Stem Cell Research (2010) 4, 129–139

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REGULAR ARTICLE

N-Cadherin is expressed on human hematopoietic progenitor cells and mediates interaction with human mesenchymal stromal cells

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Received 29 May 2009; received in revised form 11 December 2009; accepted 22 December 2009

Abstract Specific cell–cell junctions between hematopoietic stem cells (HSC) and their niche have been shown to regulate stem cell function. N-cadherin was suggested to play a central role in this process, whereas other studies indicated that it did not play an essential role in the murine model. We have analyzed the role of N-cadherin for interaction between hematopoietic progenitor cells (HPC) and supportive mesenchymal stromal cells (MSC) in a human–human setting. Expression of N-cadherin and of cadherin-11 (osteoblast cadherin) was analyzed in HPC by quantitative RT-PCR, Western blot, and flow cytometry. N-cadherin and cadherin-11 were expressed in HPC at a moderate level, whereas they were not detectable in differentiated cells. Confocal laser scanning microscopy revealed that N-cadherin and β-catenin are colocalized at the junction of HPC and MSC. siRNA knockdown of N-cadherin or cadherin-11 as well as treatment with the blocking function antibody decreased adhesive interaction of HPC to MSC. Furthermore, knockdown of N-cadherin or blocking function antibody impaired maintenance of long-term culture-initiating cells (LTC-IC) on coculture of HPC and MSC. These results indicate that N-cadherin is involved in the bidirectional interaction of human HPC with their cellular determinants in the niche.

Introduction

Maintenance, self-renewal, and differentiation of hematopoietic stem cells (HSC) are regulated by their microenvironment, the so-called "stem cell niche." Evidence from the literature indicated that HSC might reside in two different niches in the bone marrow: (i) an "osteoblastic niche" where HSC are associated with osteoblasts at the inner surface of the bone cavity (Calvi et al., 2003; Zhang et al., 2003; Arai and Suda, 2007; Kollet et al., 2006; Xie et al., 2008) and (ii) a "vascular niche" that is formed by endothelial cells which line the sinusoids (Kiel et al., 2005). The osteoblastic niche is thought to maintain HSC in a quiescent state over a long time, whereas the vascular niche supports expansion of progenitor cells and differentiation (Zhang and Li, 2008).

Much of the knowledge on the stem cell niche has been gained from studies in model organisms such as *Drosophila*

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doi:10.1016/j.scr.2009.12.004

and *Caenorhabditis elegans* (Knoblich, 2008). Self-renewal of stem cell function and differentiation toward specific lineages are determined by a process called "asymmetric cell division." Niche signaling, cellular polarity, and asymmetric segregation of centrosomes and spindles, as well as cell cycle regulators, are involved in the self-renewing asymmetry during stem cell division (Knoblich, 2008; Ho and Wagner, 2007; Yamashita et al., 2007). Recent evidence indicates that these concepts for asymmetric cell division also hold true for regulation of stem cell function in the mammalian hematopoietic system (Ho and Wagner, 2007). Specific cell–cell junctions might retain one daughter cell in the stem cell niche for self-renewal of the HSC pool, whereas the other one becomes more differentiated toward a specific hematopoietic lineage. Cell–cell junctions are therefore of central interest for maintenance and regulation of stem cell function as well as for processes such as homing and mobilization from their niche.

N-cadherin (neuronal; also known as cadherin 2, type 1 [CDH2]) and cadherin-11 (also named osteoblast cadherin; OB-cadherin) resemble both adhesion proteins of the cadherin superfamily. They mediate cell–cell adhesion in a calcium-dependent manner by homophilic binding of cadherins between two neighboring cells and they are connected via α-catenin and β-catenin to the actin cytoskeleton. Furthermore, interaction between cadherins and β-catenin is indirectly involved in signal cascades such as the Wnt-signaling pathway (Fleming et al., 2008). The tissue-specific expression of N-cadherin and the connection to the β-catenin/Wnt-pathway have early indicated that N-cadherin plays a central role in interaction of HSC and their niche (Arai and Suda, 2007). Specialized spindle-shaped N-cadherin-expressing osteoblasts located in the endosteum were suggested to be essential components of the niche in the bone marrow (Zhang et al., 2003; Xie et al., 2008; Wilson et al., 2004). Cadherin-11 is also expressed in osteoblastic cell lines, and it is up-regulated during differentiation (Okazaki et al., 1994). It has been shown that c-Myc and Rbm15 overexpression can down-regulate expression of N-cadherin and hence, it has been speculated that down-regulation of N-cadherin is essential for the release from the stem cell pool (Wilson et al., 2004; Niu et al., 2009). The role of N-cadherin in controlling the interactions between stem cells and their niche has been controversial. Some authors did not detect any N-cadherin expression by polymerase chain reaction in primitive murine HSC ($CD150^+$ $CD48^-$ $CD41^-$ $c\text{-}kit^+$ $Sca\text{-}1^+$ $lineage^-$; SLAM code) nor by Western blot in HSC or whole bone marrow cells (Kiel et al., 2007, 2009) and conditional deletion of N-cadherin did not impair hematopoiesis in a mouse model (Kiel et al., 2009), while other authors have reported that N-cadherin low ($N\text{-}cadherin^{low}$) expressing cells include the HSC fraction and that populations expressing N-cadherin at intermediate levels ($N\text{-}cadherin^{int}$) are held in reserve (Haug et al., 2008).

Observations in the murine model need to be verified for the human system. Puch et al. have previously indicated that N-cadherin is expressed on human $CD34^+$ cells (Puch et al., 2001). However, it remains to be demonstrated if this adhesion protein is expressed at higher levels in HPC than in differentiated cells and if it has functional implications for the cell–cell interaction. The stromal feeder layer (e.g., mesenchymal stromal cells (MSC) from bone marrow) represents an *in vitro* model for analyzing this interaction. Thereby, molecular mechanisms can be addressed in a human–human setting (Dexter et al., 1977; Wagner et al., 2007a). In this study, we analyzed N-cadherin expression in human HPC and its role for HPC–MSC interaction.

Results

N-cadherin and cadherin-11 are expressed on human MSC and HPC

N-cadherin expression was analyzed in $CD34^+$ cells (HPC fraction) and $CD34^-$ cells (majority of MNC) from human cord blood (CB) or mobilized peripheral blood (PB). Quantitative RT-PCR analysis revealed that N-cadherin was significantly higher expressed in $CD34^+$ cells than in $CD34^-$ cells derived from CB or PB. Cadherin-11 was also significantly higher expressed in $CD34^+$ cells from CB, whereas no difference was observed in PB. In the myeloid cell line KG1a expression of N-cadherin was detected at high levels, whereas cadherin-11 was not expressed. MSC expressed both adhesion proteins at high levels. N-cadherin expression was 3.4-fold and 8.3-fold higher in MSC than in HPC from CB and PB, respectively. Cadherin-11 concentration was found at about 230-fold more elevated in MSC than in $CD34^+$ CB cells (Fig. 1A). Additional analysis of HPC subfractions by RT-PCR revealed that N-cadherin is much higher expressed in $CD34^+CD38^-$ cells than in $CD34^+CD38^+$ cells. Similarly aldehydedehydrogenase $(ALDH)^+$ cells contained much more N-cadherin than $ALDH^-$ cells (data not shown).

We have reanalyzed our previously published microarray data with a focus on N-cadherin and cadherin-11 expression. We have compared gene expression profiles of $CD34^+$ cells from human cord blood that adhered to MSC *versus* those that were nonadherent (Wagner et al., 2007a). N-cadherin and cadherin-11 were higher expressed in the fraction of $CD34^+$ cells that adhered to MSC (Fig. 1B). Expression of N-cadherin in HPC was also analyzed on the protein level by immunoblot. A specific band at 130 kDa for N-cadherin demonstrated that it is expressed at low levels in $CD34^+$ cells from CB and PB. A clear N-cadherin signal was also detected in all three available samples of $CD34^+$ cells from bone marrow (BM). In contrast, N-cadherin was barely detectable in MNC from CB, PB, or BM. KG1a cells and MSC were highly positive for N-cadherin and on a protein level N-cadherin expression was much higher in MSC than in HPC. Cadherin-11 was also detected in $CD34^+$ cells from CB and PB, although the expression level varied between different samples and the specific band indicated that cadherin-11 has a higher molecular weight in HPC. This variation and differences in the loading control made a reliable quantification of protein expression impossible. However, cadherin-11 was clearly expressed at lower levels in HPC than in MSC and it was not detectable in KG1a cells (Fig. 1C). Overall these data demonstrate that human HPC express N-cadherin and cadherin-11 on mRNA and the protein level.

Flow cytometric analysis of adhesion proteins

Flow cytometry might discern $N\text{-}cadherin^+$ or $cadherin\text{-}11^+$ subpopulations of HPC for subsequent analysis. Therefore,

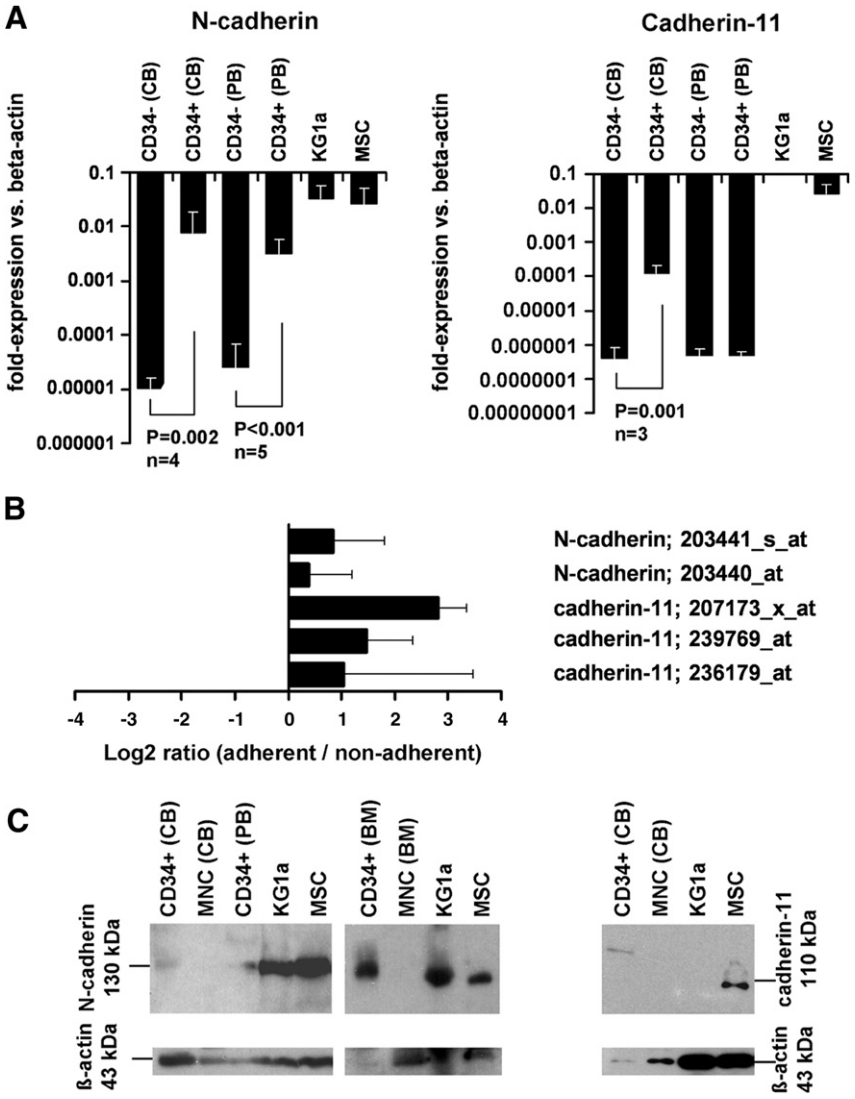

Figure 1 Human HPC express N-cadherin and cadherin-11. (A) Expression of N-cadherin and cadherin-11 was analyzed by quantitative RT-PCR. $CD34^+$ cells from cord blood (CB) or mobilized peripheral blood (PB) expressed significantly more N-cadherin than $CD34^-$ cells. Cadherin-11 was also significantly higher expressed in $CD34^+$ cells from CB, whereas no difference was observed in PB. (B) Furthermore, our previously published microarray data were reanalyzed with a focus on N-cadherin and cadherin-11: $CD34^+$ CB cells have been divided into a fraction that adhered to MSC and into a nonadherent fraction (Wagner et al., 2007a). N-cadherin and cadherin-11 were always higher expressed in the adherent fraction than in the nonadherent fraction. Log_2 rations of differential gene expression (±SD) and corresponding affymetrix IDs are provided. (C) Immunoblot analysis revealed that N-cadherin and cadherin-11 are expressed in $CD34^+$ cells from CB and PB. $CD34^+$ cells from bone marrow (BM) were also positive for N-cadherin. N-cadherin, but not cadherin-11, were detected in KG1a cells and both adhesion proteins were present in MSC (representative blots of three).

we have used the GC-4 antibody that has previously been used for flow cytometric analysis (Hayashi et al., 2007) or the MNCD2 antibody (Haug et al., 2008). Only a small fraction of MNC and $CD34^+$ from CB or PB was positive for N-cadherin, whereas it was detected on most KG1a cells and MSC. The percentage of N-$cadherin^+$ HPC varied between different samples (5–50%), but the signal intensity was low and this might be due to the available antibodies. Similar results were observed by intracellular staining of N-cadherin using the monoclonal antibody 32 or the directly labled clone 8C11 that also did not discern N-cadherin-positive HPC (results not shown). Expression of cadherin-11 was analyzed using the antibody 5B2H5 that is directed against the intracellular domain of the protein. Cadherin-11 was detected at low levels in the above-noted cell types. Overall, flow cytometry demonstrated that N-cadherin and cadherin-11 are expressed at low levels on HPC, but did not reliably discern N-$cadherin^+$ or cadherin-11^+ subpopulations. In fact, recent studies demonstrated that the MNCD2 anti-N-cadherin antibody does not recognize N-cadherin by flow cytometry (Kiel et al., 2009; Foudi et al., 2009) and also the GC-4 antibody has not been shown to clearly discern N-cadherin-positive subpopulations before (Hayashi et al., 2007). In contrast, expression of CD29 and CD44 clearly demarcated subpopulations of MNC and these adhesion proteins are highly expressed in

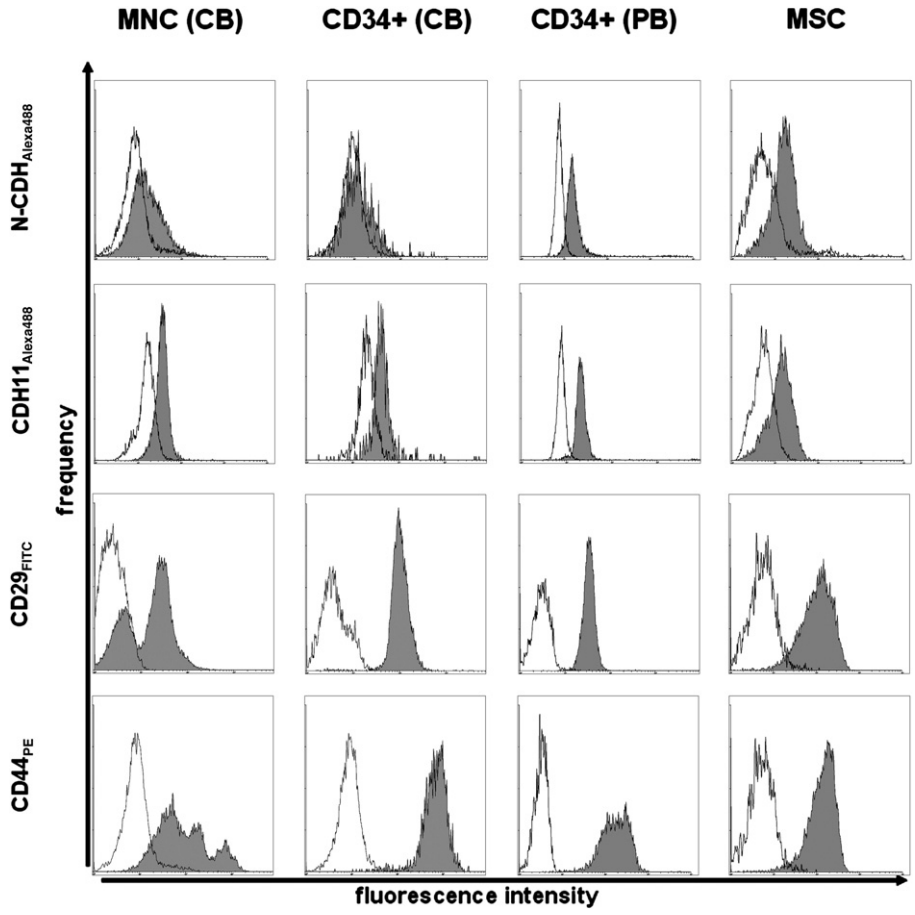

Figure 2 Flow cytometric analysis of adhesion proteins. Expression of adhesion proteins was analyzed by flow cytometry in MNC, $CD34^+$ cells from CB or PB and MSC using different antibodies. Here, we have used the GC-4 antibody against N-cadherin or the 5B2H5 antibody directed against cadherin-11. For controls we have used the secondary anti-mouse-Alexa 488 antibody (black line). This technique did not reliably discern N-$cadherin^+$ or $cadherin\text{-}11^+$ subpopulations and this might be attributed to the available antibodies. Furthermore, we have analyzed expression of integrin beta-1 (ITGB1, CD29) and CD44 (black lines demonstrate autofluorescence controls). Representative histograms are presented.

HPC (Fig. 2). It is also conceivable, that there is a dynamic turnover of cadherins in suspension culture and hence it might be difficult to apply flow cytometry assays to cadherin family proteins.

N-cadherin is localized at the junction of HPC and MSC

To investigate the role of N-cadherin for cell–cell interaction, we have cocultured $CD34^+$ cells from CB with MSC and characterized the junctions by confocal microscopy. The homotypic junctions among MSC were highly positive for N-cadherin as described before (Wuchter et al., 2007). Heterotypic junctions between HPC and MSC were analyzed after repeated washing steps which are due to the staining protocol and inevitably remove some of the HPC from the cell feeder. In 7 independent experiments N-cadherin was detected at $36.2 \pm 9.1\%$ at the point of contact of heterotypic junctions. Beta-catenin was colocalized at the point of contact between the two cell types. Furthermore, N-cadherin was often enriched at the uropod at the trailing edge of the cell. The uropod has been shown to be involved in the intermittent cell–cell adhesion of HPC on MSC (Wagner et al., 2007a). These data support the notion that cell–cell junctions exist and that they involve N-cadherin (Fig. 3).

N-cadherin is involved in adhesion of HPC on MSC

The role of N-cadherin for adhesion of HPC on MSC was further analyzed on specific knockdown of cadherins with siRNA using our recently described adhesion assay (Wagner et al., 2007a). Usually cadherins within one class will bind only to themselves and therefore homophilic adhesion might be impaired on knockdown in either HPC or MSC. In this study, cadherins were knocked down in MSC prior to coculture, since this was more efficient than transfection of HPC and the effect could be verified by immunoblot analysis. At 14 h after siRNA treatment these adhesion proteins were hardly detectable and this observation suggests a high protein turnover. The siRNA effect lasted about 1 week and thereafter the expression levels slowly normalized again. Knockdown of either N-cadherin or cadherin-11 did not affect protein expression of the other cadherin (Fig. 4). Adhesion of $CD34^+$ cells from cord blood was not affected by

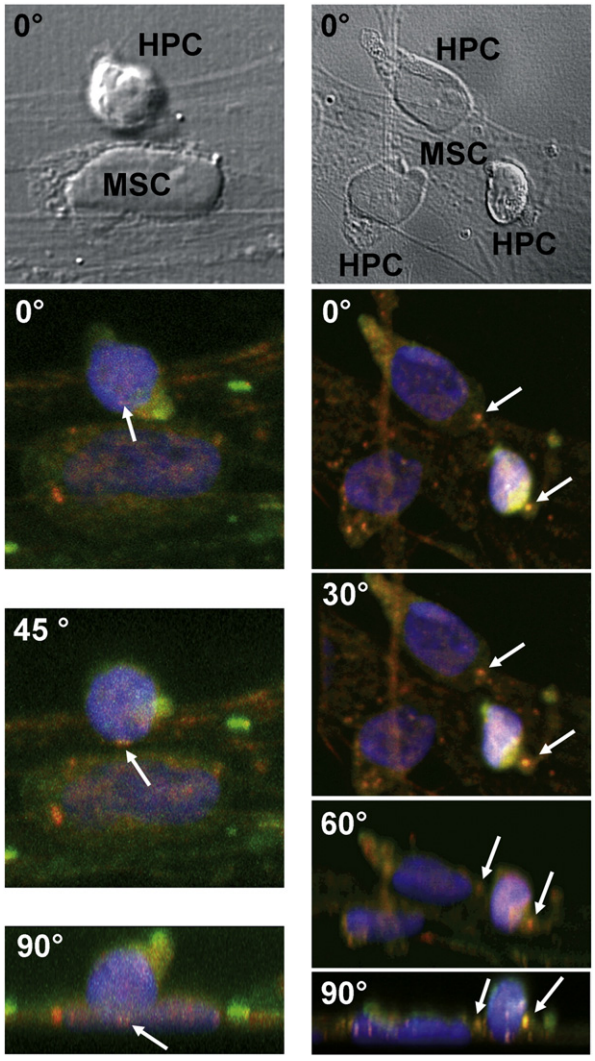

Figure 3 N-cadherin is localized at the junction of HPC and MSC. $CD34^+$ cells from human CB were cocultured with human MSC for 48 h. Subsequently, expression and localization of N-cadherin (green), β-catenin (red), and DNA (DAPI; blue) was analyzed by confocal laser scanning microscopy. N-cadherin was predominantly localized at the uropod of HPC that usually mediates cell–cell adhesion. In the panels on the left side the uropod demonstrates high N-cadherin expression, although it is not attached to the MSC feeder layer. Colocalization of N-cadherin and β-catenin (yellow) was often observed at the point of contact between HPC and MSC (white arrows).

siRNA knockdown of MAPK1 that was used as a reference (untreated MSC, 63 ± 12% adherent cells; MAPK1-siRNA treatment, 60 ± 9%). However, knockdown of N-cadherin resulted in a significant reduction of adhesion of HPC (46 ± 9%, $n = 7$, $P = 0.004$) and there was a nonsignificant tendency for reduced cell–cell adhesion on knockdown of cadherin-11 (53 ± 11%, $n = 8$, $P = 0.06$; Fig. 5A). In addition, we have analyzed the role of N-cadherin for cell–cell adhesion using the functional blocking antibody GC-4 (Puch et al., 2001; Li et al., 2001). Adhesion of $CD34^+$ CB cells to MSC was significantly reduced (control antibody, 65 ± 5%; anti-N-cadherin (GC-4), 58 ± 5%, $n = 3$, $P = 0.01$) and there was a similar although nonsignificant tendency for $CD34^+$ cells from PB (control antibody, 71 ± 14%; anti-N-cadherin (GC-4), 59 ± 18%, $n = 4$, $P = 0.06$; Fig. 5B). These results demonstrate that N-cadherin is involved in cell–cell interaction of HPC and MSC, although this adhesion protein is not the exclusive mechanism for cell–cell adhesion.

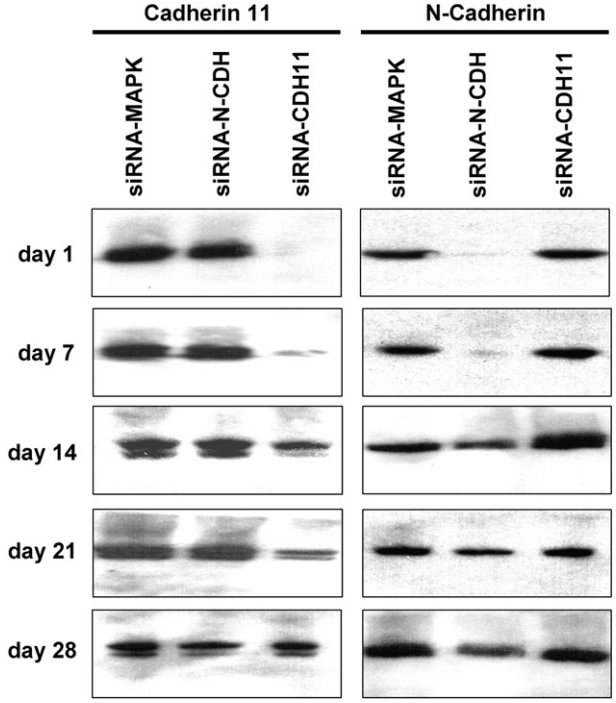

Figure 4 N-cadherin and cadherin-11 are efficiently knocked down by siRNA. MSC were transfected with siRNA for N-cadherin, cadherin-11, or MAPK1. The specific knockdown is demonstrated by immunoblot analysis. Expression of cadherins was specifically inhibited already 14 h after the transfection and the effect lasted about 1 week in irradiated feeder layers. Thereafter the expression normalized again.

N-cadherin supports maintenance of long-term culture-initiating cells

The role of N-cadherin for the hematopoiesis-supportive activity of MSC was further analyzed using the long-term culture-initiating cell (LTC-IC) assay. The LTC-IC frequency of the $CD34^+$ cells cocultured on untreated MSC was 7.3 ± 0.5%, whereas there was a nonsignificant tendency for lower frequency on treatment of MSC with N-cadherin-siRNA (4.1 ± 1.9%, $n = 3$, $P = 0.13$). Furthermore, LTC-IC frequency on treatment with an isotype antibody (8.7 ± 3.0%) was significantly higher than on treatment with the function blocking anti-N-cadherin antibody (GC-4; 5.2 ± 2.0%, $n = 4$, $P = 0.03$). These results indicated that N-cadherin is involved in maintenance of LTC-IC in coculture with MSC (Fig. 6).

Discussion

The molecular mechanisms that regulate stem cell self-renewal are of central interest for stem cell biology. Chemokines and specific cell–cell junctions have been

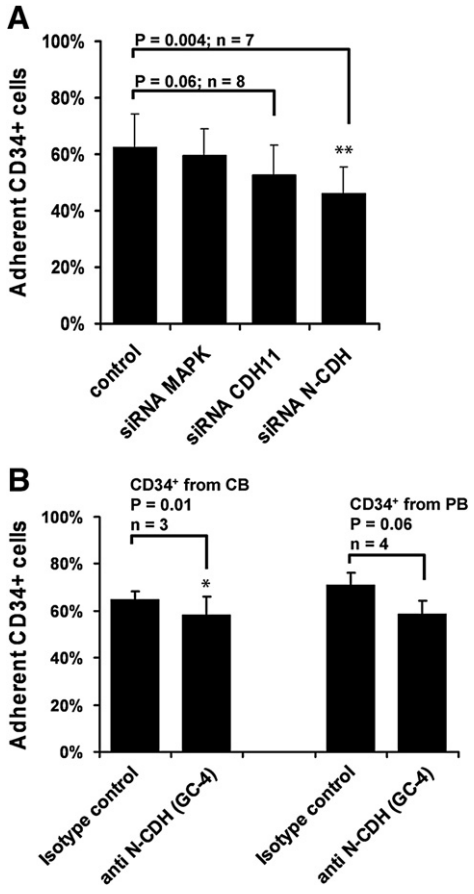

Fig. 5 N-cadherin and cadherin-11 are involved in cell–cell interaction of HPC and MSC. (A) The role of N-cadherin and cadherin-11 for adhesive interaction of $CD34^+$ cells and MSC was analyzed using our adhesion assay based on gravity force (Wagner et al., 2007a). Knockdown of N-cadherin and cadherin-11 in MSC resulted in a decreased adhesion of $CD34^+$ HPC from CB. (B) Furthermore, treatment with blocking function antibody for N-cadherin (GC-4) resulted in a decreased adhesion of $CD34^+$ cells from CB or PB (* $P<0.01$; ** $P<0.001$; two-sided t test).

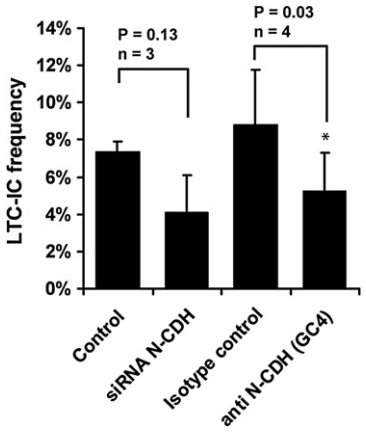

Fig. 6 N-cadherin sustains maintenance of LTC-IC. Maintenance of long-term culture-initiating cells (LTC-IC) was analyzed after coculture of HPC and MSC for 5 weeks. Knockdown of N-cadherin expression in MSC resulted in a reduced maintenance of LTC-IC compared to untreated MSC, although this tendency was not significant. Furthermore, treatment with N-cadherin blocking function antibody (GC-4) reduced LTC-IC frequency compared to treatment with an unspecific control antibody (* $P<0.01$; two-sided t test).

associated with homing and adhesion to the niche, subsequently leading to regulation of self-renewing divisions of stem cells (Ho and Wagner, 2007; Gottig et al., 2006). However, research on the hematopoietic stem cell niche is hampered by the hidden localization in the bone marrow and by the complex composition of different cell types and extracellular matrix compounds in this environment. Recently, Xie and co-workers described an *ex vivo* real-time imaging technology demonstrating that several HSC attached directly to $N\text{-cadherin}^+$ cells in the murine bone marrow (Xie et al., 2008). Here, we have used MSC as a surrogate model for a cellular milieu to address the role of N-cadherin in a human–human system.

HPC demonstrate directed migration toward feeder layer cells and adhere to them with their uropod at the trailing edge (Wagner et al., 2005a; Freund et al., 2006). We have previously shown that a significantly higher proportion of primitive HPC adhered to MSC than the more differentiated counterparts ($CD34^+$ vs $CD34^-$; $CD34^+CD38^-$ vs $CD34^+CD38^+$; slow dividing fraction vs fast dividing fraction of $CD34^+CD38^-$ cells) and LTC-IC frequency is higher in the adherent fraction than in the nonadherent fraction of $CD34^+$ cells (Wagner et al., 2007a). We have focused on standardization and characterization of MSC preparations. Osteoblasts, endothelial cells, or a mixture of these different cell types might provide an even more suitable environment and this will be addressed in the future. The origin of MSC (bone marrow, cord blood, or adipose tissue), culture media, and the number of passages have a tremendous impact on the molecular composition of MSC preparations and the latter affects their stromal function (Walenda et al., 2009). Using standardized culture conditions, we have demonstrated that gene expression profile, proteome, and hematopoiesis-supportive function are reproducible even in samples derived from different donors (Wagner et al., 2005b, 2006; Wagner and Ho, 2007; Wagner et al., 2008a, in press). MSC from BM and CB have a higher hematopoiesis-supportive function than MSC from AT and this correlated with a higher adhesive propensity for $CD34^+$ HPC (Wagner et al., 2007b). This indicated that specific cell–cell junctions between HPC and MSC are essential for the hematopoiesis-supportive function.

Therefore, we reasoned that the more supportive MSC preparations derived from BM and CB might express higher levels of specific adhesion molecules. In fact, N-cadherin and cadherin-11 were significantly higher expressed in these MSC preparations in comparison to nonsupportive AT-MSC and this has been confirmed by both mRNA and protein levels (Wagner et al., 2007b). The molecular profiles of supportive and nonsupportive feeder layer cells have been analyzed by other authors before (Weisel et al., 2006; Oostendorp et al., 2005; Hackney et al., 2002). AFT024 cells, a murine fetal liver cell line with a high hematopoiesis-supportive potential, have been compared to nonsupportive cell lines and cadherin-11, NCAM, and thrombospondin 2 were among the genes that were significantly higher expressed in AFT024 cells (Hackney et al., 2002; Charbord and Moore, 2005).

Cadherin-11 was also higher expressed in supportive OP-9 cells in comparison to nonsupportive NIH3T3 cells (Lagergren et al., 2007). Furthermore, it has been demonstrated that N-cadherin is higher expressed in stromal cells from fetal liver that might provide a more conducive microenvironment to support HSC than those from adult BM (Martin and Bhatia, 2005). These gene expression profiles suggest that cadherins may play a role in the hematopoiesis-supportive function of stromal feeder layer cells.

It is intriguing that N-cadherin and cadherin-11 are higher expressed in the adherent fraction of HPC as well as in adhesive MSC from bone marrow. In this study we have demonstrated that N-cadherin is expressed in HPC from CB and PB, whereas BM samples have an even higher expression of N-cadherin. The decreased level of N-cadherin in CB and PB might be attributed to their immediate removal from the natural niche. It would have been advantageous to perform these studies with HPC from BM, but there was only a very limited number of samples from healthy bone marrow donors available. Puch et al. have previously shown that human $CD34^+$ HPC express N-cadherin (Puch et al., 2001). In their study, these authors demonstrated that N-cadherin is involved in homophilic interaction of KG1a cells and that GC-4 antibody impaired colony formation of $CD34^+$ HPC. In contrast, other authors could not detect the presence of N-cadherin in murine HSC or if present, only at low or intermediate levels (Kiel et al., 2007; Haug et al., 2008). In analogy to CD34 expression, it is conceivable that N-cadherin is initially not expressed in long-term repopulating HSC and then up-regulated in progenitor cells. On the other hand, it is surprising that the myeloid cell line KG1a expresses high levels of N-cadherin (Puch et al., 2001). Whereas N-cadherin was not found in other myeloid cell lines including KG1 cells. Therefore the subline KG1a might have acquired some additional mutations that increased expression of N-cadherin. The vast majority of myeloid cells seem to be N-cadherin negative. Recently, Kiel et al. reported that they did not detect N-cadherin expression within 100 000 whole bone marrow cells by Western blot (Kiel et al., 2009). These differences might arise from variation between mice and man as well as by the absence of N-cadherin expression in the majority of MNC. Furthermore, this might be caused by a dynamic turnover of cadherins as soon as HPC depart from their niche. N-cadherin might be rapidly degraded if it is not involved in stable cell–cell junctions. This thesis is supported by the fast down-regulation of N-cadherin expression only 14 h after siRNA treatment in MSC.

Cadherins are glycoproteins which are thought to mediate cell–cell adhesion by a homophilic binding mechanism. A widely held view is that segregation of cells into tissue-specific subpopulations during development is largely attributed to the discrimination between different cadherins on cell surfaces. However, there is recent evidence that the homophilic selectivity of cadherin–cadherin binding is not very stringent and that there is cross-reactivity between different cadherin subtypes (Prakasam et al., 2006). Furthermore, it has been demonstrated that N-cadherin and cadherin-11 occur in heterotypic ("mixed") complexes in the fiber cells of the eye lense (Straub et al., 2003). Different subtypes of cadherins may form mixed *cis*-dimers in the same cells to further activate adhesive function or they might form heterotypic *trans*-cellular complexes. Therefore, it is possible that inhibition or knockdown of N-cadherin in the cellular microenvironment (respectively the MSC) affects cell–cell adhesion even if it was not or only at low levels expressed on HPC. Notably, N-cadherin was expressed at a lower level in HPC than in MSC and it is conceivable that enrichment of N-cadherin-positive HPC would reveal a stronger effect on cell adhesion or colony-forming unit frequency. Either way, our results support the notion that N-cadherin is involved in the junction between HPC and MSC as well as in their adhesive and supportive interaction.

Apart from N-cadherin and cadherin-11, it has been demonstrated that various other adhesion proteins might play a role in the interactions of HPC with their microenvironment. For example, there is clear evidence that CD44 (Avigdor et al., 2004; Wagner et al., 2007c), integrin beta-1 (ITGB1) (Gottschling et al., 2007), VCAM-1 (Priestley et al., 2007), CD164 (Forde et al., 2007), and Jagged/Notch (Stier et al., 2002), among others, form an orchestra of adhesion proteins that mediate adhesion of HPC to other cells or to extracellular matrix components. Furthermore, chemokines such as the SDF-1α/CXCR4 axis play an essential role in homing and adhesion (Bonig et al., 2006; Dar et al., 2006). It is likely that for the vital process of adhesion of HPC to the niche many redundant mechanisms are involved. This is compatible with the observation that inhibition of N-cadherin resulted in a moderate decrease of cell–cell adhesion.

Conclusion

We have used for the first time a human–human model to analyze the role of N-cadherin for interaction with a supportive cellular microenvironment. N-cadherin is expressed in human HPC on RNA and protein level, whereas it was not detected in the fraction of differentiated MNC. Furthermore, it is localized at the cell–cell junction between HPC and MSC and it is involved in cell–cell adhesion and maintenance of LTC-IC. These results are in alignment with the assumption that N-cadherin anchors HSC to the osteoblastic niche and that it might be involved in the control of self-renewal of stem cells. The recent study by Kiel and coworkers indicated that conditional deletion of N-cadherin in the murine system does not affect hematopoiesis (Kiel et al., 2009). However, it is conceivable that there are differences between mice and men and this study does not rule out that N-cadherin is involved in the orchestra of adhesion molecules for specific cell–cell interaction. The precise composition of potentially mixed *cis*- and *trans*-binding complexes with other cadherins and the relative significance with regard to other adhesion proteins and downstream signal cascades remain to be elucidated.

Design and methods

Isolation of hematopoietic progenitor cells

HPC were collected from fresh umbilical cord blood (CB) of 16 donors. $CD34^+$ and $CD34^-$ cell fractions were also isolated from the peripheral blood (PB) of 5 different healthy donors for allogeneic transplantation after G-CSF treatment for 5

days. Furthermore, we received bone marrow aspirates (BM) from 3 healthy bone marrow donors for isolation of HPC. All samples were taken after written consent using guidelines approved by the Ethic Committee on the Use of Human Subjects at the University of Heidelberg. Mononuclear cells (MNC) were isolated after density gradient centrifugation on Ficoll-Hypaque (Biochrom KG, Berlin, Germany). $CD34^+$ cells were enriched by labeling with a monoclonal anti-CD34 antibody conjugated with magnetic MicroBeads and passing them over an affinity column in an AutoMACS system (MACS, Miltenyi Biotec, Bergisch-Gladbach, Germany). After additional staining with anti-CD34-phycoerythrin (PE; Becton Dickinson, San Jose, CA, USA [BD]) further purification was achieved by sorting the $CD34^+$ cells with a FACS-Vantage-SE flow cytometry cell sorting system (BD). Staining with propidium iodide (PI) was performed to allow exclusion of nonviable cells. Reanalysis of isolated cells revealed a purity of more than 98% in all separated fractions.

Cultivation of mesenchymal stromal cells

Mesenchymal stromal cells were isolated from human bone marrow and characterized as described in our previous work (Wagner et al., 2005b, 2006, 2008b). In this study we have used MSC from four individual donors (25, 28, 37, and 50 years old). In brief, MNC were seeded in tissue culture flasks that have been coated with 10 ng/ml fibronectin (Sigma) in culture medium as described by Reyes and colleagues (Reyes et al., 2001). The medium consists of 58% Dulbecco's modified Eagles medium–low glucose (DMEM-LG, Cambrex, Apen, Germany) and 40% MCDB201 (Sigma, Deisenhofen, Germany), 2% FCS (HyClone, Bonn, Germany), supplemented with 2 mM L-glutamine, 100 U/ml Pen/Strep (Cambrex), 1% insulin transferrin selenium, 1% linoleic acid bovine serum albumin, 10 nM dexamethasone, 0.1 mM L-ascorbic acid-2-phosphate (all from Sigma), PDGF-bb, and EGF (10 ng/ml each, R&D Systems, Wiesbaden, Germany). For these studies, we have used a subconfluent MSC feeder layer (70–80%) of the third to sixth passage.

RT-PCR analysis

Quantitative real-time PCR was used to assess mRNA expression of N-cadherin, cadherin-11, and β-actin. Total RNA was isolated using TRIzol reagent (Invitrogen, Paisley, Scotland) and cDNA was generated with the high-capacity reverse-transcription kit (Applied Biosystems). Quantification of mRNA expression was performed with predesigned gene expression assays using TaqMan TAMR probes for N-cadherin (Hs00169953_m1), cadherin-11 (Hs00156438_m1), and β-actin (Hs00181698_m1, all from Applied Biosystems, Applera Deutschland GmbH, Darmstadt, Germany), the PCR mastermix (Eurogentec, Seraing, Belgium), and the ABI PRISM 7700HT Sequence Detection System Instrument (Applied Biosystems). Differential gene expression was normalized to β-actin.

Microarray data

Expression of N-cadherin and cadherin-11 was reanalyzed in our previously published microarray data. Gene expression data of adherent and nonadherent HPC have been described in detail (Wagner et al., 2007a). In brief, $CD34^+$ cells were cocultured on human MSC from bone marrow for 1 h and subsequently divided into adherent and nonadherent fractions. All microarray data have been deposited according to Minimal Information About Microarray Experiments (MIAME) requirements at the public microarray database ArrayExpress (http://www.ebi.ac.uk/arrayexpress/; accession number: E-MEXP-586).

Immunoblot

Cell pellets from 50 000 MSC or 500 000 HPC were treated for 15 min in 50 μl lysis buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 5 mM EDTA, 1% Triton, 1% protease inhibitor cocktail [Complete; Roche, Mannheim, Germany]). Equal amounts of protein were resolved on each lane of 4–12% TRIS-glycine gradient gels (Anamed, Darmstadt, Germany). Proteins were transferred on a polyvinylidene difluoride membrane (Millipore, Billerica, USA), labeled with murine monoclonal anti-cadherin-11 antibody (1 μg/ml; clone 5H2H5, Zymed Laboratories, San Francisco, USA) or anti-N-cadherin (0.5 μg/ml; clone 32, BD) and detected with a secondary antibody (sc-2005, Santa Cruz) by chemiluminescence (ECL, Amersham Biosciences, UK). Protein concentration was determined by Bradford analysis (60 μg protein lysate per lane), or we used the anti-β-actin antibody (0.08 μg/ml; sc-47778, Santa Cruz) for loading control.

Flow cytometry

Expression of adhesion proteins was analyzed by flow cytometry in MNC, $CD34^+$ cells (CB or PB), in the myeloid cell line KG1a and in MSC. N-cadherin and cadherin-11 antibodies were not directly fluorochrome-coupled and secondary anti-mouse antibodies might bind to residual murine CD34 antibody linked to the MACS beads. Therefore, these experiments were performed on $CD34^+$-enriched cells using the Dynal CD34 Progenitor Cell Selection System (Invitrogen, Karlsruhe, Germany) that allows detachment of both beads and antibodies prior to the staining procedure. Cells were then stained with monoclonal antibody for human N-cadherin antibody (clone GC-4, Sigma), detected by a secondary chicken anti-mouse-Alexa 488 antibody (A21200; Molecular probes, OR, USA). Alternatively, we have used a biotinylated rat anti-mouse N-cadherin antibody (MNCD2; kindly provided by Linheng Li (Matsunami and Takeichi, 1995)) or a phycoerythrin-labled monoclonal muine antibody (clone 8C11). For analysis of intracellular epitopes, cells were fixed and permeabilized using the Fix and Perm kit (Caltag, Burlingame, CA, USA). Subsequently the cells were stained with the murine N-cadherin antibody (Clone 32, BD) or the murine antibody for cadherin-11 (Clone 5B2H5, Invitrogen) detected by a secondary antibody labeled with Alexa 488 (A21200). For controls, some cells were only stained with the secondary antibody or left unstained. All antibodies were used in a final concentration of 4 μg/ml at 4 °C for 30 min. In addition, we have used the anti-CD44-PE (clone G44-26; BD) or anti-CD29-FITC (MG101; BD) antibodies. Analysis was performed on a FACScan flow cytometer (BD).

Confocal laser scanning microscopy

CD34$^+$ cells from CB were cocultured with MSC for 48 h and subsequently fixed with 3% paraformaldehyde for 5 min and permeabilized with 0.2% Triton X-100 for 5 min. N-cadherin was detected using the mouse anti-human antibody (clone 32, BD) and a secondary chicken anti-mouse antibody labeled with Alexa 488 (A21200). β-Catenin was analyzed using the goat anti-rabbit antibody (C2206; Sigma) and a secondary goat anti-rabbit antibody labeled with Cy3 (111-165-144; Jackson Laboratories). Nuclei were counterstained with DAPI (4′,6-diamidino-2-phenylindol). Images were taken with the Nikon C1Si-CLEM spectral imaging confocal laser scanning system at the Nikon Imaging Center (Heidelberg, Germany) using a 60x objective.

siRNA knockdown of N-cadherin and cadherin-11

The role of N-cadherin or cadherin-11 for homophilic interaction of HPC and MSC was analyzed by specific siRNA knockdown in MSC using the RNAi starter kit (301799; Qiagen, Hilden, Germany) and validated siRNA constructs for N-cadherin (SI00028441) and cadherin-11 (SI00028511). On passaging MSC were transfected with 5 nM siRNA and 3 μl HiPerFect transfection reagent according to the manufacturer's instructions. Knockdown efficiency was verified by immunoblot analysis as described above.

Treatment with blocking function antibody

The N-cadherin GC-4 antibody (Sigma) has previously been demonstrated to have blocking function (Puch et al., 2001; Li et al., 2001). CD34$^+$ cells were incubated with 50 μg/ml of this antibody for 30 min at 4 °C and then transferred to the adhesion assay (Turner et al., 1998). As a control we have used goat anti-human Ig's (AHI0701; Biosource, Carmarillo, USA) in the same concentration.

Adhesion assay

Cell-cell adhesion was analyzed using our previously described assay based on gravity force (Wagner et al., 2007a). In brief, adhesive press-to-seal silicone isolators with eight wells, 9 mm diameter, 1.0 mm deep (Invitrogen), were fixed on glass slides and confluent feeder layers were grown in these wells. CD34$^+$ HPC were stained with the fluorescent membrane dye PKH26 (Sigma, Saint Louis, MO, USA) according to the manufactures instructions and about 10 000 HPC were seeded in each well. After an attachment period of 1 h, the adhesion array was inverted (180° flip) and after 1 h adherent cells remained attached to feeder layer cells, whereas nonadherent cells dropped down and could be observed on the focus level of the cover slide. Single images were acquired of the same region on the adhesion array before the inversion (all cells) as well as after the inversion in the focus level of feeder layer cells (adherent cells) and on the lower glass slide level (nonadherent cells) (Wagner et al., 2007a). Mean and standard deviation (SD) of independent experiments are presented. We have adopted the two-sided, paired Student's *t* test to estimate the probability of differences in adherence under different experimental conditions. A *P* value $P<0.05$ was considered to be statistically significant.

LTC-IC assay

To estimate the role of N-cadherin for maintenance of primitive HPC, we have used the long-term culture-initiating cell assay (Sutherland et al., 1989). LTC-IC frequency was analyzed on treatment with either blocking function antibody (GC-4) or for control with goat anti-human antibody (AHI0701) at an initial concentration of 10 μg/ml and subsequently 5 μg/ml with every medium exchange. Alternatively, we have knocked down N-cadherin in MSC before coculture using siRNA as described before. CD34$^+$ cells were plated in limiting dilutions (150, 50, 15, and 5 cells per well and 22 replicates per concentration for each experiment) on irradiated confluent MSC feeder layers (20 Gy) in 96-well plates. Cells were cultured in long-term culture medium (LTBMC; (Sutherland et al., 1990)) supplemented with 10 ng/ml Flt-3L and 10 ng/ml TPO (both R&D Systems; Minneapolis, MN, USA) for 5 weeks, and then overlaid with clonogenic methylcellulose medium as described before (Punzel et al., 1999; Giebel et al., 2006). Cultures were scored for secondary colony-forming cells (CFC) after 10 additional days of culture. LTC-IC frequency was determined using the L-Calc Software for Limiting Dilution Analysis (Stem Cell Technologies) and results of independent experiments were compared using the two-sided, paired Student's *t* test.

Acknowledgments

We thank Kerstin Wörner and Katrin Miesala for excellent technical assistance in cell culture and cell sorting. This work was supported by the German Ministry of Education and Research (START-MSC and CB-HERMES), the German Research Foundation DFG (HO 914/7-1), the KIND Stiftung, the Joachim Siebeneicher-Stiftung, Germany, the Heidelberg Academy of Sciences and Humanities (WIN-Kolleg), and the Stem Cell Network North Rhine Westphalia.

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