



## Hematopoietic and endothelial progenitor cell trafficking in patients with myeloproliferative diseases

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**Background and Objectives.** The presence of circulating hematopoietic progenitor cells in patients with myeloproliferative diseases (MPD) has been described. However, the exact nature of such progenitor cells has not been specified until now. The aim of this work was to investigate the presence of endothelial precursor cells in the blood of patients with MPD and to assess the role of the endothelial cell lineage in the pathophysiology of this disease.

**Design and Methods.** Endothelial progenitor cell marker expression (CD34, prominin (CD133), kinase insert domain receptor (KDR) or vascular endothelial growth factor receptor 2 (VEGFR2), and von Willebrand factor) was assessed in the blood of 53 patients with MPD by quantitative polymerase chain reaction. Clonogenic stem cell assays were performed with progenitor cells and monocytes to assess differentiation towards the endothelial cell lineage. The patients' were divided according to whether they had essential thrombocythemia (ET, n=17), polycythemia vera (PV, n=21) or chronic idiopathic myelofibrosis (CIMF, n=15) and their data compared with data from normal controls (n=16) and patients with secondary thrombo- or erythrocytosis (n=17).

**Results.** Trafficking of CD34-positive cells was increased above the physiological level in 4/17 patients with ET, 5/21 patients with PV and 13/15 patients with CIMF. A subset of patients with CIMF co-expressed the markers CD34, prominin (CD133) and KDR, suggesting the presence of endothelial precursors among the circulating progenitor cells. Clonogenic stem cell assays confirmed differentiation towards both the hematopoietic and the endothelial cell lineage in 5/10 patients with CIMF. Furthermore, the molecular markers trisomy 8 and JAK2 V617F were found in the grown endothelial cells of patients positive for trisomy 8 or JAK2 V617F in the peripheral blood, confirming the common clonal origin of both hematopoietic and endothelial cell lineages.

**Interpretation and Conclusions.** Endothelial precursor cells are increased in the blood of a subset of patients with CIMF, and peripheral endothelial cells bear the same molecular markers as hematopoietic cells, suggesting a primary role of pathological endothelial cells in this disease.

Key words: myeloproliferative diseases, trafficking, progenitor cells, PCR, endothelial, hemangioblasts.

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It has been shown by several groups that there is an increased number of circulating CD34-positive cells in patients with myeloproliferative diseases.<sup>1-4</sup> Increased numbers of circulating CD34-positive cells have been found in patients with chronic idiopathic myelofibrosis, polycythemia vera and essential thrombocythemia, and their presence is associated with disease progression and poor outcome. Recently, a common ancestor to the hematopoietic and endothelial cell lineage, the hemangioblast, has been identified. Hemangioblasts are characterized by the expression of CD34, CD133 (prominin), and vascular endothelial growth factor-receptor 2 (VEGFR2 or KDR).<sup>5-7</sup> These cells are able to differentiate into both hematopoietic and endothelial cells. Circulating CD34-positive cells were recently characterized by Massa *et al.* in patients with myelofibrosis with myeloid metaplasia, and endothelial progenitor cells were found.<sup>8</sup> Myeloproliferative diseases are stem cell disorders for which the mechanism responsible for cell proliferation has not yet been determined. The level of the stem (or progenitor) cell defect

is not known. While cell proliferation is restricted to the megakaryocytic lineage in essential thrombocythemia (isolated hyperplasia of the megakaryocytic cell lineage in the bone marrow according to the 2001 WHO classification of tumors), the erythroid, myeloid and megakaryocytic cell lineages are involved to various degrees in polycythemia vera and chronic idiopathic myelofibrosis. Furthermore, endothelial cell proliferation is present in the bone marrow and spleen of patients with polycythemia vera and chronic idiopathic myelofibrosis. Such proliferation might be a secondary accompanying phenomenon (caused, for example, by stimulation from cytokines) but it might also be primarily associated with the pathophysiology of myeloproliferative diseases, caused by a dysregulation of the endothelial lineage by the same mechanism that leads to hematopoietic cell proliferation. The dysregulation might happen at the progenitor cell level involving both lineages. However, work by two different groups showed the ability of subsets of peripheral monocytes to acquire endothelial-like functional capacity,

opening the way to a completely new explanation for endothelial cell proliferation.<sup>9,10</sup> Recently, the gain-of-function mutation V617F in the *JAK2* gene has been described in myeloproliferative disorders, providing a genetic marker for cell analysis.<sup>11,12</sup> Based on the facts that both CD34 positive cells and monocytes are increased in the blood of subsets of patients with myeloproliferative diseases, we planned a prospective study aimed to quantify the presence of endothelial precursor cells in the blood of these patients by quantitative polymerase chain reaction (PCR). We also tested the differentiation towards the endothelial cell lineage of both the endothelial precursor and the monocytic cell population by clonogenic stem cell assays and looked for the genetic marker of myeloproliferative disease, *JAK2* V617F, in the grown endothelial cells.

## Design and Methods

### Study design

Blood samples were taken from healthy controls and from patients with myeloproliferative diseases at the time of diagnosis for quantitative PCR and clonogenic stem cell assays. All participants signed informed consent to the study and the protocols were approved by our hospital's ethic committee. The study group was composed of patients with myeloproliferative diseases diagnosed according to the WHO criteria. Patients were not under treatment at the time of analysis. The 53 patients in the study group were divided into patients with essential thrombocythemia (n=17, male/female=8/9) with a median age of 68 years (range 24-87); (ii) patients with polycythemia vera (n=21, male/female=14/7) with a median age of 60 years (range 34-81), and (iii) patients with chronic idiopathic myelofibrosis (n=15, male/female=7/8) with a median age of 70 years (range 42-77). Sixteen patients (male/female=8/8) investigated at our center for thrombocytosis (n=4) or erythrocytosis (n=13) and found to have secondary erythrocytosis or thrombocytosis served as a control group; the median age of these patients was 48 years (range 27-74). Sixteen healthy donors (male/female=11/5) with a median age of 36 years (range 21-57) served as controls.

### Quantitative RT-PCR for CD34, CD133, KDR and vWF

Total cellular RNA was extracted from 10<sup>7</sup> peripheral blood leukocytes using the QIAmp RNA Blood Mini Kit

(Qiagen, Basel, Switzerland). cDNA was synthesized from 2 µg total RNA applying the Superscript II system (Invitrogen, Basel, Switzerland) and random hexamer primers in a total volume of 40 µL. CD34, CD133, KDR and vWF mRNA expression was determined by quantitative real-time PCR based on specific primer and probe sets (TaqManGene Expression Assays, <http://myscience.applied-biosystems.com>) supplied by Applied Biosystems (Rotkreuz, Switzerland). The primer and probe sets are listed in Table 1. All assays were designed to span exon-exon boundaries. A specific primer and probe set (TaqManGene Expression Assays, Hs00609297\_m1) was used for the internal control gene, porphobilinogendeaminase (*PBGD*). All PCR reactions were performed in duplicate. PCR reactions contained 125 ng cDNA, 12.5 µL of 2x universal master mix (Applied Biosystems) and 1.25 µL of 20x primer-probe mix in a final reaction volume of 25 µL. Amplification was carried out at 50°C for 2 minutes and 95°C for 10 minutes, followed by 45 cycles at 95°C for 15 seconds and 60°C for 1 minute. All reactions were performed in Micro-Amp optical 96-well plates using an ABI PRISM 7700 sequence detection system (Applied Biosystems). A mean cycle threshold (Ct) was calculated for each duplicate. Only values under Ct 32 were accepted for the housekeeping gene, *PBGD*. The relative expression of the target mRNA was normalized to *PBGD* expression and was calculated using the equation: relative expression level = 2<sup>-CtTARGET-CtPBGD</sup>.

### Correlation of CD34 quantification by flow cytometry and RT-PCR

The correlation between flow cytometry and RT-PCR measurements of CD34 has been described before.<sup>4</sup> In a dilution experiment, a linear correlation between CD34 protein and mRNA was obtained down to a dilution of 1:100. However, while the linear range for quantitative CD34 measurements by flow cytometry was limited to 2 log, the linear range for quantification by RT-PCR was extended to 4 log.

### Clonogenic stem cell assays

Clonogenic stem cell assays for the determination of colony forming units-erythroid (CFU-E) and colony forming units-granulocyte monocyte (CFU-GM) were performed as described elsewhere.<sup>14</sup> Clonogenic stem cell assays for the determination of colony forming units-megakaryocyte (CFU-Mega) were performed as follows: mononuclear cells (0.50×10<sup>6</sup>cells/well) were plated into

**Table 1.** TaqMan gene expression assays used for quantitative real-time RT-PCR.

TaqMan gene expression assay	Gene	Exon boundary	PCR Product	Reference sequence
Hs00156373_m1	CD34	Exon 4 to 5	63 bp	NM_001773
Hs00195682_m1	Prominin (CD133)	Exon 2 to 3	107 bp	NM_002253
Hs00176676_m1	KDR, VEGFR2	Exon 15 to 16	84 bp	NM_006017
Hs00368522_m1	vWF	Exon 17 to 18	79 bp	NM_000552
Hs00609297_m1	PBGD	5' UTR	64 bp	NM_000190

sterile 35 mm diameter culture dishes. Unstimulated assays contained 100  $\mu$ L L-asparagine (Fluka/Sigma), 100  $\mu$ L IMDM Supplemental Medium (Sigma), 550  $\mu$ L IMDM and 100  $\mu$ L Bacto™ Agar (Difco 214050) 2.2%. Stimulated assays contained 100  $\mu$ L L-asparagine (Fluka/Sigma), 100  $\mu$ L IMDM Supplemental Medium (Sigma), 445.5  $\mu$ L IMDM and 100  $\mu$ L Bacto™ Agar (Difco 214050) 2.2%, 22  $\mu$ L erythropoietin (Roche Diagnostics, Rotkreuz, Switzerland) 1.1 U, 27.5  $\mu$ L stem cell factor (R&D Systems, Bülmann Laboratories) 5.5 ng, 27.5  $\mu$ L interleukin-3 (R&D Systems) 5.5 ng and 27.5  $\mu$ L MGDF (Amgen) 11 ng. The culture dishes were allowed to cool at room temperature for 20 minutes and were covered with 2.79 mL IMDM +0.31 mL AB-Plasma. The cultures were incubated for 12 days at 37°C with 4.3% CO<sub>2</sub>. Cultures were fixed, stained and counted. Clonogenic stem cell assays for the determination of colony forming units-endothelial cells (CFU-Endo) were performed using the procedure described by Hill *et al.*<sup>15</sup> Briefly, a mononuclear cell suspension was prepared by Ficoll density separation using the Lymphoprep™ solution (Axis-Shield PoC AS Company, Oslo, Norway) and 2.5×10<sup>6</sup> mononucleated cells per well were added to a fibronectin-coated 4-well culture slide (Biocoat, Becton Dickinson Labware, Franklin Lakes, USA). The cells were incubated for 2 days at 37°C in 5% CO<sub>2</sub> with 95% humidity. After 48 hours, the non-adherent cells from each well were transferred into a 10 mL polypropylene tube and 1×10<sup>6</sup> cells per well were plated in a 24-well dish (Biocoat), incubated for 3 days, and counted using an inverse microscope.

#### **Cell sorting for endothelial precursor cells and monocytes**

Cell sorting was performed on peripheral blood cells from a patient diagnosed as having polycythemia vera. Mononuclear cells were enriched by Ficoll gradient (Lymphoprep™, Axis-Shield PoC AS Company). CD14<sup>+</sup> cells were isolated with anti-human CD14 (Clone M5E2, BD Pharmingen, Basel, Switzerland) and pan-mouse immunoglobulin Dynabeads (Dyna, LuBioScience, Lucerne, Switzerland). Alternatively, CD45<sup>+</sup>CD146<sup>+</sup> were isolated by negative selection using anti-human CD45/pan-mouse immunoglobulin Dynabeads (Clone 2D1; Santa Cruz Biotechnology, LabForce AG, Nunningen, Switzerland) followed by positive selection using anti-human CD146/pan-mouse immunoglobulin Dynabeads (Clone P1H12; BD Pharmingen, Basel, Switzerland). Purified cells were cultured as described above. *JAK2* V617F mutational status was determined from genomic DNA extracted from all subsets of cells before and after endothelial cell culture.

#### **Cytogenetic analysis**

Cytogenetic analysis was done on cultures derived from the peripheral blood of a 77-year old female patient with chronic idiopathic myelofibrosis, who had been found to

have trisomy 8 in both her unstimulated blood and bone marrow, 6 years earlier. *Non-adhering* cells from 5 to 6-day old multiwell cultures in Endocult liquid medium were collected, washed once and reincubated in suspension culture with either RPMI 1640/15% FCS or PB max karyotyping medium (Invitrogen Life Technologies) containing phytohemagglutinin (PHA) as a T-lymphocyte mitogen.

A standard chromosome preparation, as with peripheral blood cultures, was made 3 days later. The chromosome preparation of adhering cells from the same 5 to 6-day old multiwell cultures and from a 4-well culture slide was made after a 2-hour or overnight metaphase arrest with colcemide (50 to 100 ng/mL), and either subsequent enzymatic (standard trypsin/EDTA-mix±collagenase) plus additional mechanical detachment of the cells, or *in situ* without cell detachment.

Chromosomes were counted and identified after Q- or Distamycin/DAPI banding. Dual color fluorescence *in situ* hybridization (FISH) was done with all types of chromosome preparations, using standard procedures. We used centromere-specific  $\alpha$  satellite probes (Oncor, labeled with digoxigenin or biotin; Vysis, labeled with spectrum orange or spectrum green). Fluorescent signals were visualized and processed using PSI powergene software (now at Abbott Cy).

#### **JAK2 V617F mutational analysis**

For analysis of *JAK2* V617F mutational status, genomic DNA was isolated using the EZ-1 bioroboter and EZ-1 Blood DNA Kit (Qiagen, Basel, Switzerland) and an allele-specific quantitative PCR assay, developed based on a published method.<sup>16</sup> Briefly, two separate reactions for the amplification of mutated *JAK2* and total *JAK2* were performed on an ABI PRISM® 7900 Sequence Detection System (primer and probe sequences are available on request). Results were analyzed by the  $\Delta$ Ct method, normalized to the expression in the *JAK2* V617F positive cell line HEL and the percentage of *JAK2* V617F alleles was calculated.

#### **Statistical analysis**

The data were analyzed using SigmaStat® software from Jandell Corporation (San Rafael, CA, USA). Groups were compared using multiple comparison procedures (Kruskal-Wallis one way analysis of variance on ranks) and pairwise multiple comparison procedures (Dunn's method). Linear regression analysis was used to determine correlations.

## **Results**

#### **Definition of physiological levels of CD34, CD133, KDR and vWF mRNA expression**

The levels of expression of CD34, CD133, KDR and vWF mRNA were assessed in peripheral blood samples from 16 healthy donors. The highest level of expression

found in this group defined the cut-off for the evaluation of expression levels in the different groups of patients.

### CD34 mRNA expression

All normal controls expressed detectable levels of CD34 mRNA, ranging from 0.0004-0.08 CD34 copies/PBGD copy, with a median value of 0.02. The highest value of 0.08 measured within this group was chosen as the *cut-off* value for physiological CD34 mRNA expression. According to this cut-off, 13 of 15 patients (87%) with chronic idiopathic myelofibrosis overexpressed CD34 mRNA. The increase of CD34 mRNA expression was 10- to 100-fold in most cases (Figure 1). A more moderate increase was detected in 5/21 patients with polycythemia vera (24%) and in 4/17 patients with essential thrombocythemia (24%) but in none of the 16 patients with secondary thrombocytosis or erythrocytosis. Statistical analysis revealed a significant increase of CD34 mRNA expression in the group of patients with chronic idiopathic myelofibrosis when compared with the groups of healthy individuals, patients with secondary thrombocytosis or erythrocytosis, and patients with essential thrombocythemia and polycythemia vera ( $p < 0.05$ ).

### CD133 mRNA expression

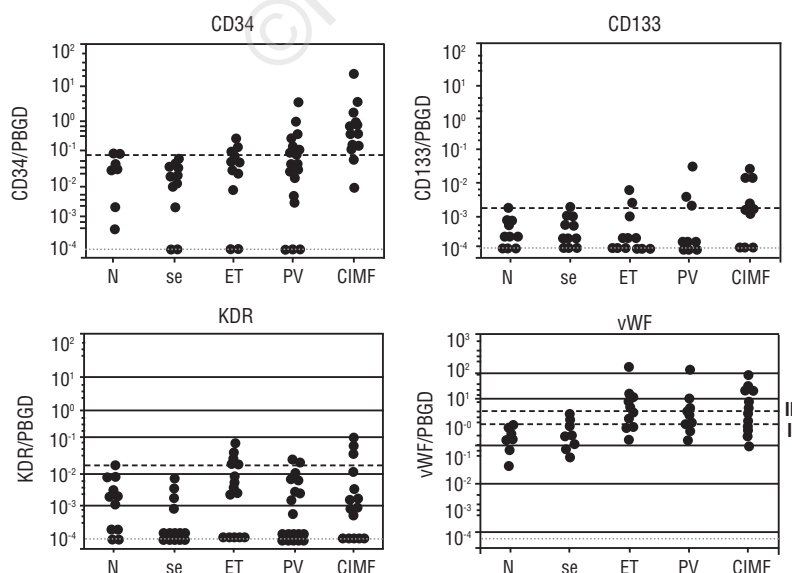
The highest value of 0.0016 measured within the normal control group was chosen as the *cut-off* value for physiological CD133 mRNA expression. Five of 15 patients (33%) with chronic idiopathic myelofibrosis overexpressed CD133 (with a 10-fold increase in most cases) (Figure 1). CD133 mRNA overexpression was also seen in 3/21 patients with polycythemia vera (14%), and in 2/17 patients with essential thrombocythemia (12%) but in none of the 16 patients with secondary thrombocytosis or erythrocytosis. Statistical analysis showed no significant increase of CD133 mRNA expression in any of the groups tested ( $p = 0.058$ ).

### KDR (VEGFR2) mRNA expression

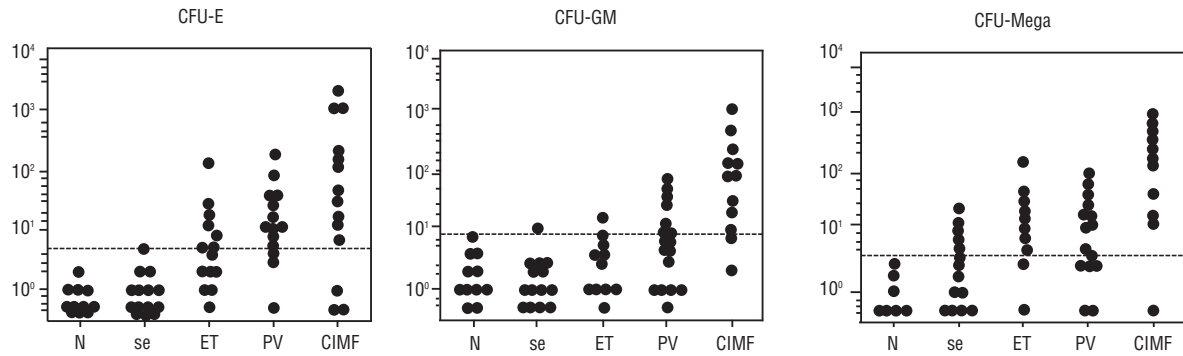
The highest value of 0.019 measured within the normal control group was chosen as the *cut-off* value for physiological KDR expression. Three of 15 patients (20%) with chronic idiopathic myelofibrosis showed KDR overexpression (Figure 1). Overexpression of KDR was also detected in 4/17 patients with essential thrombocythemia (24%), but in none of the 21 patients with polycythemia vera and in none of the 16 patients with secondary thrombocytosis or erythrocytosis. Statistical analysis showed that KDR expression was significantly increased in the group of patients with essential thrombocythemia when compared with the groups of healthy individuals, patients with secondary thrombocytosis or erythrocytosis, polycythemia vera and chronic idiopathic myelofibrosis ( $p < 0.05$ ).

### vWF mRNA expression

The highest value of 1.18 measured within the normal control group was chosen as the *cut-off* value (cut-off I) for physiological vWF expression. Nine of 15 patients (60%) with chronic idiopathic myelofibrosis overexpressed vWF mRNA (Figure 1). vWF overexpression was detected in 15/21 patients with polycythemia vera (67%), in 13/17 patients with essential thrombocythemia (77%) but also in 4/16 patients with secondary thrombocytosis or erythrocytosis (25%). When another cut-off value was set above the highest expression of the patients with secondary thrombocytosis or erythrocytosis (2.96, cut-off II), vWF overexpression was still detected in 6/15 patients with chronic idiopathic myelofibrosis (40%), 9/21 patients with polycythemia vera (43%) and 9/17 patients with essential thrombocythemia (53%). Statistical analysis showed that vWF expression was significantly increased in the groups of patients with essential thrombocythemia, polycythemia vera and chronic idiopathic myelofibrosis when compared to the groups of healthy individuals and patients with secondary thrombocytosis or erythrocytosis ( $p < 0.05$ ).



**Figure 1.** Expression of each marker in individual patients and controls CD34, prominin (CD133), KDR and vWF expression in normal controls (N), patients with secondary thrombocytosis or erythrocytosis (se), essential thrombocythemia (ET), polycythemia vera (PV) and chronic idiopathic myelofibrosis (CIMF). The dashed line indicates the *cut-off* value for physiological expression of the marker. The dotted line indicates the defined detection limit of 0.0001 copies of CD34, CD133, KDR or vWF/copy of PBGD.



**Figure 2.** Clonogenic stem cell assays number of CFU-E, CFU-GM and CFU-Mega for normal controls (N), patients with secondary thrombocytosis or erythrocytosis (se), essential thrombocythemia (ET), polycythemia vera (PV) and chronic idiopathic myelofibrosis (CIMF). The dotted line indicates the cut-off value of the assay.

### Clonogenic stem cell assays

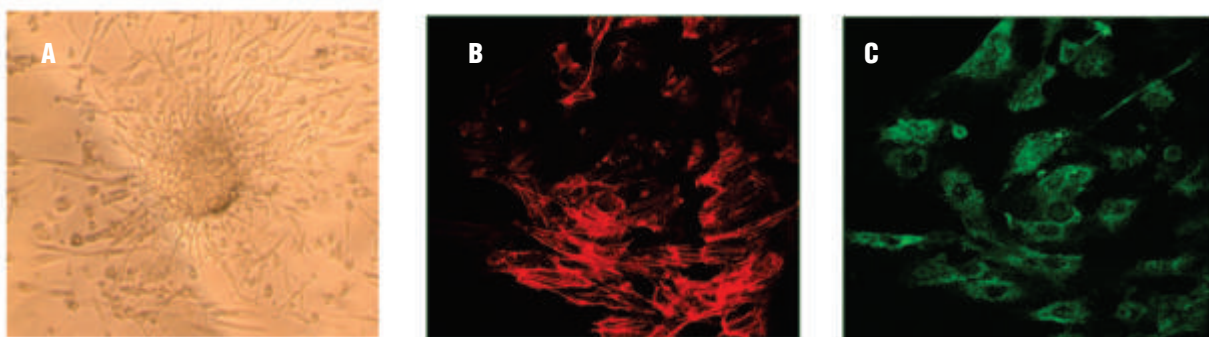
Clonogenic hematopoietic stem cell assays were performed in 14 healthy individuals, in all patients with secondary thrombocytosis or erythrocytosis ( $n=16$ ), polycythemia vera ( $n=21$ ) and chronic idiopathic myelofibrosis ( $n=15$ ) as well as in 16/17 patients with essential thrombocythemia. Significant endogenous erythroid colony growth ( $>5$  CFU-E) was seen in none of the patients with secondary thrombocytosis or erythrocytosis (0%), in 6/16 patients with essential thrombocythemia (38%), in 18/21 patients with polycythemia vera (86%), and in 12/15 patients with chronic idiopathic myelofibrosis (80%) (Figure 2). Significant endogenous myeloid growth ( $>10$  CFU-GM) was present in 0/16 of the patients with secondary thrombocytosis or erythrocytosis (0%), in 3/16 patients with essential thrombocythemia (19%), in 7/21 patients with polycythemia vera (33%) and in 12/15 patients with chronic idiopathic myelofibrosis (80%) (Figure 2). Significant endogenous megakaryocytic growth ( $>4$  CFU-Mega) was present in 6/15 patients with secondary thrombocytosis or erythrocytosis (30%), in 14/16 patients with essential thrombocythemia (88%), in 12/20

patients with polycythemia vera (60%) and in 13/14 patients with chronic idiopathic myelofibrosis (93%) (Figure 2). Endogenous growth of all three lineages was massive in chronic idiopathic myelofibrosis.

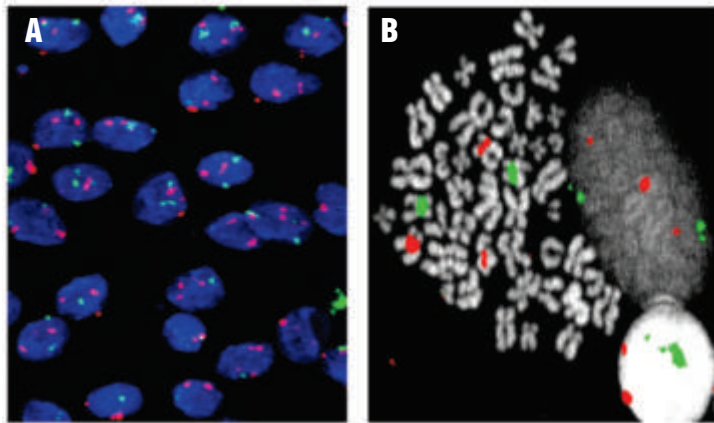
### Endothelial colony assays

Endothelial colony assays were performed using peripheral blood mononuclear cells from 18 healthy individuals and 13 patients with chronic idiopathic myelofibrosis. Significant numbers of endothelial colonies (Figure 3A) were obtained from 7/13 patients with chronic idiopathic myelofibrosis (54%), but from none of the 18 controls. The endothelial cell origin of these cells was confirmed by the presence of F-actin and vWF demonstrated by immunostaining (Figure 3B and 3C).

Endothelial cell cultures were also performed with sorted cells from a patient with polycythemia vera known to bear the *JAK2* V617F mutation. Endothelial colonies were obtained both from the endothelial precursor cell population ( $CD45^+/CD146^+$ ) and the  $CD14^+$  monocytic population. Both endothelial cell cultures were positive for the *JAK2* V617F mutation.



**Figure 3.** Endothelial colony assays **A.** A typical endothelial cell colony is shown. Immunostaining confirms the endothelial cell origin. **B.** Staining of F-actin. **C.** Staining of the vWF-containing granules.



**Figure 4.** *In situ* FISH of adherent (endothelial) cells. Red: Centromere 8; green: centromere 9; blue: DAPI chromosome counterstain: **A.** The majority of the interphase nuclei show three red centromere-8-specific signals. **B.** One metaphase and one interphase nucleus with trisomy 8 and another interphase nucleus with possibly only two chromosomes 8.

### JAK2 V617F mutation analysis

JAK2 V617F mutation analysis was performed in 8/15 patients with chronic idiopathic myelofibrosis (5 positive, 3 negative), 15/21 patients with polycythemia vera (13 positive, 2 negative), 11/17 patients with essential thrombocythemia (8 positive, 3 negative) and 8/17 patients with secondary erythrocytosis or thrombocytosis (8 negative).

### Cytogenetics

In one patient with chronic idiopathic myelofibrosis and known trisomy 8 (diagnosed 6 years earlier, in the unstimulated leukocytes from peripheral blood and in bone marrow), trisomy 8 was found in the *adherent* (endothelial) cells grown in the endothelial colony assays: 56% of the interphase nuclei (194/349 nuclei from four cultures) and 64% of the metaphases (25/39 metaphases from two cultures) had trisomy 8 (Figure 4). The constitutional karyotype, obtained from *non-adherent* cells reincubated with PHA for three days, was a normal 46,XX. The result of interphase FISH was consistent: 168 of 170 nuclei (98%) were disomic for chromosome 8 and only two nuclei were trisomic.

### Discussion

It has been shown that circulating progenitor cells are increased in the blood of patients with myeloproliferative diseases, i.e. mainly in patients with chronic idiopathic myelofibrosis and in a subset of patients with polycythemia vera.<sup>1-4</sup> CD34 expression was quantified in these studies by flow cytometry, and has recently been confirmed by quantitative PCR.<sup>4</sup> The present study confirms a massive (10- to 100-fold) increase of CD34 expression in the blood of 87% of patients with chronic idiopathic myelofibrosis using a quantitative PCR technique. A more moderate CD34 overexpression was seen in about one fourth (24%) of patients with polycythemia vera and essential thrombocytosis. The significance of such an increase is not yet known; it has been associated with a bad prognosis by certain groups but not by others.<sup>1-3,17</sup> This

issue deserves further investigation in larger prospective studies, and these patients are currently being followed at our clinic for signs of disease progression. The identification of the hemangioblast, the common precursor of the hematopoietic and endothelial cell lineages might shed new light on the pathophysiology of myeloproliferative disease. Firstly, hemangioblasts are known to express CD34, AC133, and VEGFR2 (KDR) and to be able to differentiate towards the hematopoietic and endothelial cell lineages when cultured in clonogenic stem cell assays.<sup>5-7</sup> Secondly, endothelial cell proliferation in the bone marrow and spleen of patients with myeloproliferative diseases is a frequent finding, particularly in patients with chronic idiopathic myelofibrosis and progressive essential thrombocytosis and polycythemia vera. However, whether endothelial proliferation is a secondary phenomenon (induced, for example, by cytokines) or is primarily caused by a defect at the level of hemangioblast is not known. Furthermore, as monocytes have recently been shown to be involved in endothelial repair, endothelial cell proliferation might be caused or aided by endothelial cells differentiated from monocytes.<sup>9,10</sup>

With the aim of investigating the presence of endothelial precursor cells in the blood of our patients, we quantified mRNA expression of CD34, CD133, KDR, and vWF and performed clonogenic progenitor cell assays with mononuclear cells from the same blood samples. Co-expression of the hemangioblast markers, CD34, CD133 and KDR, was seen in three patients (PV1, CIMF7 and CIMF12), suggesting the presence of endothelial progenitor cells among the circulating cells. This is consistent with the findings recently published by Massa *et al.*<sup>8</sup> Clonogenic stem cell assays showed differentiation towards both hematopoietic and endothelial cell lineages in about half of our patients with CIMF (54% of the patients tested), confirming the presence of endothelial precursor cells in their peripheral blood.

Cell sorting experiments showed that both the CD45<sup>+</sup>/CD146<sup>-</sup> cell fraction (progenitors) and the CD14<sup>+</sup> monocytic fraction produced endothelial cell colonies. These are preliminary experiments, and we are aware that CD146 may be aberrantly expressed on different types of

cells in the peripheral blood of cancer patients. Furthermore, the double staining of CD45<sup>+</sup> and CD146<sup>+</sup> cells does not ensure complete exclusion of monocyte contamination. Whether the endothelial cells are derived from progenitor cells or from monocytes (or both) cannot yet be answered and this issue needs well-conducted, prospective studies and further cell-sorting experiments to be resolved. However, trisomy 8 and the *JAK2* V617F mutation were found in the grown endothelial cells of patients in whom these molecular markers were diagnosed earlier in their peripheral blood. These results confirm the preliminary data from Bergamashi *et al.* and Barosi *et al.*, who demonstrated a clonal origin of endothelial cells in three patients with chronic idiopathic myelofibrosis and one patient with essential thrombocythemia.<sup>18,19</sup> Our findings provide strong evidence that the endothelial cells in subsets of patients with myeloproliferative diseases originate from the same pathologic clone as the hematopoietic cells, as they bear the same molecular markers. This possible primary involvement of the endothelial cell lineage in myeloproliferative diseases is important and worth further investigation, given the therapeutic potential of anti-endothelial drugs in such patients.

The results of the clonogenic stem cell assays confirmed the high specificity of CFU-E for the diagnosis of myeloproliferative diseases, as no false positive result was observed in the control groups.<sup>14</sup>

Although the analysis of CFU-Mega results was not the primary goal of this study, we observed that endogenous CFU-Mega colonies were present in 20% of the control group with secondary erythrocytosis or thrombocytosis, conferring this analysis a low specificity for the diagnosis of essential thrombocythemia. It is, however, interesting to note the growth pattern of the three lineages in the subtypes of myeloproliferative diseases as they appeared to be affected differently: patients with essential thrombocythemia showed less bi- or tri-lineage involvement than

did patients with polycythemia vera or chronic idiopathic myelofibrosis. Furthermore, patients with chronic idiopathic myelofibrosis showed massive numbers of endogenous colonies of all three lineages as well as endothelial cell colony growth, further suggesting a primary involvement of endothelial precursor cells in this disease.

In conclusion, we show that circulating CD34-expressing cells are increased in the blood of patients with myeloproliferative diseases, particularly in patients with chronic idiopathic myelofibrosis. In a subset of these patients, CD34-expressing cells also express the hemangioblast-specific markers prominin (CD133) and KDR (VEGFR2). Endothelial colony growth was obtained in parallel to hematopoietic growth in about half of the patients with chronic idiopathic myelofibrosis, and the presence of genetic markers (trisomy 8 and the *JAK2* V617F mutation) in both lineages confirmed the presence of endothelial and hematopoietic cells of the same clonal origin. This suggests a primary involvement of the endothelial cell lineage in the pathophysiology of a subset of myeloproliferative diseases. The exact pathway of differentiation (through differentiation of pathologic endothelial progenitors or differentiation of pathologic monocytes) remains to be elucidated in prospective cell-sorting studies.

*EOL: experimental design and supervision of molecular biology, critical revision of the manuscript; MPH: flow cytometry analysis and cell sorting, molecular biology, critical revision of the manuscript; CB: cell culture work; BP, DM: molecular biology; HH: FISH and classical cytogenetics; AT: supervision and critical revision of the manuscript; CZ: responsibility from conception to submitted manuscript, experimental design and supervision, manuscript preparation and revision, statistical analysis, management of clinical data. The authors declare that they have no potential conflicts of interest.*

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