AB cells.[82] In Par mutant embryos, in which both arising daughter cells acquire identical cell fates,[84,85] the asymmetric distribution of these endosomes is lost.[82]

Conclusion:

These data, in the connection with the finding that the 'endosomal sorting complex required for transport' (ESCRT) machinery which participates in the transition of early to late endosomes, and especially in the formation of multi vesicular bodies (MVBs), has tumour suppressor functions which among others are required to shut down Notch, Decapentaplegic and receptor tyrosine kinase signalling,[86-91] underscore the importance of the endosomal compartment in cell fate specification processes as well as in tumorigenesis. Furthermore, these data demonstrate that during mitosis the segregation of certain endosomes needs to be highly controlled, most likely in either a symmetrical or an asymmetrical manner. According to our opinion it is challenging within the next few years to learn more about the connection of the endosomal compartment, the mitotic segregation of endosomes and the mechanisms governing cell fate specification processes, i.e. those controlling self-renewal *verus* differentiation processes of somatic stem cells.

Acknowledgments

We thank Dr. Johannes Fischer, Simon Magin and Dr. Verdon Taylor for discussion and critics on the manuscript. Our studies were supported by grants from the Deutsche Forschungsgemeinschaft (SPP1109 GI 336/1-4) and from the Forschungskommission of the HHU-Duesseldorf.

Literature:

1. Al-Hajj M. Cancer stem cells and oncology therapeutics. Curr Opin Oncol. 2007;19:61-64.
2. Beachy PA, Karhadkar SS, Berman DM. Tissue repair and stem cell renewal in carcinogenesis. Nature. 2004;432:324-331.
3. Reya T, Morrison SJ, Clarke MF, Weissman IL. Stem cells, cancer, and cancer stem cells. Nature. 2001;414:105-111.
4. Passegue E. Hematopoietic stem cells, leukemic stem cells and chronic myelogenous leukemia. Cell Cycle. 2005;4:266-268.
5. Wagers AJ, Conboy IM. Cellular and molecular signatures of muscle regeneration: current concepts and controversies in adult myogenesis. Cell. 2005;122:659-667.
6. Taupin P, Gage FH. Adult neurogenesis and neural stem cells of the central nervous system in mammals. J Neurosci Res. 2002;69:745-749.
7. Kamminga LM, de Haan G. Cellular memory and hematopoietic stem cell aging. Stem Cells. 2006;24:1143-1149.
8. Anversa P, Rota M, Urbanek K, Hosoda T, Sonnenblick EH, Leri A, Kajstura J, Bolli R. Myocardial aging--a stem cell problem. Basic Res Cardiol. 2005;100:482-493.
9. Muller-Sieburg CE, Sieburg HB. The GOD of hematopoietic stem cells: a clonal diversity model of the stem cell compartment. Cell Cycle. 2006;5:394-398.
10. Walkley CR, McArthur GA, Purton LE. Cell division and hematopoietic stem cells: not always exhausting. Cell Cycle. 2005;4:893-896.
11. Giebel B, Zhang T, Beckmann J, Spanholtz J, Wernet P, Ho AD, Punzel M. Primitive human hematopoietic cells give rise to differentially specified daughter cells upon their initial cell division. Blood. 2006;107:2146-2152.
12. Beckmann J, Scheitza S, Wernet P, Fischer JC, Giebel B. Asymmetric cell division within the human hematopoietic stem and progenitor cell compartment: identification of asymmetrically segregating proteins. Blood. 2007;109:5494-5501.
13. Bonifer C. Epigenetic plasticity of hematopoietic cells. Cell Cycle. 2005;4:211-214.
14. Iscove NN, Nawa K. Hematopoietic stem cells expand during serial transplantation in vivo without apparent exhaustion. Curr Biol. 1997;7:805-808.
15. Nolta JA, Thiemann FT, Arakawa-Hoyt J, Dao MA, Barsky LW, Moore KA, Lemischka IR, Crooks GM. The AFT024 stromal cell line supports long-term ex vivo maintenance of engrafting multipotent human hematopoietic progenitors. Leukemia. 2002;16:352-361.
16. Moore KA, Ema H, Lemischka IR. In vitro maintenance of highly purified, transplantable hematopoietic stem cells. Blood. 1997;89:4337-4347.
17. Shih CC, Hu MC, Hu J, Medeiros J, Forman SJ. Long-term ex vivo maintenance and expansion of transplantable human hematopoietic stem cells. Blood. 1999;94:1623-1636.
18. Punzel M, Moore KA, Lemischka IR, Verfaillie CM. The type of stromal feeder used in limiting dilution assays influences frequency and maintenance assessment of human long-term culture initiating cells. Leukemia. 1999;13:92-97.
19. Thiemann FT, Moore KA, Smogorzewska EM, Lemischka IR, Crooks GM. The murine stromal cell line AFT024 acts specifically on human CD34+CD38- progenitors to maintain primitive function and immunophenotype in vitro. Exp Hematol. 1998;26:612-619.
20. Shimizu Y, Ogawa M, Kobayashi M, Almeida-Porada G, Zanjani ED. Engraftment of cultured human hematopoietic cells in sheep. Blood. 1998;91:3688-3692.
21. Conneally E, Cashman J, Petzer A, Eaves C. Expansion in vitro of transplantable human cord blood stem cells demonstrated using a quantitative assay of their lympho-myeloid repopulating activity in nonobese diabetic-scid/scid mice. Proc Natl Acad Sci U S A. 1997;94:9836-9841.

22. Bhatia M, Bonnet D, Kapp U, Wang JC, Murdoch B, Dick JE. Quantitative analysis reveals expansion of human hematopoietic repopulating cells after short-term ex vivo culture. J Exp Med. 1997;186:619-624.
23. Schofield R. The relationship between the spleen colony-forming cell and the haemopoietic stem cell. Blood Cells. 1978;4:7-25.
24. Haylock DN, Nilsson SK. Stem cell regulation by the hematopoietic stem cell niche. Cell Cycle. 2005;4:1353-1355.
25. Calvi LM, Adams GB, Weibrecht KW, Weber JM, Olson DP, Knight MC, Martin RP, Schipani E, Divieti P, Bringhurst FR, Milner LA, Kronenberg HM, Scadden DT. Osteoblastic cells regulate the haematopoietic stem cell niche. Nature. 2003;425:841-846.
26. Zhang J, Niu C, Ye L, Huang H, He X, Tong WG, Ross J, Haug J, Johnson T, Feng JQ, Harris S, Wiedemann LM, Mishina Y, Li L. Identification of the haematopoietic stem cell niche and control of the niche size. Nature. 2003;425:836-841.
27. Kiel MJ, Yilmaz OH, Iwashita T, Terhorst C, Morrison SJ. SLAM family receptors distinguish hematopoietic stem and progenitor cells and reveal endothelial niches for stem cells. Cell. 2005;121:1109-1121.
28. Arai F, Hirao A, Ohmura M, Sato H, Matsuoka S, Takubo K, Ito K, Koh GY, Suda T. Tie2/Angiopoietin-1 signaling regulates hematopoietic stem cell quiescence in the bone marrow niche. Cell. 2004;118:149-161.
29. Karanu FN, Murdoch B, Gallacher L, Wu DM, Koremoto M, Sakano S, Bhatia M. The notch ligand jagged-1 represents a novel growth factor of human hematopoietic stem cells. J Exp Med. 2000;192:1365-1372.
30. Karanu FN, Murdoch B, Miyabayashi T, Ohno M, Koremoto M, Gallacher L, Wu D, Itoh A, Sakano S, Bhatia M. Human homologues of Delta-1 and Delta-4 function as mitogenic regulators of primitive human hematopoietic cells. Blood. 2001;97:1960-1967.
31. Suzuki T, Yokoyama Y, Kumano K, Takanashi M, Kozuma S, Takato T, Nakahata T, Nishikawa M, Sakano S, Kurokawa M, Ogawa S, Chiba S. Highly efficient ex vivo expansion of human hematopoietic stem cells using Delta1-Fc chimeric protein. Stem Cells. 2006;24:2456-2465.
32. Lauret E, Catelain C, Titeux M, Poirault S, Dando JS, Dorsch M, Villeval JL, Groseil A, Vainchenker W, Sainteny F, Bennaceur-Griscelli A. Membrane-bound delta-4 notch ligand reduces the proliferative activity of primitive human hematopoietic CD34+CD38low cells while maintaining their LTC-IC potential. Leukemia. 2004;18:788-797.
33. Ohishi K, Varnum-Finney B, Bernstein ID. Delta-1 enhances marrow and thymus repopulating ability of human CD34(+)CD38(-) cord blood cells. J Clin Invest. 2002;110:1165-1174.
34. Varnum-Finney B, Wu L, Yu M, Brashem-Stein C, Staats S, Flowers D, Griffin JD, Bernstein ID. Immobilization of Notch ligand, Delta-1, is required for induction of notch signaling. J Cell Sci. 2000;113 Pt 23:4313-4318.
35. Stier S, Cheng T, Dombkowski D, Carlesso N, Scadden DT. Notch1 activation increases hematopoietic stem cell self-renewal in vivo and favors lymphoid over myeloid lineage outcome. Blood. 2002;99:2369-2378.
36. Carlesso N, Aster JC, Sklar J, Scadden DT. Notch1-induced delay of human hematopoietic progenitor cell differentiation is associated with altered cell cycle kinetics. Blood. 1999;93:838-848.
37. Ye Q, Shieh JH, Morrone G, Moore MA. Expression of constitutively active Notch4 (Int-3) modulates myeloid proliferation and differentiation and promotes expansion of hematopoietic progenitors. Leukemia. 2004;18:777-787.
38. Vercauteren SM, Sutherland HJ. Constitutively Active Notch4 Promotes Early Human Hematopoietic Progenitor Cell Maintenance While Inhibiting Differentiation and Causes Lymphoid Abnormalities In Vivo. Blood. 2004

39. Morrison SJ, Kimble J. Asymmetric and symmetric stem-cell divisions in development and cancer. Nature. 2006;441:1068-1074.
40. Leary AG, Strauss LC, Civin CI, Ogawa M. Disparate differentiation in hemopoietic colonies derived from human paired progenitors. Blood. 1985;66:327-332.
41. Suda T, Suda J, Ogawa M. Disparate differentiation in mouse hemopoietic colonies derived from paired progenitors. Proc Natl Acad Sci U S A. 1984;81:2520-2524.
42. Suda J, Suda T, Ogawa M. Analysis of differentiation of mouse hemopoietic stem cells in culture by sequential replating of paired progenitors. Blood. 1984;64:393-399.
43. Mayani H, Dragowska W, Lansdorp PM. Lineage commitment in human hemopoiesis involves asymmetric cell division of multipotent progenitors and does not appear to be influenced by cytokines. J Cell Physiol. 1993;157:579-586.
44. Brummendorf TH, Dragowska W, Zijlmans J, Thornbury G, Lansdorp PM. Asymmetric cell divisions sustain long-term hematopoiesis from single-sorted human fetal liver cells. J Exp Med. 1998;188:1117-1124.
45. Huang S, Law P, Francis K, Palsson BO, Ho AD. Symmetry of initial cell divisions among primitive hematopoietic progenitors is independent of ontogenic age and regulatory molecules. Blood. 1999;94:2595-2604.
46. Punzel M, Zhang T, Liu D, Eckstein V, Ho AD. Functional analysis of initial cell divisions defines the subsequent fate of individual human CD34(+)CD38(-) cells. Exp Hematol. 2002;30:464-472.
47. Takano H, Ema H, Sudo K, Nakauchi H. Asymmetric division and lineage commitment at the level of hematopoietic stem cells: inference from differentiation in daughter cell and granddaughter cell pairs. J Exp Med. 2004;199:295-302.
48. Ema H, Takano H, Sudo K, Nakauchi H. In vitro self-renewal division of hematopoietic stem cells. J Exp Med. 2000;192:1281-1288.
49. Beckmann J, Scheitza S, Wernet P, Fischer JC, Giebel B. Asymmetric cell division within the human hematopoietic stem and progenitor cell compartment: Identification of asymmetrically segregating proteins. Blood. 2007
50. Yin AH, Miraglia S, Zanjani ED, Almeida-Porada G, Ogawa M, Leary AG, Olweus J, Kearney J, Buck DW. AC133, a novel marker for human hematopoietic stem and progenitor cells. Blood. 1997;90:5002-5012.
51. Miraglia S, Godfrey W, Yin AH, Atkins K, Warnke R, Holden JT, Bray RA, Waller EK, Buck DW. A novel five-transmembrane hematopoietic stem cell antigen: isolation, characterization, and molecular cloning. Blood. 1997;90:5013-5021.
52. Krause DS, Fackler MJ, Civin CI, May WS. CD34: structure, biology, and clinical utility. Blood. 1996;87:1-13.
53. Arribas M, Cutler DF. Weibel-Palade body membrane proteins exhibit differential trafficking after exocytosis in endothelial cells. Traffic. 2000;1:783-793.
54. Escola JM, Kleijmeer MJ, Stoorvogel W, Griffith JM, Yoshie O, Geuze HJ. Selective enrichment of tetraspan proteins on the internal vesicles of multivesicular endosomes and on exosomes secreted by human B-lymphocytes. J Biol Chem. 1998;273:20121-20127.
55. Kobayashi T, Vischer UM, Rosnoblet C, Lebrand C, Lindsay M, Parton RG, Kruithof EK, Gruenberg J. The tetraspanin CD63/lamp3 cycles between endocytic and secretory compartments in human endothelial cells. Mol Biol Cell. 2000;11:1829-1843.
56. Qian ZM, Li H, Sun H, Ho K. Targeted drug delivery via the transferrin receptor-mediated endocytosis pathway. Pharmacol Rev. 2002;54:561-587.
57. Shen Q, Temple S. Creating asymmetric cell divisions by skewing endocytosis. Sci STKE. 2002;2002:PE52.
58. Gho M, Bellaiche Y, Schweisguth F. Revisiting the Drosophila microchaete lineage: a novel intrinsically asymmetric cell division generates a glial cell. Development. 1999;126:3573-3584.

59. Guo M, Jan LY, Jan YN. Control of daughter cell fates during asymmetric division: interaction of Numb and Notch. Neuron. 1996;17:27-41.
60. Frise E, Knoblich JA, Younger-Shepherd S, Jan LY, Jan YN. The Drosophila Numb protein inhibits signaling of the Notch receptor during cell-cell interaction in sensory organ lineage. Proc Natl Acad Sci U S A. 1996;93:11925-11932.
61. Hutterer A, Knoblich JA. Numb and alpha-Adaptin regulate Sanpodo endocytosis to specify cell fate in Drosophila external sensory organs. EMBO Rep. 2005;6:836-842.
62. Santolini E, Puri C, Salcini AE, Gagliani MC, Pelicci PG, Tacchetti C, Di Fiore PP. Numb is an endocytic protein. J Cell Biol. 2000;151:1345-1352.
63. Berdnik D, Torok T, Gonzalez-Gaitan M, Knoblich JA. The endocytic protein alpha-Adaptin is required for numb-mediated asymmetric cell division in Drosophila. Dev Cell. 2002;3:221-231.
64. Skeath JB, Doe CQ. Sanpodo and Notch act in opposition to Numb to distinguish sibling neuron fates in the Drosophila CNS. Development. 1998;125:1857-1865.
65. Verdi JM, Schmandt R, Bashirullah A, Jacob S, Salvino R, Craig CG, Program AE, Lipshitz HD, McGlade CJ. Mammalian NUMB is an evolutionarily conserved signaling adapter protein that specifies cell fate. Curr Biol. 1996;6:1134-1145.
66. Zhong W, Feder JN, Jiang MM, Jan LY, Jan YN. Asymmetric localization of a mammalian numb homolog during mouse cortical neurogenesis. Neuron. 1996;17:43-53.
67. Zhong W, Jiang MM, Weinmaster G, Jan LY, Jan YN. Differential expression of mammalian Numb, Numblike and Notch1 suggests distinct roles during mouse cortical neurogenesis. Development. 1997;124:1887-1897.
68. French MB, Koch U, Shaye RE, McGill MA, Dho SE, Guidos CJ, McGlade CJ. Transgenic expression of numb inhibits notch signaling in immature thymocytes but does not alter T cell fate specification. J Immunol. 2002;168:3173-3180.
69. Berezovska O, McLean P, Knowles R, Frosh M, Lu FM, Lux SE, Hyman BT. Notch1 inhibits neurite outgrowth in postmitotic primary neurons. Neuroscience. 1999;93:433-439.
70. McGill MA, McGlade CJ. Mammalian numb proteins promote Notch1 receptor ubiquitination and degradation of the Notch1 intracellular domain. J Biol Chem. 2003;278:23196-23203.
71. Cayouette M, Whitmore AV, Jeffery G, Raff M. Asymmetric segregation of Numb in retinal development and the influence of the pigmented epithelium. J Neurosci. 2001;21:5643-5651.
72. Shen Q, Zhong W, Jan YN, Temple S. Asymmetric Numb distribution is critical for asymmetric cell division of mouse cerebral cortical stem cells and neuroblasts. Development. 2002;129:4843-4853.
73. Conboy IM, Rando TA. The regulation of Notch signaling controls satellite cell activation and cell fate determination in postnatal myogenesis. Dev Cell. 2002;3:397-409.
74. Chang JT, Palanivel VR, Kinjyo I, Schambach F, Intlekofer AM, Banerjee A, Longworth SA, Vinup KE, Mrass P, Oliaro J, Killeen N, Orange JS, Russell SM, Weninger W, Reiner SL. Asymmetric T lymphocyte division in the initiation of adaptive immune responses. Science. 2007;315:1687-1691.
75. Dho SE, Trejo J, Siderovski DP, McGlade CJ. Dynamic regulation of mammalian numb by G protein-coupled receptors and protein kinase C activation: Structural determinants of numb association with the cortical membrane. Mol Biol Cell. 2006;17:4142-4155.
76. Metzelaar MJ, Wijngaard PL, Peters PJ, Sixma JJ, Nieuwenhuis HK, Clevers HC. CD63 antigen. A novel lysosomal membrane glycoprotein, cloned by a screening procedure for intracellular antigens in eukaryotic cells. J Biol Chem. 1991;266:3239-3245.
77. Bampton ET, Goemans CG, Niranjan D, Mizushima N, Tolkovsky AM. The dynamics of autophagy visualized in live cells: from autophagosome formation to fusion with endo/lysosomes. Autophagy. 2005;1:23-36.

78. Duffield A, Kamsteeg EJ, Brown AN, Pagel P, Caplan MJ. The tetraspanin CD63 enhances the internalization of the H,K-ATPase beta-subunit. Proc Natl Acad Sci U S A. 2003;100:15560-15565.
79. Tsukazaki T, Chiang TA, Davison AF, Attisano L, Wrana JL. SARA, a FYVE domain protein that recruits Smad2 to the TGFbeta receptor. Cell. 1998;95:779-791.
80. Bokel C, Schwabedissen A, Entchev E, Renaud O, Gonzalez-Gaitan M. Sara endosomes and the maintenance of Dpp signaling levels across mitosis. Science. 2006;314:1135-1139.
81. Panopoulou E, Gillooly DJ, Wrana JL, Zerial M, Stenmark H, Murphy C, Fotsis T. Early endosomal regulation of Smad-dependent signaling in endothelial cells. J Biol Chem. 2002;277:18046-18052.
82. Andrews R, Ahringer J. Asymmetry of Early Endosome Distribution in C. elegans Embryos. PLoS ONE. 2007;2:e493.
83. Rose LS, Kemphues KJ. Early patterning of the C. elegans embryo. Annu Rev Genet. 1998;32:521-545.
84. Kemphues KJ, Priess JR, Morton DG, Cheng NS. Identification of genes required for cytoplasmic localization in early C. elegans embryos. Cell. 1988;52:311-320.
85. Watts JL, Etemad-Moghadam B, Guo S, Boyd L, Draper BW, Mello CC, Priess JR, Kemphues KJ. par-6, a gene involved in the establishment of asymmetry in early C. elegans embryos, mediates the asymmetric localization of PAR-3. Development. 1996;122:3133-3140.
86. Giebel B, Wodarz A. Tumor suppressors: control of signaling by endocytosis. Curr Biol. 2006;16:R91-92.
87. Bache KG, Slagsvold T, Stenmark H. Defective downregulation of receptor tyrosine kinases in cancer. Embo J. 2004;23:2707-2712.
88. Slagsvold T, Pattni K, Malerod L, Stenmark H. Endosomal and non-endosomal functions of ESCRT proteins. Trends Cell Biol. 2006;16:317-326.
89. Moberg KH, Schelble S, Burdick SK, Hariharan IK. Mutations in erupted, the Drosophila ortholog of mammalian tumor susceptibility gene 101, elicit non-cell-autonomous overgrowth. Dev Cell. 2005;9:699-710.
90. Vaccari T, Bilder D. The Drosophila tumor suppressor vps25 prevents nonautonomous overproliferation by regulating notch trafficking. Dev Cell. 2005;9:687-698.
91. Thompson BJ, Mathieu J, Sung HH, Loeser E, Rorth P, Cohen SM. Tumor suppressor properties of the ESCRT-II complex component Vps25 in Drosophila. Dev Cell. 2005;9:711-720.

10.6 Cell polarity is a prerequisite for migration and homing of primitive human hematopoietic cells (Beckmann et al., 2008, eingereicht)

Cell polarity is a prerequisite for migration and homing of primitive human hematopoietic cells

Short title: Cell Polarity and Migration of $CD34^+$ cells

Julia Beckmann[1], Lawrence Rajendran[2] and Bernd Giebel[1]

[1] From the Institute for Transplantation Diagnostics and Cellular Therapeutics, Heinrich-Heine-University Duesseldorf, Duesseldorf, Germany

[2] Max Planck Institute of Molecular Cell Biology and Genetics, Dresden, Germany.

Correspondence:

Bernd Giebel, Ph.D. Institute for Transplantation Diagnostics and Cell Therapeutics, Heinrich-Heine-University Duesseldorf, D-40225 Duesseldorf, Germany
Tel.: +49 211 8119443
FAX: +49 211 8117643
e-mail: giebel@itz.uni-duesseldorf.de

Abstract Word Count: 196
Total Text Word Count: 4864
Scientific Heading: Stem Cells in Hematology

ABSTRACT

Several signal transducing molecules, the chemokine receptor CXCR4, the phosphoinositol-3-kinase (PI3K), an atypical protein kinase C (aPKC) and Rho-GTPases, have been associated with homing of hematopoietic stem and progenitor cells (HSC/HPC) and control their migration. Since cell polarization is a prerequisite for cell migration, we analyzed the effect of these signaling molecules and the role of the cytoskeleton on the establishment and maintenance of cell polarity in human umbilical cord blood derived $CD34^+$ cells.

Studying sub-cellular distributions of lipid raft associated proteins, i.e. of the inter-cellular adhesion molecule 3 (ICAM-3), flotillin-1 and -2, we distinguish cues of intrinsic and morphological cell polarities. Using this discrimination, we show that protein translation, actin polymerization and activities of PI3K, aPKCs and the Rho-GTPases are required for establishing and maintaining polarized cell morphologies. In contrast, establishment of the intrinsic polarity mainly depends on protein translation and PI3K and aPKC activities, while its maintenance was strongly disturbed only in the presence of a PI3K inhibitor. Inhibition of CXCR4 signaling did not detectably affect cell polarity but interfered with *in vitro* migratory capabilities of HSC/HPC. Altogether we qualified cell polarization as an essential first step in the cascade that orchestrates HSC/HPC homing.

Introduction

Hematopoietic stem cells (HSC) are defined as cells with high self-renewal capacities which can give rise to cells of all blood lineages during the entire life. Additionally they contain the capacity to home to the BM, the main site of adult hematopoiesis. Homing is a naturally occurring process which comprises the capability of circulating primitive hematopoietic cells to find their way into the BM, to actively cross the endothelium which separates the blood from the BM, and to lodge to appropriate niches.[1,2]

Homing can be sectioned in a variety of different processes. It involves selectin-mediated tethering and rolling of circulating HSC/HPC on endothelial cells[3-5] as well as interactions of chemokines with their corresponding receptors, i.e. the binding of the chemokine stromal derived factor-1 (SDF-1α) to its receptor CXCR4.[6-11] Due to this interaction, a tight attachment of HSC/HPC to the endothelial wall is established through α4 and α5 integrins and CD44, thus providing the basis for the transendothelial migration which subsequently occurs.[10,12-19] Once arrived in the adjacent tissue HSC/HPC migrate along the BM stroma to finally lodge into their niches that have been discovered in the endosteum of the bone marrow and in the vicinity of endothelial cells in the BM and in the spleen.[20-22]

According to our current understanding, the SDF-1α binding to CXCR4, which is expressed on the majority of primitive hematopoietic cells, is implicated to play a central role during homing.[7] Mechanistically, binding of SDF-1α activates CXCR4 and amongst others results in the activation of the PI3K.[23-25] One of the downstream targets of the PI3K is the aPKC nPKCζ,[26,27] whose activity in combination with that of the Rho-GTPases Cdc42 and Rac1 regulates the assembly of components of the cytoskeleton.[28-33] Inhibition of any of these signaling transducing activities interferes with the *in vitro* migratory and homing capabilities of HSC/HPC.[7,11,34-41]

Since the acquisition of polarized morphologies is an essential prerequisite for cell migration,[42] the adoption of polarized morphologies should be one of the earliest events in processes that orchestrate HSC/HPC homing. Indeed, in our previous work, we have demonstrated that upon cultivation HSC/HPC increase in size and acquire a polarized cell shape, forming a leading edge at the front and a specialized structure, the uropod, at the rear.[35] These morphological changes coincide with redistributions of lipids and lipid-raft associated proteins, e.g. CXCR4 and the ganglioside GM3 become highly enriched at the leading edge and CD43, CD44, ICAM-3 (CD50), ICAM-1 (CD54) as well as Prominin-1/CD133 at the uropod.[35]

At the example of resting T- and B-lymphocytes, we have shown that the lipid raft associated flotillins, flotillin-1 (Flot-1) and flotillin-2 (Flot-2) become distributed in a polarized manner prior to all other investigated cell polarity-associated molecules and prior to a morphological polarization.[43] According to this observation, we suggested that flotillin polarization provides one of the earliest events in the course of cellular polarization of primitive hematopoietic cells.

In order to dissect the polarization process of primitive hematopoietic cells we have studied the flotillin distributions in combination with that of the lipid raft associated uropod marker ICAM-3 in freshly isolated as well as in cultivated, human umbilical cord blood derived $CD34^+$ cells. To study whether signal transducing molecules that participate in HSC/HPC homing, already control HSC/HPC polarization, we have also analyzed the distribution of the flotillins together with that of ICAM-3 in $CD34^+$ cells that had been cultured in the presence of drugs which are known to inhibit HSC/HPC homing.

MATERIALS AND METHODS

Cell preparation and cell culture

Human umbilical cord blood (CB) samples were obtained from unrelated donors and are used after informed consent of the mothers according to the declaration of Helsinki. The usage of these materials was approved by our University internal ethics commission. Following isolation of MNC via Ficoll density gradient centrifugation (Biocoll Separating Solution; Biochrom AG, Berlin, Germany), $CD34^+$ cells were enriched by magnetic cell sorting (MACS; Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's instructions. We yielded an average purity of $CD34^+$ cells of 78.0% ± 10.8% (n=24).

Freshly enriched $CD34^+$ cells were cultured in a humidified atmosphere at 37°C and 5% CO_2 at a density of approximately $1x10^5$ cells/mL in Iscove's modified Dulbecco's medium (IMDM; Invitrogen GmbH, Karlsruhe, Germany) supplemented with 20% fetal calf serum (FCS; Biochrom AG), 1000 U/mL penicillin, and 100 U/mL streptomycin (Invitrogen GmbH) in the presence of early-acting cytokines, i.e. fetal liver tyrosine kinase 3 ligand (FLT3L), SCF, and thrombopoietin (TPO), each at 10 ng/mL final concentration (all from PeproTech, Rocky Hill, NJ, USA).

To inhibit the PI3K-activity cells were incubated in the presence of 100 µM Ly294002 (Calbiochem, Bad Soden, Germany). The aPKC inhibitor Chelerythrine chloride (Calbiochem) was used at a final concentration of 10 µg/mL. To inhibit signaling via G_i-protein coupled receptors cells were incubated with 2 µg/mL Pertussistoxin (Calbiochem) and to inhibit Rho-GTPases with 200 ng/mL *Clostridium difficile* toxin B (Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany). Translation was blocked by Cycloheximide (Sigma-Aldrich Chemie GmbH) at a final concentration of 20 µg/mL. Nocodazole (Sigma-Aldrich Chemie GmbH) was used at 20 µg/mL to promote microtubule depolymerization, and Cytochalasin B (Sigma-Aldrich Chemie GmbH) at 10 µg/mL to inhibit actin polymerization.

Immunostainings and microscopy

Freshly isolated $CD34^+$ cells were either stained immediately or after cultivation for 1-2 days in the presence of early acting cytokines. To conserve their morphology, cultivated $CD34^+$ cells were prefixed for 5 minutes (min) at room temperature in 1% paraformaldehyde (PFA; Sigma-Aldrich Chemie GmbH). After washing in PBS cells were resuspended in a small volume of PBS (~100 µL) and transferred in 10 µL aliquots to special coated adhesion slides (Squarix biotechnology; Marl, Germany) to which they adhere within 10 min. Adhered cells were then fixed with ice-cold methanol for 5 min in -20°C and then washed 3 times with PBS. After 30 min blocking in 10% donkey-serum (Jackson Immuno Research Laboratories Inc, West Grove, PA, USA), cells were stained for 45 min at room temperature with either 1:150 diluted anti-flotillin-1 antibodies (BD PharMingen, Heidelberg, Germany) or with 1:1000 diluted anti-flotillin-2 antibodies (BD PharMingen). After a washing step, cells were counterstained for 30 min with 1:200 diluted Cy3-conjugated donkey-anti-mouse AffiniPure Fab fragments (Jackson Immuno Research Laboratories Inc). Remaining mouse epitopes were then saturated with unconjugated rabbit-anti-mouse AffiniPure Fab fragments for 30 min (Jackson Immuno Research Laboratories Inc). Next, cells were incubated for 30 min at room temperature with anti-ICAM-3-FITC antibodies (TU41; BD PharMingen). Following the antibody incubation cells were washed 2 times with PBS and counterstained either Alexa Fluor488-conjugated goat-anti-mouse IgG (1:100; Invitrogen) secondary antibodies.

For the CD133 Flotillin double-staining, cells were fixed in 0.2% PFA for 5 min, washed in PBS, and stained for 20 min with PE conjugated anti-CD133 antibodies (1:10; AC133/1-PE; Miltenyi Biotec). After washing, cells were incubated in 1% PFA for 5 minutes, washed and transferred to adhesion slides. Following methanol treatment cells were blocked for 30 min in 10% donkey-serum (Jackson Immuno Research Laboratories Inc.) and counterstained with Cy3-conjugated Fab-fragments (1:200 Jackson Immuno Research Laboratories Inc). Free

murine epitops were then blocked with unconjugated Fab-fragments (1:100; Jackson Immuno Research Laboratories Inc). The following anti-flotillin staining was performed as described above. For the counterstaining of the Flotillins Alexa Fluor488-conjugated goat-anti-mouse IgG (1:400; Invitrogen) secondary antibodies were used. Labeled cells were washed with PBS thrice, and mounted in 75% glycerin containing propylgallat (50 mg/mL) and DAPI (4,6 diamidino-2- phenylindole; 200 ng/mL; Roche, Mannheim, Germany). Cells were observed with an Axioplan 2 fluorescence microscope (Carl Zeiss, Goettingen, Germany) using x63/1.25 or x100/1.30 oil immersion lenses. For evaluation of the immunostaining at least 80 labeled cells per sample were counted and classified into one of the defined categories.

Migration assays

The migratory potential of cultivated cells was analyzed by transmigration assays using 3 µm pore filters (Costar Transwell, 6.5-mm diameter; Corning Incorporated, Corning, NY, USA) as described previously.[35] Briefly, after a culture period of 2 days, either in the presence or absence of specific inhibitors, approximately $5x10^4$ CB-derived non-adherent $CD34^+$ cells were loaded onto filters and were allowed to migrate over night under culture conditions. In selected cases SDF-1α (R&D SystemsInc, Minneapolis, MN, USA) was added to the lower chamber in a final concentration of 100 ng/mL. For migration analysis of cells which were preincubated for 2h with one of the inhibitors, the inhibitors were also added to the cell suspensions in the upper as well as to the lower chambers. After carefully removing the filters the percentage of transmigrated cells was evaluated flow-cytometrically using a Cytomics FC 500 flow-cytometer equipped with the RXP software (Beckman Coulter, Krefeld, Germany). Since the initial cell number and the volume of all recovered cell suspensions were equalized before, flow-cytometric analyses and the time and flow rate were kept constant, we could set the number of measured transmigrated cells in relation to the number of measured cells that were cultured without the transwell filters.

Results

Morphologic and intrinsic cell polarity of freshly isolated and cultivated CD34$^+$ cells

To study whether flotillins are pre-compartmentalized in HSC/HPC like in resting T- and B-cells, we analyzed the flotillin distributions in freshly isolated as well as in cultivated CB-derived CD34$^+$ cells. While the vast majority of the freshly isolated CD34$^+$ cells which lack any indication of morphological polarities displayed random Flot-1 and Flot-2 distributions, approximately 25% of them showed cap like flotillin localizations. This observation suggests that only a small proportion of these cells contain manifested, intrinsic cell polarity cues. However, in agreement to our previous data most of the cultivated CD34$^+$ cells exhibited a polarized morphology (degree of cells containing a polarized morphology: 72.3% ± 15.3% of the cells at culture day 1, n=9; 71.6% ± 10.6% at culture day 2, n=7). Of the morphological round cells about one 3rd still showed random Flot-1 and Flot-2 distribution (Fig. 1 A, D), in the remaining round cells the flotillins where concentrated in a cap like structure (Fig. 1 B, E), like in T and B lymphocytes.[43] In all morphologically polarized CD34$^+$ cells both, Flot-1 and Flot-2 were highly enriched in the tip of the uropod (Fig. 1C; F). Occasionally an additional flotillin-positive domain was found at the base of the uropod (Fig. 1 F), the region in which we recently observed endosomes to bud into the interior of the cells.[44]

Counterstaining of cultivated CD34$^+$ cells with antibodies recognizing the lipid raft associated uropod marker ICAM-3 revealed that the ICAM-3 distribution was similar to that of both flotillins. In morphological round cells the flotillins and ICAM-3 were either distributed in a random fashion (Fig. 1 A, D) or both, the flotillins as well as ICAM-3 were partially co-localized and displayed polarized distributions (Fig. 1 B, E). A partial co-localization of the flotillins and ICAM-3 is also found in morphological polarized CD34$^+$ cells, regularly at the tip of the uropod (Fig. 1 C, E), suggesting that flotillins and ICAM-3 localize to the same or closely adjacent sites. Since we recently qualified the stem cell surrogate marker

CD133/Prominin-1 as a specific uropod marker in polarized $CD34^+$ cells, we also studied its distribution in relation to that of both flotillins. According to our staining both, the flotillins and CD133/Prominin-1, are highly enriched in the tip of the uropod, but localize to different microdomains (Fig. 2).

Cell polarity cues in inhibitor treated $CD34^+$ cells

To analyze whether pharmacological substances that have been found to interfere with HSC/HPC homing have any impact on the cell polarity of cultivated $CD34^+$ cells, we have analyzed morphological as well as intrinsic cell polarity cues (measured by the flotillin and ICAM-3 distributions) of treated as well as of non-treated $CD34^+$ cells. In this context, we have chosen two settings: i) to study the effects of the drugs on the establishment of the intrinsic and morphological cell polarity, freshly isolated $CD34^+$ cells were immediately cultured in the presence of the drugs. In those cases in which the drug treatment was toxic to the cells, cells were analyzed at culture day 1 at which the vast majority of the treated cells were still alive, otherwise cells were examined at culture day 2. ii) to investigate whether drug treatment interferes with the maintenance of cell polarity and/or the cell motility of already polarized $CD34^+$ cells, we further added these drugs for 2 hours to $CD34^+$ cells that had been cultured for 2 days in a drug free-environment before, and studied the effect on the cell polarity thereafter. Additionally, we transferred aliquots of these cells in the presence of the drugs to Transwell assays and estimated the rate of transmigrated cells 12 hours later.

Protein synthesis is required for the polarization process of $CD34^+$ cells

We first asked, if new protein synthesis is essential for setting up intrinsic and morphological cell polarities. To this end, we cultured freshly isolated $CD34^+$ cells in the presence or absence of the protein translation inhibiting drug Cycloheximide (CHX). Due to the toxicity of the CHX treatment, which killed most cells between culture day 1 and day 2, we analyzed $CD34^+$ cells at culture day 1. In contrast to control $CD34^+$ cells, most CHX treated

$CD34^+$cells remained small and did not adopt polarized morphologies (Fig. 3A; Supplement Tab. 1). Furthermore, the content of $CD34^+$ cells displaying a polarized distribution of the flotillins or of ICAM-3 was significantly reduced as well (Fig. 3A; Supplement Tab. 1).

Next we tested whether protein translation is also required to maintain established intrinsic and morphological cell polarity in cultured $CD34^+$ cells. Remarkably, most of the 2 hours treated $CD34^+$ cells lost their polarized morphology (Fig. 3A'; Supplement Tab. 2). Additionally, the polar distribution of ICAM-3 and both flotillins was slightly but significantly decreased as well (Fig. 3A'; Supplement Tab. 2). Therefore, in $CD34^+$ cells protein translation is required for both, to set up and to maintain intrinsic and morphological cell polarity cues. In agreement with its effect on the cell morphology, significantly less CHX treated $CD34^+$ cells migrated through the Transwell filters than non-treated control $CD34^+$ cells (Fig. 4A; Supplement Tab. 3).

Pertussis toxin decreases *in vitro* migration but not the polarization of $CD34^+$ cells

Since SDF-1α mediated activation of CXCR4 triggers homing of HSC/HPC, we analyzed whether inhibition of SDF-1α mediated chemotaxis would affect polarization of CD34 cells. Surprisingly, in our analyses pertussis toxin (PTX) treatment, which inhibits SDF-1α-induced chemotaxis,[6] did not reveal any effect on the cell polarization process of $CD34^+$ cells in both settings. Neither the establishment nor the maintenance of morphological or intrinsic cell polarities was recognizable altered in comparison to non-treated $CD34^+$ cells (Fig. 3B, B'; Supplement Tab. 1, 2). According to these results and due to the fact that PTX inhibits the $G_{\alpha i}$ mediated signaling which acts downstream of the CXCR4 receptor,[45] we conclude that at least in the presence of the early acting cytokines $G_{\alpha i}$-mediated signaling is dispensable for the establishment and maintenance of the cell polarity in cultivated $CD34^+$ cells. However, in contrast to the cell polarization process, we found that PTX treatment interferes with the

transmigration rate of cultivated $CD34^+$ cells and significantly reduced the proportion of transmigrated cells (Fig. 4B; Supplement Tab. 3).

Pointing into a similar direction we realized in some control experiments that SDF-1α only marginally triggered the cell polarization process of $CD34^+$ cells that were cultured for 1 day in the presence or absence of SDF-1α under otherwise serum and cytokine free conditions (with SDF-1α: 39.3% ± 13.5% *versus* SDF-1α-free control: 30.0% ± 8.8%, p=0.05, n=6). Furthermore, in good agreement to the published data addition of SDF-1α to the Transwell assays increased the transmigration rate of $CD34^+$ cells that were cultured for 2 days in the presence early acting cytokines before (+SDF-1α: 28.2% ± 8.9% *versus* control: 15.1% ± 3.1, $p_{SDF\text{-}1\alpha}<0.05$; n=4); this migration stimulating effect was completely blocked in the presence of PTX (PTX2h+SDF-1α: 6.7% ± 2.5% versus SDF-1α: $p_{PTX2h,\ SDF\text{-}1\alpha}<0.05$; n=4).

The activity of PI3K is essentially required to set up and maintain cell polarity

Due to the fact that CXCR4 signaling has been reported to activate the PI3K,[23-25] we studied the role of the PI3K on the cell polarization process of $CD34^+$ cells next. In agreement to our previous data,[35] $CD34^+$ cells which are cultivated in the presence of the PI3K inhibitor Ly294002 remain small and round and die between culture day 1 and day 2. Accompanied by the absence of morphological polarization at culture day 1 (Fig. 3C; Supplement Tab. 1), most of the Ly294002 treated $CD34^+$ cells lacked a polarized distribution of ICAM-3 and the flotillins (Fig. 3C; Supplement Tab. 1). Demonstrating that the PI3K activity is not only required to establish cell polarity but also for their maintenance, we found that most of the cultivated $CD34^+$ cell which were incubated for 2 hours with Ly294002 lost their polarized morphologies (Fig. 3B'; Supplement Tab. 2) and their polarized ICAM-3 and flotillin distributions (Fig. 3B'; Supplement Tab. 2). Along with that the transmigration capability of these cells was significantly reduced (Fig. 4B; Supplement Tab. 3).

Activities of atypical protein kinases C are required to establish and maintain cell polarity in CD34$^+$ cells

One of the downstream targets of the PI3K that have been implicated in the cell polarization processes of numerous of different cell types are the aPKCs.[26,27,46] Their activities can be inhibited in the presence of Chelerythrine chloride (CHEL) which interacts with the catalytic domain of the aPKCs and affects the translocation of aPKCs from the cytosol to the membrane.[47,48] To gain insight into the role of the aPKCs in the cell polarization process of human HSC/HPC, we cultured CD34$^+$ cells in the presence and absence of CHEL. Similar to the CHX and Ly294002 treatment we observed that CHEL is toxic to the cells and that many treated CD34$^+$ cells die between culture day 1 and day 2. Thus, we have analyzed the effect of CHEL treatment on CD34$^+$ cells on culture day 1.

At culture day 1 almost all CHEL treated CD34$^+$ cells had a round morphology and the content of CD34$^+$ cells revealing an intrinsic cell polarity was significantly reduced in comparison to non-treated cells (Fig. 3D; Supplement Tab. 1). Short term treatment of already polarized CD34$^+$ cells with CHEL resulted in a loss of morphological but not intrinsic cell polarity (Fig. 3D'; Supplement Tab. 2). Along with the loss of morphological cell polarity the transmigration rate of treated CD34$^+$ cells was highly and significantly suppressed (Fig. 4D; Supplement Tab. 3).

Small GTPases control morphological but only marginally affect intrinsic cell polarities

In combination with the aPKCs, the Rho-GTPases Cdc42 and Rac1 regulate the assembly of components of the cytoskeleton.[28-33] To investigate the role of the Rho-GTPases in the cell polarization process of HSC/HPC, we treated CD34$^+$ cells with the Rho-GTPase inhibitor *Clostridium difficile* Toxin B (CDTxB) which acts on the effector domain of the Rho-GTPases and thus blocks further downstream signaling.[50]

The addition of CDTxB to freshly isolated or to cultivated $CD34^+$ cells inhibited both, the establishment as well as the maintenance of polarized cell morphologies. Remarkably, it only marginally interferes with the establishment of intrinsic cell polarity cues and showed no effect on their maintenance (Fig. 3E and E'; Supplement Tab. 1 and 2). Again, in consistence with its effect on the polarized morphology and in agreement to the published data, CDTxB treatment significantly reduced the transmigration rate of cultivated $CD34^+$ cells (Fig. 4E; Supplement Tab. 3).

The role of cytoskeleton dynamics for cell polarity of $CD34^+$ cells

All external stimuli and internal signaling cascades that control cell movements become integrated and finally govern rearrangements of the cytoskeleton. In this context it has been shown that cell movement is tightly coupled to the formation of new and the depolymerization of old actin filaments (F-actin).[51] Furthermore, actin polymerization is required to establish polarized cell shapes e.g. of neutrophils.[52] To determine the role of the actin cytoskeleton dynamics during establishment and maintenance of the cell polarity in HSC/HPC, we treated $CD34^+$ cells with the fungal toxin Cytochalasin B (CytB) which inhibits actin polymerization.[53,54] Consistent to the data reported for the neutrophils,[52] addition of CytB to freshly isolated $CD34^+$ cells completely blocked the establishment of polarized cell shapes (Fig. 3F; Supplement Tab. 1). However, although the level of $CD34^+$ cells displaying intrinsic cell polarity cues at culture day 2 was significantly below that of corresponding control cells, the vast majority of the treated $CD34^+$ cells established polarized ICAM-3 and flotillin distributions (Fig. 3F; Supplement Tab. 1). Similarly, addition of CytB to $CD34^+$ cells at culture day 2 suppressed polarized cell morphologies but had no major effect on the intrinsic cell polarity (Fig. 3F'; Supplement Tab. 2). And again, in correlation to the CytB's effect on the cell morphology the transmigration rate of treated $CD34^+$ cells was nearly completely suppressed (Fig. 4F; Supplement Tab. 3).

Apart from the actin cytoskeleton the microtubule cytoskeleton is involved in governing cell polarity and motility processes.[55-58] Since microtubuli polymerization can be blocked by the addition of Nocodazole (R 17934),[59] we have studied the effect of Nocodazole (Noco) treatment on $CD34^+$ cells. Most of the $CD34^+$ cells that were cultured for 2 days in the presence of Noco did not display a polarized morphology (Fig. 3G; Supplement Tab. 1). Similar to the situation observed in CytB treated $CD34^+$ cells the percentage of cells that established an intrinsic cell polarity was nearly as high as in corresponding control cells (Fig. 3G; Supplement Tab. 1). Addition of Noco for 2 hours to already polarized cells did not reveal any significant effect, neither on the morphological nor on the intrinsic cell polarity (Fig. 3G'; Supplement Tab. 2). Remarkably, the transmigration rate of treated cells was markedly increased (Fig. 4G; Supplement Tab. 3).

Discussion:

Here we demonstrate that many processes known to participate in HSC/HPC homing and migration are associated with the cellular polarization of primitive hematopoietic cells. Therefore, we consider the cellular polarization process as an essential first step in the multi-process cascade that mediates homing of primitive hematopoietic cells. Furthermore, by dissecting cell polarization at both morphological and molecular levels, we gained additional insights into the intrinsic mechanisms with which homing-associated processes orchestrate the cellular polarization.

Functional levels during HSC/HPC polarization

Recently, we reported that freshly isolated human $CD34^+$ cells are small round cells that contain a non-polar appearance. Upon stimulation with early acting cytokines, i.e. with FLT3L, SCF and TPO, these cells acquire a polarized morphology, a process which depends on the activity of the PI3K.[35] Concomitantly to the morphological polarization these primitive hematopoietic cells also redistribute certain lipid-raft associated cell surface molecules to one of the two arising cellular poles.[35] Now, by studying drug treated $CD34^+$ cells we found that the lipid raft associated proteins, ICAM-3 and flotillins often acquire a polarized distribution in treated $CD34^+$ cells that fail to adopt a polarized cell shape. This is in line with the finding that flotillins form preassembled platforms in a proportion of freshly isolated $CD34^+$ cells as well as in resting cells of different hematopoietic cell lines that contain a non-polarized cell shape.[43,60] It clearly demonstrates that primitive hematopoietic cells like mature leukocytes establish an intrinsic polarity before adopting a polarized cell shape.

According to our analyses protein translation, as well as the PI3K and aPKC activities participate in setting up intrinsic cell polarity cues. The establishment of this polarity also partially depends on the activities of the small Rho-GTPases and on actin polymerization. Inhibition of $G_{\alpha i}$ mediated signaling had no significant effect on the establishment of intrinsic

cell polarity while destabilization of microtubules had a mild effect. In contrast, all drugs except PTX strongly reduced the establishment of polarized cell morphologies.

Once established, only short term inhibition of PI3K activity resulted in a major loss of the intrinsic cell polarity, a gentle loss was observed when protein translation, actin polymerization or aPKC activities were blocked. Neither the inhibition of the small Rho-GTPases or of the $G_{\alpha i}$ mediated signaling pathway nor the short term destabilization of microtubules revealed a recognizable effect on the intrinsic cell polarity. However, all treatments but the drug mediated destabilization of microtubules or inhibition of the $G_{\alpha i}$ mediated signaling strongly disturbed the maintenance of polarized cell morphologies and concomitantly inhibited the migration rate of cultivated $CD34^+$ cells. These findings demonstrate that comparable to other cell types[42] $CD34^+$ cells can only migrate if they are able to adopt a polarized cell shape.

Apart from demonstrating that most of the drugs that are known to inhibit hematopoietic stem cell homing already interfere with the cellular polarization process, our data reveal mechanistically parallels to the cellular polarization processes of neutrophils as well as to that of other leukocytes.

Common mechanisms of leukocyte polarization

In neutrophils, like in other migrating cell systems stimulation with chemotactic active substances induces filamentous actin (F-actin) polymerization near the cell cortex, initially throughout the cells and later mainly at one site of the cell, at the leading edge.[52] This F-actin polymerization can effectively be blocked by cytochalasin or latrunculin which like in $CD34^+$ cells concomitantly inhibit the establishment of polarized cell shapes.[61,62] Therefore, it became evident that F-actin polymerization is one of the earliest events that triggers cellular polarization. Meanwhile it is well established that upon exposure to a chemoattractant gradient, products of phosphatidylinositol-3-OH kinases (PI3Ks), especially $PtdIns(3,4,5)P_3$,

accumulate at the site of the neutrophils which are located closest to the chemo-attractant source. To amplify the initial PtdIns(3,4,5)P_3 response a positive feedback loop, involving the Rho-GTPase Rac as well as F-actin, recruits additional PI3K to the plasma membrane, finally resulting in the establishment of an robust intra cellular PtdIns(3,4,5)P_3 gradient.[62-67]

Depending on PtdIns(3,4,5)P_3, the nPKCζ translocates to the plasma membrane and gets transiently activated at the leading edge.[26,68] Inhibition of the nPKCζ activity by Chelerythrine treatment almost completely abolished F-actin polymerization.[68] Since nPKCζ activity depends on PI3K activity and is required for F-actin polymerization it is tempting to speculate that nPKCζ essentially participates within the positive feedback loop, too.

In addition to this positive feedback loop, a negative feedback loop has been defined that depends on the activation of a Rho-dependent pathway. This pathway stimulates the activation of myosin II formation, of contractile actin-myosin complexes and myosin-dependent inhibition of Rac- and PI3K-dependent responses. While this complex inhibits formation of leading edge specific structures, the Rac activity at the front inhibits the Rho-dependent activation of myosin II.[66] Destabilization of microtubules has been shown to activate the Rho-kinase pathway[69,70] and is sufficient to initiate the polarization process and induce/enhance motility of neutrophils,[71,72] which is consistent with the increased motility that we observed in Nocodazole treated $CD34^+$ cells.

Our observation that inhibition of actin polymerization as well as that of PI3K, nPKCζ or Rho GTPase activities interfere with the acquisition of polarized cell morphologies, together with the recent finding that RhoH negatively regulates Rac mediated signaling in murine hematopoietic progenitor cells,[73] supports the notion that conserved positive and negative feedback loops control the polarization process of primitive hematopoietic cells, too.

Membrane rafts and cellular polarization

Supplementary to the signaling transduction pathways described before, the integrity of membrane rafts has been shown to regulate polarization and migration processes in different cell types including leukocytes. Disruption of membrane rafts by cholesterol depletion in neutrophils[74-76] and in T cells[77-79] affects polarization processes. Consistent with this, we observed that primitive hematopoietic cells loose their polarized morphology after cholesterol depletion (data not shown). Together these data suggest that the raft integrity is a major determinant to control morphological cell polarization in stimulated leukocytes including primitive hematopoietic cells. Interestingly, antibody mediated cross-linking of lipid raft associated proteins like CD43, CD44 and ICAM-3 triggers capping of corresponding antigens, the morphological cell polarization as well as the locomotion of neutrophils and T cells.[79-84] Therefore, it is tempting to speculate that the clustering of membrane rafts precedes an activation of corresponding signaling cascades. Our findings that under most conditions tested the majority of the cells which did not adopt a polarized cell shape showed a polarized distribution of ICAM-3 and the two flotillins strongly support this hypothesis. Furthermore, our observation that the rate of $CD34^+$ cells displaying polarized ICAM-3 and flotillin distributions was significantly decreased in the presence of the PI3K inhibitor Ly294002, supports a model according to which lipid raft clustering and PI3K activities act in a positive feedback loop to set up and maintain leukocyte polarities,[75] a feedback loop that according to our data might initially depend on protein translation and aPKC activities.

Potential clinical impact

In summary, our data reveal that together with many signaling cascades that have been associated with HSC/HPC homing, the lipid raft organization controls cellular polarization processes in primitive hematopoietic cells. Since cellular polarization is an essential prerequisite for cell migration the disturbance of either signaling cascades or lipid raft

organization not only interferes with the cellular polarization process itself, but also alters their migration and homing capabilities. In this context it is interesting to note that antibodies which block the function of CD44, not only block the homing capacity of HSC/HPC but also that of AML and CML cells.[14,16,17,85,86] Remarkably, the treatment of AML and CML cells with blocking anti-CD44 antibodies also strongly reduces their tumorgenicity.[85,86] Therefore, we hypothesize that any process which interferes with the polarization or migration capacity of leukaemic cells should alter their malignant capacity as well. Screening for drugs or antibodies that specifically inhibit cellular polarization of leukaemic cells might thus provide new avenues for anti-leukaemia treatment strategies.

Acknowledgments

The authors thank Johannes Fischer, Michael Punzel, Ingmar Bruns and Rainer Haas for general discussion and critics on the manuscript; Svenja Jünger, Jan Spanholtz and Gregor von Levetzow for general experimental support. Umbilical cord blood samples were kindly provided by Gesine Kögler of our institute (ITZ). This study was supported by grants from the Deutsche Forschungsgemeinschaft (SPP1109 GI 336/1-4 to B.G.), from the Forschungskommission of the HHU-Duesseldorf (B.G.) and the Leukämieliga e.V. (B.G.).

J.B. designed the study, performed experiments and wrote the manuscript; L.R. designed experiments, discussed data and assisted in writing; B.G. designed the study, analyzed the data and wrote the manuscript.

Authors declare no competing financial interests.

Literature

1. Lapidot T, Dar A, Kollet O. How do stem cells find their way home? Blood. 2005;106:1901-1910.
2. Chute JP. Stem cell homing. Curr Opin Hematol. 2006;13:399-406.
3. Frenette PS, Subbarao S, Mazo IB, von Andrian UH, Wagner DD. Endothelial selectins and vascular cell adhesion molecule-1 promote hematopoietic progenitor homing to bone marrow. Proc Natl Acad Sci U S A. 1998;95:14423-14428.

4. Mazo IB, Gutierrez-Ramos JC, Frenette PS, Hynes RO, Wagner DD, von Andrian UH. Hematopoietic progenitor cell rolling in bone marrow microvessels: parallel contributions by endothelial selectins and vascular cell adhesion molecule 1. J Exp Med. 1998;188:465-474.
5. Katayama Y, Hidalgo A, Furie BC, Vestweber D, Furie B, Frenette PS. PSGL-1 participates in E-selectin-mediated progenitor homing to bone marrow: evidence for cooperation between E-selectin ligands and alpha4 integrin. Blood. 2003;102:2060-2067.
6. Aiuti A, Webb IJ, Bleul C, Springer T, Gutierrez-Ramos JC. The chemokine SDF-1 is a chemoattractant for human CD34+ hematopoietic progenitor cells and provides a new mechanism to explain the mobilization of CD34+ progenitors to peripheral blood. J Exp Med. 1997;185:111-120.
7. Peled A, Petit I, Kollet O, Magid M, Ponomaryov T, Byk T, Nagler A, Ben-Hur H, Many A, Shultz L, Lider O, Alon R, Zipori D, Lapidot T. Dependence of human stem cell engraftment and repopulation of NOD/SCID mice on CXCR4. Science. 1999;283:845-848.
8. Jo DY, Rafii S, Hamada T, Moore MA. Chemotaxis of primitive hematopoietic cells in response to stromal cell-derived factor-1. J Clin Invest. 2000;105:101-111.
9. Kawabata K, Ujikawa M, Egawa T, Kawamoto H, Tachibana K, Iizasa H, Katsura Y, Kishimoto T, Nagasawa T. A cell-autonomous requirement for CXCR4 in long-term lymphoid and myeloid reconstitution. Proc Natl Acad Sci U S A. 1999;96:5663-5667.
10. Peled A, Kollet O, Ponomaryov T, Petit I, Franitza S, Grabovsky V, Slav MM, Nagler A, Lider O, Alon R, Zipori D, Lapidot T. The chemokine SDF-1 activates the integrins LFA-1, VLA-4, and VLA-5 on immature human CD34(+) cells: role in transendothelial/stromal migration and engraftment of NOD/SCID mice. Blood. 2000;95:3289-3296.
11. Kollet O, Spiegel A, Peled A, Petit I, Byk T, Hershkoviz R, Guetta E, Barkai G, Nagler A, Lapidot T. Rapid and efficient homing of human CD34(+)CD38(-/low)CXCR4(+) stem and progenitor cells to the bone marrow and spleen of NOD/SCID and NOD/SCID/B2m(null) mice. Blood. 2001;97:3283-3291.
12. Potocnik AJ, Brakebusch C, Fassler R. Fetal and adult hematopoietic stem cells require beta1 integrin function for colonizing fetal liver, spleen, and bone marrow. Immunity. 2000;12:653-663.
13. Hirsch E, Iglesias A, Potocnik AJ, Hartmann U, Fassler R. Impaired migration but not differentiation of haematopoietic stem cells in the absence of beta1 integrins. Nature. 1996;380:171-175.
14. Khaldoyanidi S, Denzel A, Zoller M. Requirement for CD44 in proliferation and homing of hematopoietic precursor cells. J Leukoc Biol. 1996;60:579-592.
15. Williams DA, Rios M, Stephens C, Patel VP. Fibronectin and VLA-4 in haematopoietic stem cell-microenvironment interactions. Nature. 1991;352:438-441.
16. Avigdor A, Goichberg P, Shivtiel S, Dar A, Peled A, Samira S, Kollet O, Hershkoviz R, Alon R, Hardan I, Ben-Hur H, Naor D, Nagler A, Lapidot T. CD44 and hyaluronic acid cooperate with SDF-1 in the trafficking of human CD34+ stem/progenitor cells to bone marrow. Blood. 2004;103:2981-2989.
17. Vermeulen M, Le Pesteur F, Gagnerault MC, Mary JY, Sainteny F, Lepault F. Role of adhesion molecules in the homing and mobilization of murine hematopoietic stem and progenitor cells. Blood. 1998;92:894-900.
18. Papayannopoulou T, Craddock C, Nakamoto B, Priestley GV, Wolf NS. The VLA4/VCAM-1 adhesion pathway defines contrasting mechanisms of lodgement of transplanted murine hemopoietic progenitors between bone marrow and spleen. Proc Natl Acad Sci U S A. 1995;92:9647-9651.
19. van der Loo JC, Xiao X, McMillin D, Hashino K, Kato I, Williams DA. VLA-5 is expressed by mouse and human long-term repopulating hematopoietic cells and mediates adhesion to extracellular matrix protein fibronectin. J Clin Invest. 1998;102:1051-1061.

20. Calvi LM, Adams GB, Weibrecht KW, Weber JM, Olson DP, Knight MC, Martin RP, Schipani E, Divieti P, Bringhurst FR, Milner LA, Kronenberg HM, Scadden DT. Osteoblastic cells regulate the haematopoietic stem cell niche. Nature. 2003;425:841-846.
21. Zhang J, Niu C, Ye L, Huang H, He X, Tong WG, Ross J, Haug J, Johnson T, Feng JQ, Harris S, Wiedemann LM, Mishina Y, Li L. Identification of the haematopoietic stem cell niche and control of the niche size. Nature. 2003;425:836-841.
22. Kiel MJ, Yilmaz OH, Iwashita T, Terhorst C, Morrison SJ. SLAM family receptors distinguish hematopoietic stem and progenitor cells and reveal endothelial niches for stem cells. Cell. 2005;121:1109-1121.
23. Ganju RK, Brubaker SA, Meyer J, Dutt P, Yang Y, Qin S, Newman W, Groopman JE. The alpha-chemokine, stromal cell-derived factor-1alpha, binds to the transmembrane G-protein-coupled CXCR-4 receptor and activates multiple signal transduction pathways. J Biol Chem. 1998;273:23169-23175.
24. Vicente-Manzanares M, Rey M, Jones DR, Sancho D, Mellado M, Rodriguez-Frade JM, del Pozo MA, Yanez-Mo M, de Ana AM, Martinez AC, Merida I, Sanchez-Madrid F. Involvement of phosphatidylinositol 3-kinase in stromal cell-derived factor-1 alpha-induced lymphocyte polarization and chemotaxis. J Immunol. 1999;163:4001-4012.
25. Sotsios Y, Whittaker GC, Westwick J, Ward SG. The CXC chemokine stromal cell-derived factor activates a Gi-coupled phosphoinositide 3-kinase in T lymphocytes. J Immunol. 1999;163:5954-5963.
26. Nakanishi H, Brewer KA, Exton JH. Activation of the zeta isozyme of protein kinase C by phosphatidylinositol 3,4,5-trisphosphate. J Biol Chem. 1993;268:13-16.
27. Le Good JA, Ziegler WH, Parekh DB, Alessi DR, Cohen P, Parker PJ. Protein kinase C isotypes controlled by phosphoinositide 3-kinase through the protein kinase PDK1. Science. 1998;281:2042-2045.
28. Etienne-Manneville S, Hall A. Cdc42 regulates GSK-3beta and adenomatous polyposis coli to control cell polarity. Nature. 2003;421:753-756.
29. Johansson A, Driessens M, Aspenstrom P. The mammalian homologue of the Caenorhabditis elegans polarity protein PAR-6 is a binding partner for the Rho GTPases Cdc42 and Rac1. J Cell Sci. 2000;113 (Pt 18):3267-3275.
30. Joberty G, Petersen C, Gao L, Macara IG. The cell-polarity protein Par6 links Par3 and atypical protein kinase C to Cdc42. Nat Cell Biol. 2000;2:531-539.
31. Adams AE, Johnson DI, Longnecker RM, Sloat BF, Pringle JR. CDC42 and CDC43, two additional genes involved in budding and the establishment of cell polarity in the yeast Saccharomyces cerevisiae. J Cell Biol. 1990;111:131-142.
32. Nobes CD, Hall A. Rho, rac, and cdc42 GTPases regulate the assembly of multimolecular focal complexes associated with actin stress fibers, lamellipodia, and filopodia. Cell. 1995;81:53-62.
33. Cau J, Hall A. Cdc42 controls the polarity of the actin and microtubule cytoskeletons through two distinct signal transduction pathways. J Cell Sci. 2005;118:2579-2587.
34. Wang JF, Park IW, Groopman JE. Stromal cell-derived factor-1alpha stimulates tyrosine phosphorylation of multiple focal adhesion proteins and induces migration of hematopoietic progenitor cells: roles of phosphoinositide-3 kinase and protein kinase C. Blood. 2000;95:2505-2513.
35. Giebel B, Corbeil D, Beckmann J, Hohn J, Freund D, Giesen K, Fischer J, Kogler G, Wernet P. Segregation of lipid raft markers including CD133 in polarized human hematopoietic stem and progenitor cells. Blood. 2004;104:2332-2338.
36. Whetton AD, Lu Y, Pierce A, Carney L, Spooncer E. Lysophospholipids synergistically promote primitive hematopoietic cell chemotaxis via a mechanism involving Vav 1. Blood. 2003;102:2798-2802.

37. Petit I, Goichberg P, Spiegel A, Peled A, Brodie C, Seger R, Nagler A, Alon R, Lapidot T. Atypical PKC-zeta regulates SDF-1-mediated migration and development of human CD34+ progenitor cells. J Clin Invest. 2005;115:168-176.
38. Gottig S, Mobest D, Ruster B, Grace B, Winter S, Seifried E, Gille J, Wieland T, Henschler R. Role of the monomeric GTPase Rho in hematopoietic progenitor cell migration and transplantation. Eur J Immunol. 2006;36:180-189.
39. Yang L, Wang L, Geiger H, Cancelas JA, Mo J, Zheng Y. Rho GTPase Cdc42 coordinates hematopoietic stem cell quiescence and niche interaction in the bone marrow. Proc Natl Acad Sci U S A. 2007;104:5091-5096.
40. Gu Y, Filippi MD, Cancelas JA, Siefring JE, Williams EP, Jasti AC, Harris CE, Lee AW, Prabhakar R, Atkinson SJ, Kwiatkowski DJ, Williams DA. Hematopoietic cell regulation by Rac1 and Rac2 guanosine triphosphatases. Science. 2003;302:445-449.
41. Bonig H, Priestley GV, Nilsson LM, Jiang Y, Papayannopoulou T. PTX-sensitive signals in bone marrow homing of fetal and adult hematopoietic progenitor cells. Blood. 2004;104:2299-2306.
42. Lauffenburger DA, Horwitz AF. Cell migration: a physically integrated molecular process. Cell. 1996;84:359-369.
43. Rajendran L, Masilamani M, Solomon S, Tikkanen R, Stuermer CA, Plattner H, Illges H. Asymmetric localization of flotillins/reggies in preassembled platforms confers inherent polarity to hematopoietic cells. Proc Natl Acad Sci U S A. 2003;100:8241-8246.
44. Beckmann J, Scheitza S, Wernet P, Fischer JC, Giebel B. Asymmetric cell division within the human hematopoietic stem and progenitor cell compartment: identification of asymmetrically segregating proteins. Blood. 2007;109:5494-5501.
45. Vila-Coro AJ, Rodriguez-Frade JM, Martin De Ana A, Moreno-Ortiz MC, Martinez AC, Mellado M. The chemokine SDF-1alpha triggers CXCR4 receptor dimerization and activates the JAK/STAT pathway. Faseb J. 1999;13:1699-1710.
46. Suzuki A, Ohno S. The PAR-aPKC system: lessons in polarity. J Cell Sci. 2006;119:979-987.
47. Herbert JM, Augereau JM, Gleye J, Maffrand JP. Chelerythrine is a potent and specific inhibitor of protein kinase C. Biochem Biophys Res Commun. 1990;172:993-999.
48. Chao MD, Chen IS, Cheng JT. Inhibition of protein kinase C translocation from cytosol to membrane by chelerythrine. Planta Med. 1998;64:662-663.
49. Wang L, Yang L, Filippi MD, Williams DA, Zheng Y. Genetic deletion of Cdc42GAP reveals a role of Cdc42 in erythropoiesis and hematopoietic stem/progenitor cell survival, adhesion, and engraftment. Blood. 2006;107:98-105.
50. Just I, Selzer J, Wilm M, von Eichel-Streiber C, Mann M, Aktories K. Glucosylation of Rho proteins by Clostridium difficile toxin B. Nature. 1995;375:500-503.
51. Theriot JA, Mitchison TJ. Actin microfilament dynamics in locomoting cells. Nature. 1991;352:126-131.
52. Coates TD, Watts RG, Hartman R, Howard TH. Relationship of F-actin distribution to development of polar shape in human polymorphonuclear neutrophils. J Cell Biol. 1992;117:765-774.
53. Fox JE, Phillips DR. Inhibition of actin polymerization in blood platelets by cytochalasins. Nature. 1981;292:650-652.
54. Casella JF, Flanagan MD, Lin S. Cytochalasin D inhibits actin polymerization and induces depolymerization of actin filaments formed during platelet shape change. Nature. 1981;293:302-305.
55. Liao G, Nagasaki T, Gundersen GG. Low concentrations of nocodazole interfere with fibroblast locomotion without significantly affecting microtubule level: implications for the role of dynamic microtubules in cell locomotion. J Cell Sci. 1995;108 (Pt 11):3473-3483.

56. Vasiliev JM, Gelfand IM, Domnina LV, Ivanova OY, Komm SG, Olshevskaja LV. Effect of colcemid on the locomotory behaviour of fibroblasts. J Embryol Exp Morphol. 1970;24:625-640.
57. Goldman RD. The role of three cytoplasmic fibers in BHK-21 cell motility. I. Microtubules and the effects of colchicine. J Cell Biol. 1971;51:752-762.
58. Siegrist SE, Doe CQ. Microtubule-induced cortical cell polarity. Genes Dev. 2007;21:483-496.
59. De Brabander MJ, Van de Veire RM, Aerts FE, Borgers M, Janssen PA. The effects of methyl (5-(2-thienylcarbonyl)-1H-benzimidazol-2-yl) carbamate, (R 17934; NSC 238159), a new synthetic antitumoral drug interfering with microtubules, on mammalian cells cultured in vitro. Cancer Res. 1976;36:905-916.
60. Langhorst MF, Reuter A, Luxenhofer G, Boneberg EM, Legler DF, Plattner H, Stuermer CA. Preformed reggie/flotillin caps: stable priming platforms for macrodomain assembly in T cells. Faseb J. 2006;20:711-713.
61. Watts RG, Crispens MA, Howard TH. A quantitative study of the role of F-actin in producing neutrophil shape. Cell Motil Cytoskeleton. 1991;19:159-168.
62. Servant G, Weiner OD, Herzmark P, Balla T, Sedat JW, Bourne HR. Polarization of chemoattractant receptor signaling during neutrophil chemotaxis. Science. 2000;287:1037-1040.
63. Wang F, Herzmark P, Weiner OD, Srinivasan S, Servant G, Bourne HR. Lipid products of PI(3)Ks maintain persistent cell polarity and directed motility in neutrophils. Nat Cell Biol. 2002;4:513-518.
64. Weiner OD, Neilsen PO, Prestwich GD, Kirschner MW, Cantley LC, Bourne HR. A PtdInsP(3)- and Rho GTPase-mediated positive feedback loop regulates neutrophil polarity. Nat Cell Biol. 2002;4:509-513.
65. Charest PG, Firtel RA. Feedback signaling controls leading-edge formation during chemotaxis. Curr Opin Genet Dev. 2006;16:339-347.
66. Xu J, Wang F, Van Keymeulen A, Herzmark P, Straight A, Kelly K, Takuwa Y, Sugimoto N, Mitchison T, Bourne HR. Divergent signals and cytoskeletal assemblies regulate self-organizing polarity in neutrophils. Cell. 2003;114:201-214.
67. Srinivasan S, Wang F, Glavas S, Ott A, Hofmann F, Aktories K, Kalman D, Bourne HR. Rac and Cdc42 play distinct roles in regulating PI(3,4,5)P3 and polarity during neutrophil chemotaxis. J Cell Biol. 2003;160:375-385.
68. Laudanna C, Mochly-Rosen D, Liron T, Constantin G, Butcher EC. Evidence of zeta protein kinase C involvement in polymorphonuclear neutrophil integrin-dependent adhesion and chemotaxis. J Biol Chem. 1998;273:30306-30315.
69. Niggli V. Microtubule-disruption-induced and chemotactic-peptide-induced migration of human neutrophils: implications for differential sets of signalling pathways. J Cell Sci. 2003;116:813-822.
70. Xu J, Wang F, Van Keymeulen A, Rentel M, Bourne HR. Neutrophil microtubules suppress polarity and enhance directional migration. Proc Natl Acad Sci U S A. 2005;102:6884-6889.
71. Dziezanowski MA, DeStefano MJ, Rabinovitch M. Effect of antitubulins on spontaneous and chemotactic migration of neutrophils under agarose. J Cell Sci. 1980;42:379-388.
72. Keller HU, Naef A, Zimmermann A. Effects of colchicine, vinblastine and nocodazole on polarity, motility, chemotaxis and cAMP levels of human polymorphonuclear leukocytes. Exp Cell Res. 1984;153:173-185.
73. Chae HD, Lee KE, Williams DA, Gu Y. Cross-talk between RhoH and Rac1 in regulation of actin cytoskeleton and chemotaxis of hematopoietic progenitor cells. Blood. 2007

74. Pierini LM, Eddy RJ, Fuortes M, Seveau S, Casulo C, Maxfield FR. Membrane lipid organization is critical for human neutrophil polarization. J Biol Chem. 2003;278:10831-10841.
75. Gomez-Mouton C, Lacalle RA, Mira E, Jimenez-Baranda S, Barber DF, Carrera AC, Martinez AC, Manes S. Dynamic redistribution of raft domains as an organizing platform for signaling during cell chemotaxis. J Cell Biol. 2004;164:759-768.
76. Niggli V, Meszaros AV, Oppliger C, Tornay S. Impact of cholesterol depletion on shape changes, actin reorganization, and signal transduction in neutrophil-like HL-60 cells. Exp Cell Res. 2004;296:358-368.
77. Gomez-Mouton C, Abad JL, Mira E, Lacalle RA, Gallardo E, Jimenez-Baranda S, Illa I, Bernad A, Manes S, Martinez AC. Segregation of leading-edge and uropod components into specific lipid rafts during T cell polarization. Proc Natl Acad Sci U S A. 2001;98:9642-9647.
78. Millan J, Montoya MC, Sancho D, Sanchez-Madrid F, Alonso MA. Lipid rafts mediate biosynthetic transport to the T lymphocyte uropod subdomain and are necessary for uropod integrity and function. Blood. 2002;99:978-984.
79. Foger N, Marhaba R, Zoller M. Involvement of CD44 in cytoskeleton rearrangement and raft reorganization in T cells. J Cell Sci. 2001;114:1169-1178.
80. Seveau S, Keller H, Maxfield FR, Piller F, Halbwachs-Mecarelli L. Neutrophil polarity and locomotion are associated with surface redistribution of leukosialin (CD43), an antiadhesive membrane molecule. Blood. 2000;95:2462-2470.
81. Seveau S, Lopez S, Lesavre P, Guichard J, Cramer EM, Halbwachs-Mecarelli L. Leukosialin (CD43, sialophorin) redistribution in uropods of polarized neutrophils is induced by CD43 cross-linking by antibodies, by colchicine or by chemotactic peptides. J Cell Sci. 1997;110 (Pt 13):1465-1475.
82. Sanchez-Mateos P, Campanero MR, del Pozo MA, Sanchez-Madrid F. Regulatory role of CD43 leukosialin on integrin-mediated T-cell adhesion to endothelial and extracellular matrix ligands and its polar redistribution to a cellular uropod. Blood. 1995;86:2228-2239.
83. Serrador JM, Nieto M, Alonso-Lebrero JL, del Pozo MA, Calvo J, Furthmayr H, Schwartz-Albiez R, Lozano F, Gonzalez-Amaro R, Sanchez-Mateos P, Sanchez-Madrid F. CD43 interacts with moesin and ezrin and regulates its redistribution to the uropods of T lymphocytes at the cell-cell contacts. Blood. 1998;91:4632-4644.
84. Campanero MR, Sanchez-Mateos P, del Pozo MA, Sanchez-Madrid F. ICAM-3 regulates lymphocyte morphology and integrin-mediated T cell interaction with endothelial cell and extracellular matrix ligands. J Cell Biol. 1994;127:867-878.
85. Jin L, Hope KJ, Zhai Q, Smadja-Joffe F, Dick JE. Targeting of CD44 eradicates human acute myeloid leukemic stem cells. Nat Med. 2006;12:1167-1174.
86. Krause DS, Lazarides K, von Andrian UH, Van Etten RA. Requirement for CD44 in homing and engraftment of BCR-ABL-expressing leukemic stem cells. Nat Med. 2006;12:1175-1180.

Figure 1: **Distribution of Flot-1, Flot-2 and ICAM-3 in morphologically non-polarized and polarized CD34$^+$ cells.** Cultivated CD34$^+$ cells can be distinguished into three categories, i) in morphological round cells that show no polarized distribution of either the flotillins or ICAM-3 (A, D), ii) in round cells containing polarized flotillin and ICAM-3 distributions (B, E) and iii) in morphological polarized cells in which the flotillins and ICAM-3 are highly concentrated in the tip of the uropod (C, F). The first column shows anti-Flot-1 (A-C) or anti-Flot-2 staining (D-F); column 2 anti-ICAM-3 staining; column 3 merges of staining of columns 1 and 2; column 4 light microscopy images of stained cells.

Figure 2: **Localization of the Prominin-1/CD133 in relation to Flot-1 and Flot-2.** In morphological polarized CD34$^+$ cells Prominin-1/CD133 (Prom-1) and Flot-1 (A) as well as Flot-2 (B) are highly concentrated in the tip of the uropod and are concentrated in non overlapping microdomains. Column 1: anti-Prominin-1 staining; column 2: anti-Flot-1 or anti-Flot-2 staining; column 3: merge of staining of columns 1 and 2; column 4 light microscopy images of stained cells.

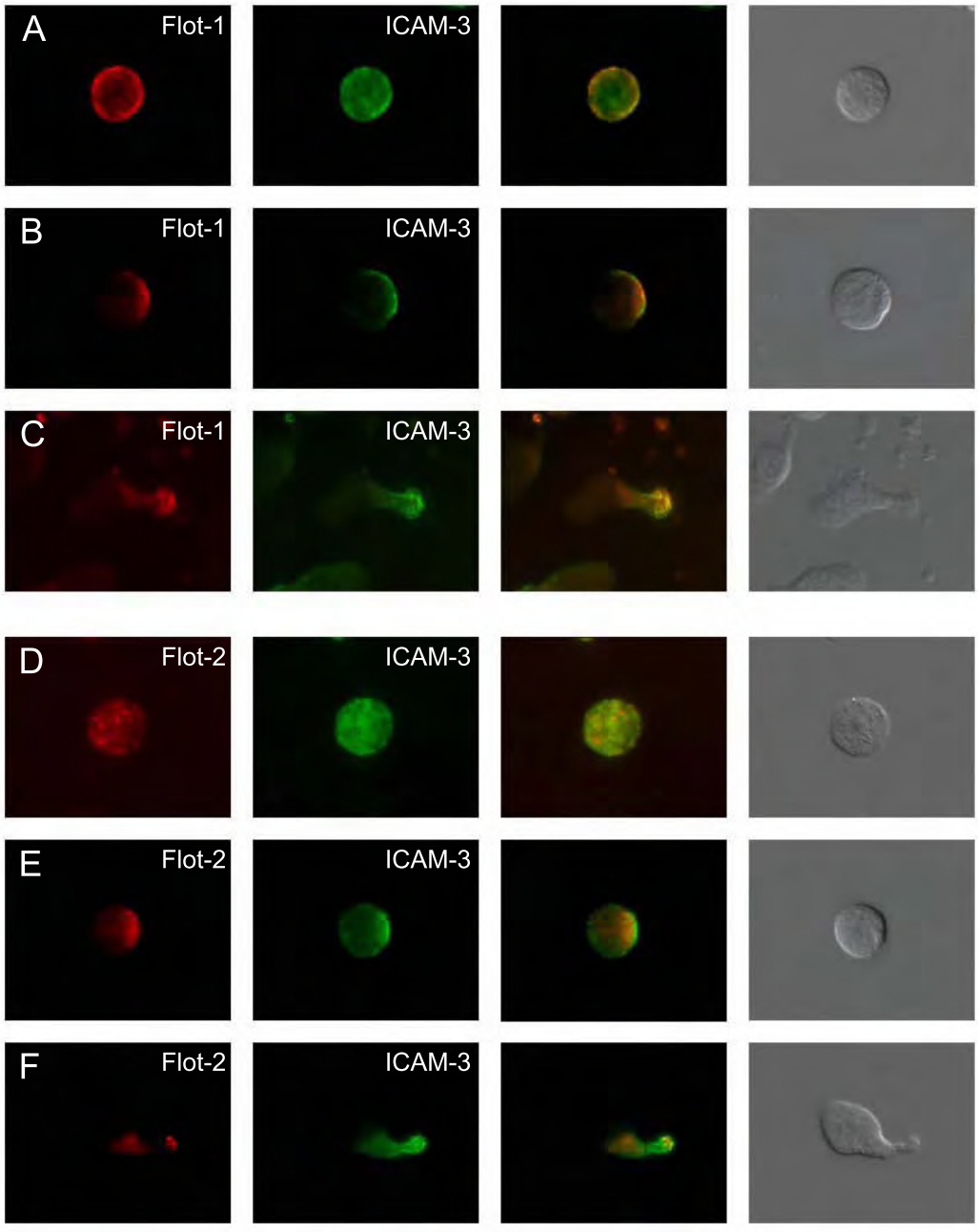
Figure 3: **Morphological and intrinsic cell polarisation rates of drug treated CD34$^+$ cells in comparison to non-treated control cells.** Freshly isolated CD34$^+$ cells were grown in the presence or absence of corresponding drugs for 1 or 2 days (A-G) or drugs were added for 2h to CD34$^+$ cells that were cultured for 2 days in the presence of early acting cytokines before (A'-G'). After incubation cultured cells were stained with anti-Flot-1 or anti-Flot-2 antibodies and counterstained with anti-ICAM-3 antibodies. Stained CD34$^+$ cells were classified according to the categories shown in Fig. 2. The percentage of drug treated CD34$^+$ cells displaying polarized morphologies and/or polarized ICAM-3, Flot-1 or Flot-2 distributions are depicted as bar charts in column 1 or 3, respectively. Representative morphologies of treated cells are shown in the light microscopy pictures of column 2 and 4. White columns reflect percentages of control, grey columns of treated cells. A, A': Cyclohexemide; B, B': Pertussis toxin; C, C': Ly294002; D, D': Chelerythrine chloride; E, E': *Clostridium difficile* Toxin B; F, F': Cytochalasin B; G, G': Nocodazole

Figure 4: **Transmigration rates of drug treated CD34$^+$ cells.** Freshly isolated CD34$^+$ cells were cultured for 2 days in the presence of early acting cytokines before individual drugs were added. After incubating cells for another 2h, they were transferred in the presence of corresponding drugs to the Transwell assays. The percentages of transmigrated cells were estimated after 12h in comparison to untreated control CD34$^+$ cells (right *versus* left columns). A: Cyclohexemide; B: Pertussis toxin; C: Ly294002; D: Chelerythrine chloride; E: *Clostridium difficile* Toxin B; F: Cytochalasin B; G: Nocodazole

Supplement Tab. 1: Statistical analyses of freshly isolated $CD34^+$ cells that were cultured for 1 or 2 days in the presence of drugs (column 1). The percentage of drug treated cells displaying a polarized morphology is depicted in column 2 (Morph), in comparison to corresponding control values which are given in brackets. Similarly, the percentage of cells displaying polarized ICAM-3, Flot-1 or Flot-2 distribution is given in columns 5 (ICAM-3), 8 (Flot-1) or 11 (Flot-2). Corresponding standard deviations (s.d.) and *p*-values that were estimated by the paired student's T-test are given in the following columns as indicated. The last column provides the number of samples that were analyzed for each condition. *p*-Values marked in green are below 0.05, reflecting statistically significant results, those in yellow are in the range between 0.05 and 0.1.

Supplement Tab. 2: Statistical analyses of freshly isolated $CD34^+$ cells that were cultured for 2 days in the presence of early acting cytokines before corresponding drugs were added for 2 hours. For details see legend of Supplement Tab. 1.

Supplement Tab. 3: Statistical analyses of freshly isolated $CD34^+$ cells that were cultured for 2 days in the presence of early acting cytokines before corresponding drugs were added for 2 hours and cells were transferred in the presence of corresponding drugs (column 1) to Transwell assays. Column 2 reflects the rate of transmigrated $CD34^+$ cells in comparison to their controls after 12h. Standard deviations, *p*-values and the number of independent experiments are given in the following columns as indicated.


A
Flot-1
ICAM-3
B
Flot-1
ICAM-3
C
Flot-1
ICAM-3
D
Flot-2
ICAM-3
E
Flot-2
ICAM-3
F
Flot-2
ICAM-3

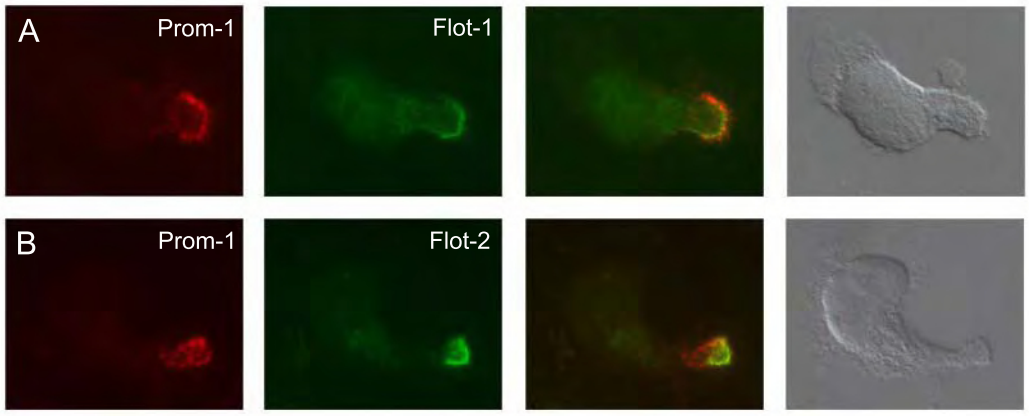

A
Prom-1
Flot-1
B
Prom-1
Flot-2

Figure 3

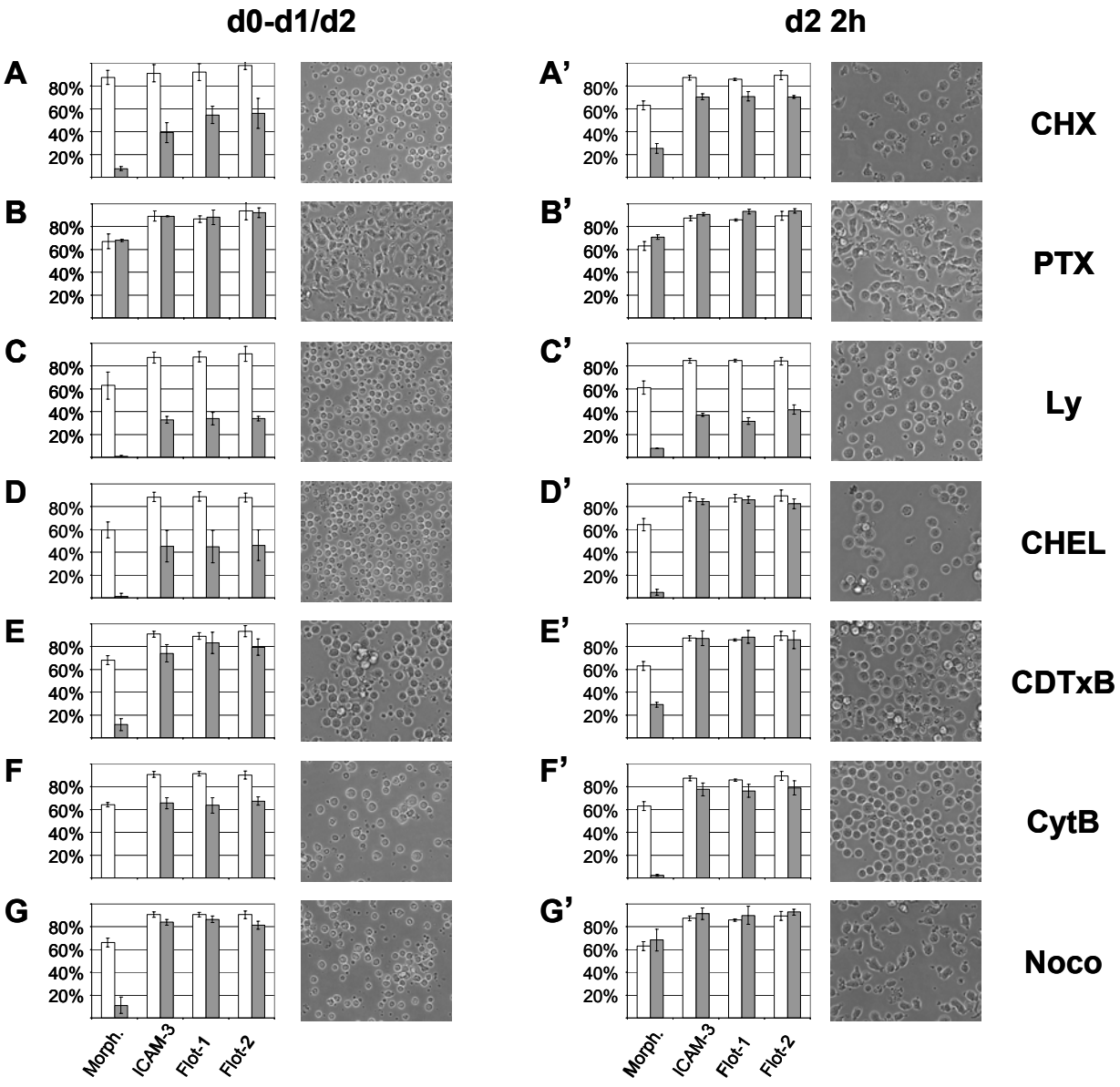
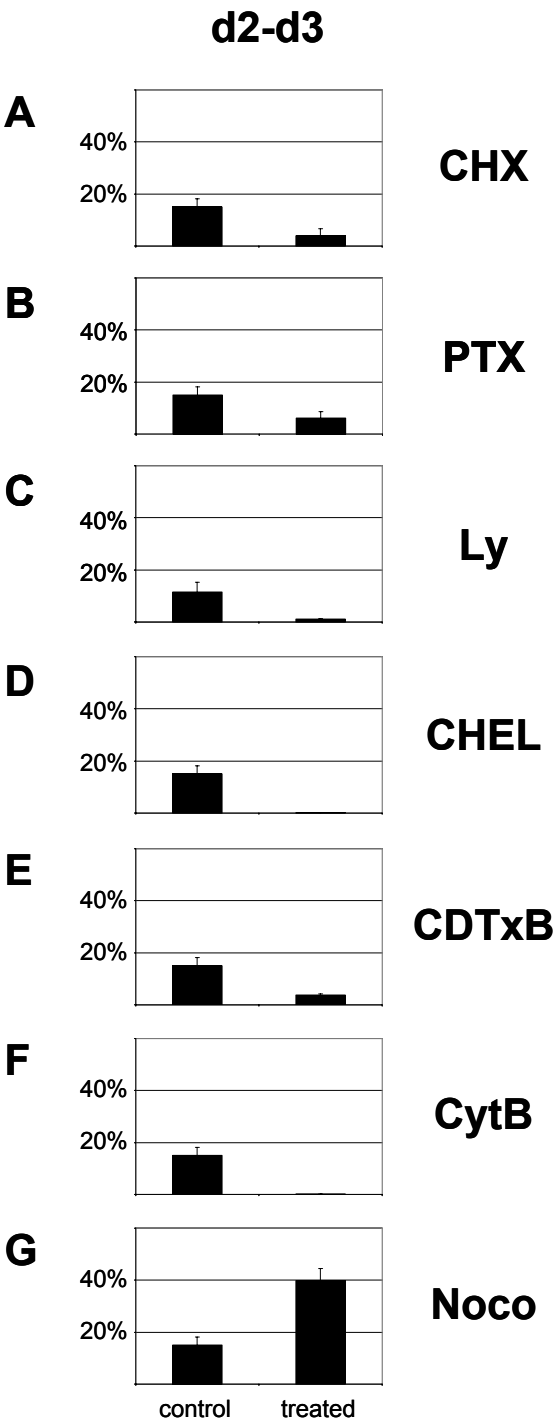

d0-d1/d2
d2 2h
A
A'
B
B'
C
C'
D
D'
E
E'
F
F'
G
G'
80%
60%
40%
20%
Morph.
ICAM-3
Flot-1
Flot-2
CHX
PTX
Ly
CHEL
CDTxB
CytB
Noco

Figure 4


d2-d3
A
40%
20%
CHX
B
40%
20%
PTX
C
40%
20%
Ly
D
40%
20%
CHEL
E
40%
20%
CDTxB
F
40%
20%
CytB
G
40%
20%
Noco
control
treated

d0-d1/d2	Morph	s.d.	pMorph	ICAM-3	s.d.	pICAM-3	Flot-1	s.d.	pFlot-1	Flot-2	s.d.	pFlot-2	n
CHX d1	7.5 (88.5)	1.7 (6.4)	0.00275	39.2 (91.2)	8.7 (7.5)	0.00018	54.6 (92.3)	7.5 (7.5)	0.04667	56.2 (98.1)	13.2 (3.4)	0.02095	3
PTX d2	68.0 (67.1)	1.0 (6.5)	0.61092	89.1 (89.2)	0.4 (4.5)	0.96885	88.2 (86.6)	6.3 (2.9)	0.74134	92.1 (93.7)	4.3 (7.7)	0.72942	3
Ly294002 d1	0.5 (63.0)	0.9 (10.5)	0.00003	27.4 (87.5)	13.7 (4.2)	0.00017	36.3 (88.3)	8.1 (4.1)	0.00004	33.7 (90.6)	2.0 (6.5)	0.00002	6
CHEL d1	1.2 (59.5)	2.6 (7.0)	0.00015	45.2 (88.5)	13.7 (4.0)	0.00230	44.9 (88.9)	14.1 (4.2)	0.00219	46.1 (88.0)	13.4 (3.9)	0.00264	5
CDTxB d2	11.5 (68.1)	5.3 (3.8)	0.00003	74.0 (90.9)	7.6 (2.5)	0.00483	83.3 (89.3)	9.5 (2.8)	0.28240	79.5 (93.5)	7.2 (4.9)	0.00683	5
CytB d1	0.0 (64.2)	0.0 (2.1)	0.00034	65.6 (90.9)	5.0 (2.7)	0.01272	83.7 (91.5)	6.8 (2.0)	0.02100	67.5 (90.3)	3.8 (3.3)	0.00532	3
Noco d2	11.0 (66.3)	7.2 (3.9)	0.00568	83.9 (90.5)	2.6 (2.3)	0.07194	86.5 (90.5)	2.9 (2.0)	0.16962	81.3 (90.6)	3.4 (3.4)	0.05582	3

Suppl. Tab. 1

2h	Morph	s.d.	pMorph	ICAM-3	s.d.	pICAM-3	Flot-1	s.d.	pFlot-1	Flot-2	s.d.	pFlot-2	n
CHX	25.2 (63.1)	4.3 (3.8)	0.00025	70.6 (87.4)	2.5 (2.1)	0.00060	71 (85.8)	4.2 (1.1)	0.01994	70.4 (89.5)	1.1 (3.8)	0.00730	3
PTX	70.8 (63.1)	2.0 (3.8)	0.05792	90.8 (87.4)	1.4 (2.1)	0.19924	93.2 (85.8)	2.1 (1.1)	0.05541	93.8 (89.5)	2 (3.8)	0.05782	3
Ly294002	7.7 (61.2)	0.1 (5.9)	0.00413	37.1 (84.7)	1.3 (2.2)	0.00159	31.6 (85.0)	2.9 (1.2)	0.00070	41.8 (84.4)	4.3 (3.3)	0.00973	3
CHEL	4.9 (64.1)	2.5 (5.4)	0.00000	84.2 (88.5)	2.5 (3.8)	0.05338	86.0 (87.5)	3.1 (3.3)	0.42712	82.5 (89.6)	4.3 (4.7)	0.03915	6
CDTxB	29.0 (63.1)	2.0 (3.8)	0.00553	87.3 (87.4)	6.4 (2.1)	0.95400	88.4 (85.8)	5.6 (1.1)	0.43520	86.1 (89.5)	7.8 (3.8)	0.34426	3
CytB	2.4 (63.1)	0.7 (3.8)	0.00088	77.7 (87.4)	5.7 (2.1)	0.05184	76.4 (85.8)	5.7 (1.1)	0.07518	79.0 (89.5)	6.3 (3.8)	0.06297	3
Noco	68.3 (63.1)	9.5 (3.8)	0.33551	91.4 (87.4)	5.1 (2.1)	0.14212	90.0 (85.8)	7.9 (1.1)	0.40904	92.8 (89.5)	2.4 (3.8)	0.08694	3

Suppl. Tab. 2

12h	Migrated	s.d.	pMigrated	n
CHX	4.0 (15.1)	2.7 (3.1)	0.04100	4
PTX	6.3 (15.1)	2.3 (3.1)	0.02640	4
Ly294002	1.0 (11.5)	0.4 (3.8)	0.04990	3
CHEL	0.1 (15.1)	0.1 (3.1)	0.04610	4
CDTxB	3.8 (15.1)	0.5 (3.1)	0.02960	4
CytB	0.1 (15.1)	0.1 (3.1)	0.04560	4
Noco	39.8 (15.1)	4.7 (3.1)	0.03360	4

Suppl. Tab. 3

Eigenständigkeitserklärung

Die hier vorgelegte Dissertation habe ich eigenständig und ohne unerlaubte Hilfe angefertigt. Die Dissertation wurde in der vorgelegten oder in ähnlicher Form noch bei keiner anderen Institution eingereicht. Ich habe bisher keine erfolglosen Promotionsversuche unternommen.

Düsseldorf, 02.05.08

_____

(Julia Beckmann)