

## Mesenchymal Stem Cells in Patients with Chronic Myelogenous Leukaemia or Bi-phenotypic Ph+ Acute Leukaemia are not Related to the Leukaemic Clone

S. WÖHRER<sup>1</sup>, W. RABITSCH<sup>1</sup>, M. SHEHATA<sup>2</sup>, R. KONDO<sup>2</sup>, H. ESTERBAUER<sup>3</sup>, B. STREUBEL<sup>4</sup>,  
C. SILLABER<sup>2</sup>, M. RADERER<sup>5</sup>, U. JAEGER<sup>2</sup>, C. ZIELINSKI<sup>5</sup> and P. VALENT<sup>2</sup>

<sup>1</sup>Department of Internal Medicine 1, Division of Bone Marrow Transplantation,

<sup>2</sup>Department of Internal Medicine 1, Division of Haematology and Haemostasiology,

<sup>3</sup>Clinical Institute of Medical and Chemical Laboratory Diagnostics, <sup>4</sup>Department of Pathology and

<sup>5</sup>Department of Internal Medicine 1, Division of Oncology, Medical University of Vienna, Vienna, Austria

**Abstract.** *Background:* Human mesenchymal stem cells (MSCs) are thought to be multipotent cells which primarily reside in the bone marrow. Besides their well-known ability to replicate as undifferentiated cells and to differentiate into diverse lineages of mesenchymal tissues, they were recently suggested to also give rise to haematopoietic and leukaemic/cancer stem cells. *In this study, the relationship between MSCs and leukemic stem cells in patients with either chronic myelogenous leukaemia (CML) or the more primitive variant, Ph+ bi-phenotypic leukaemia was investigated. Patients and Methods:* Cultured MSCs from 5 patients with CML and 3 patients with bi-phenotypic Ph+ leukaemia, all of them positive for BCR-ABL, were analysed with conventional cytogenetics, fluorescence in situ hybridisation (FISH) and polymerase chain reaction (PCR) for the presence of t(9;22) and BCR-ABL. MSCs were characterised phenotypically with surface markers (+CD73, +CD90, +CD105, -CD34, -CD45) and functionally through their potential to differentiate into both adipocytes and osteoblasts. *Results:* MSCs could be cultivated from seven patients. These cells were BCR-ABL negative when analysed with conventional cytogenetics and FISH. Further cytogenetic analysis revealed a normal set of chromosomes without any aberrations. Two patients were BCR-ABL-positive when analysed with PCR, probably as a result of MSC contamination with macrophages. *Conclusion:* MSCs in patients with CML or Ph+ bi-phenotypic leukaemia are not related to the malignant cell clone.

Human mesenchymal stem cells (MSCs), as defined by Pittenger *et al.*, are multipotent cells which primarily reside in the bone marrow. They have the ability to replicate as undifferentiated cells and the potential to differentiate into lineages of mesenchymal tissues, including bone, cartilage, fat, tendon, muscle and marrow stroma (1). Until recently, it was thought that their main role was to support the survival and lineage progression of haematopoietic stem cells (2).

One of the most stunning investigations on carcinogenesis, performed by Houghton and coworkers, has suggested that gastric carcinoma can originate from MSCs recruited from the bone marrow through chronic inflammation (3). Additionally, it was reported that cells within the MSC compartment, so-called multipotent adult progenitor cells (MAPCs), are not only capable of differentiating into mesenchymal cells, but may also develop into cells with visceral mesodermal, neuroectodermal and endodermal characteristics *in vitro* (4). When injected into an early blastocyst, single MAPCs contributed to most somatic cell types including haematopoietic cells (4).

These interesting features have raised the question whether MSCs might give rise to leukaemic stem cells. One typical example for a leukaemic stem cell disease is chronic myeloid leukaemia (CML). It is a myeloproliferative disease originating from multipotent HSCs which acquire the reciprocal translocation t(9;22)(q34;q11) resulting in the presence of the Philadelphia (Ph1) chromosome. This translocation intercalates portions of the *c-ABL* protooncogene in chromosome 9 with the *c-BCR* gene in chromosome 22, forming a hybrid *BCR-ABL* gene that encodes the BCR-ABL oncoprotein (5-7). Recent studies have suggested that CML stem cells might be more primitive stem cells than multipotent HSCs. Gunsilius *et al.* (8) have identified the *BCR-ABL* fusion gene in haemangioblastic-like cells and Fang *et al.* (9) found the *BCR-ABL* fusion gene in even more primitive progenitor cells defined as Flk1<sup>+</sup>, CD31<sup>-</sup>, CD34<sup>-</sup>. These findings,

*Correspondence to:* Stefan Wöhrer, MD, BC Cancer Centre, Terry Fox Laboratory, 675 West 10th Ave, Vancouver, BC, V5Z1L3, Canada. Tel/Fax: +1 604 675 7732, e-mail: swoehrer@bccrc.ca

**Key Words:** Chronic myelogenous leukaemia, mesenchymal stem cells, fusion protein, BCR-ABL gene, Philadelphia chromosome.

together with the aforementioned characteristics of MSCs, raised the question whether the *BCR-ABL* fusion gene can also be found in MSCs in patients with CML. Since Ph+ biphenotypic leukaemia may arise from even more immature stem cells than CML, the potential relation of such stem cells to MSCs would also be of interest. In fact, whereas some recent data suggest that MSCs in chronic phase CML may not display clonal markers of CML (10, 11), the more primitive variants of Ph+ neoplasms have not been investigated for such a relationship so far.

The objective of our study was to investigate if MSCs in patients with newly diagnosed CML or biphenotypic leukaemia harbour the *BCR-ABL* fusion gene or other chromosomal aberrations compatible with a possible relationship with the leukemic stem cell.

## Patients and Methods

**Patients.** Five patients with newly diagnosed CML as verified by molecular methods and cytogenetics and three patients with newly diagnosed *BCR-ABL*-positive bi-phenotypic leukaemia were included in the study. The patients' characteristics are summarised in Table I. After informed consent to participate in the study had been obtained from all patients, 5 ml of fresh bone marrow acquired by bone marrow biopsy were used for further study-specific analyses.

**Bone marrow preparation and cultivation of MSC.** Bone marrow mononuclear cells (BMNC) were isolated by Ficoll-Hypaque (Sigma Diagnostics, MO, USA) density gradient separation for 25 min at room temperature at 300xg. MSCs were cultured according to the protocol suggested by StemCell Technologies (StemCell Technologies, BC, Canada). One to  $1.5 \times 10^7$  BMNC were suspended in 7 ml MSC complete medium (StemCell Technologies), namely MSC basal medium (StemCell Technologies) and MSC Stimulatory Supplements (StemCell Technologies), and placed into T-25 cm<sup>2</sup> tissue culture treated flasks (TPP Tissue Culture, Trasadingen, Switzerland). Every medium described here contained additional 100 IU/mL penicillin/streptomycin. Cultures were maintained (weekly half medium change) in a humidified atmosphere at 37°C and 5% CO<sub>2</sub> until a confluent layer of about 80% was reached. The cells were then harvested with pre-warmed (37°C) trypsin, neutralized with fetal calf serum (FCS), washed with phosphate-buffered saline (PBS) and centrifuged for 7 min at room temperature at 300xg. The supernatant was then removed and the remaining cell pellet re-suspended in 21 ml MSC complete medium. The cell suspension was divided between three 25 cm<sup>2</sup> flasks, 7 ml each. This procedure was repeated until enough cells for the planned analyses were available, but only up to a maximum of three passages overall.

**Cell-surface analyses with FACS.** The culture-expanded MSCs were immediately labeled with monoclonal antibodies specific for the following surface molecules: CD90-PE (Stemcell Technologies), CD73-PE, CD105-PE (Pharmingen, CA, USA), CD34-PE, CD45-FITC (Becton Dickinson, NJ, USA). Species- and isotype-matched irrelevant antibodies were used as controls. Flow cytometry analysis of at least 5,000 events was performed using a FACScan (Becton

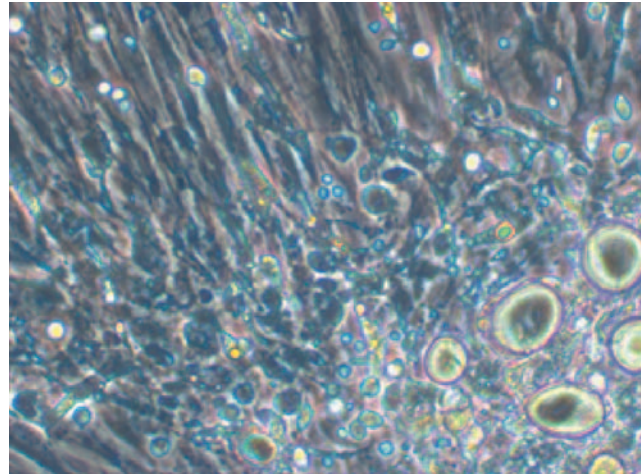


Figure 1. *Highly proliferative cell centre: Unstained image of a highly proliferative cell centre and the radiantly outgrowing MSCs before the first passage (day 7) (x60 magnification).*

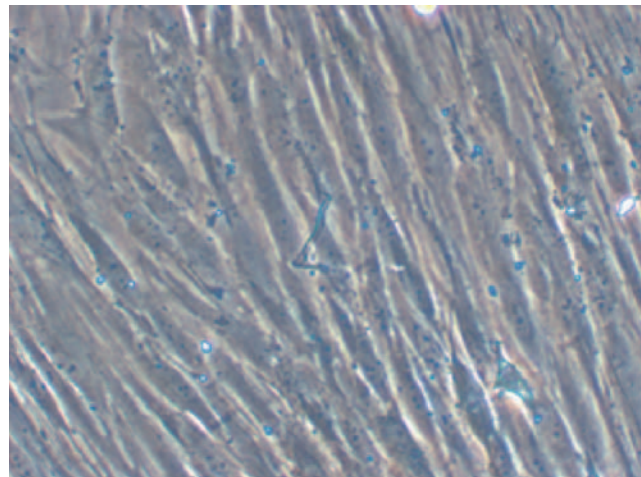


Figure 2. *Confluent mesenchymal stem cells: Unstained image showing the spindle-like MSCs growing in a confluent layer after the first passage (day 14) (x60 magnification).*

Dickinson) running CellQuest data acquisition and analysis software (Becton Dickinson). Forward scatter and light scatter were used for gating on staining cells and excluding cell debris.

**Cytogenetic analysis.** Cytogenetic analysis was performed after cell cultivation, chromosome preparation and staining by a modified GAG-banding technique as described elsewhere (12).

**Fluorescence in situ hybridisation (FISH) and reverse transcriptase-polymerase chain reaction (RT-PCR).** FISH was performed according to the manufacturers' instructions (Vysis, Downer's Grove, IL, USA) (13). Detection of the *BCR-ABL* fusion transcript by RT-PCR was performed using primers and methods described elsewhere (14, 15). The method has been thoroughly

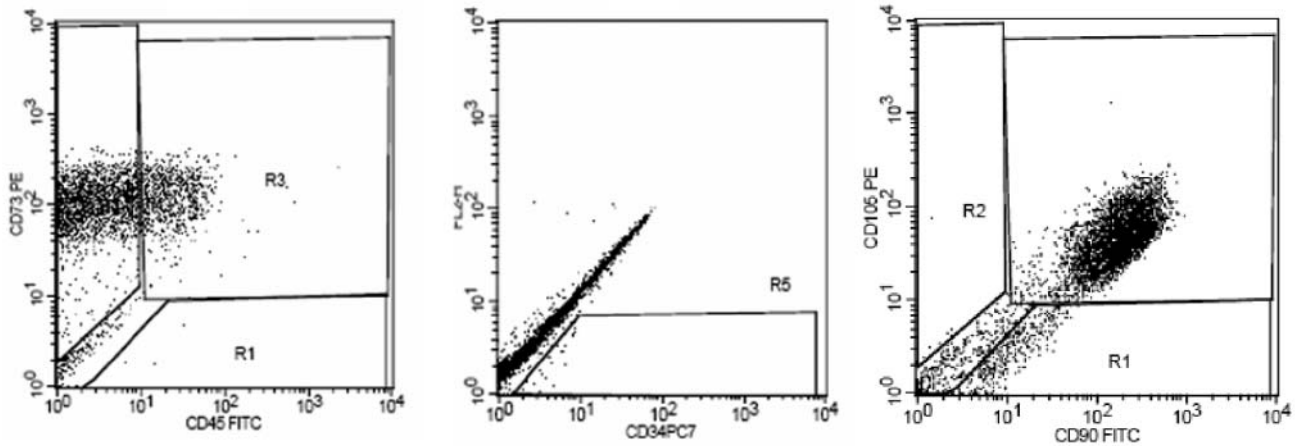


Figure 3. The fluorescence activated cell sorting (FACS) analysis shows the typical staining pattern for MSCs. Left panel: CD73+, CD45-; middle panel: CD34-; right panel: CD105+, CD90+.

evaluated for clinical use, and, due to its extraordinary high sensitivity, is still routinely used at the Clinical Institute of Medical and Chemical Laboratory Diagnostics of the Medical University of Vienna, Austria, to monitor BCR-ABL-positive leukaemias after various treatments, especially bone marrow transplantations. The method, which is based on a double-nested PCR design, reliably detects 1 BCR-ABL-positive cell in a background of  $>10^5$  normal cells.

**Differentiation assays.** To show the characteristic differentiation potential of MSCs, cells were cultured under conditions that were favourable for adipocytic or osteoblastic differentiation.

For adipocytic differentiation the medium consisted of alpha-MEM, 10% fetal calf serum (FCS), 10% horse serum (HS) and dexamethasone. For osteoblastic differentiation the medium consisted of alpha-MEM, 10% FCS, dexamethasone, ascorbic acid, and beta-glycerolphosphate. The medium was completely changed every fourth day until a confluent cell population with typical cell appearance was reached (3 to 5 weeks). Adipocytic differentiation was assessed microscopically and with RT-PCR for PPAR-gamma 1 and 2 as described elsewhere (11). Osteoblastic differentiation was assessed with RT-PCR as described elsewhere (16). Bone matrix mineralization was examined using Von Kossa staining (17).

The study was approved by the local ethical committee and conducted according to the Declaration of Helsinki.

## Results

In total, samples from 5 patients with newly diagnosed CML in chronic phase and 3 patients with bi-phenotypic Ph+ acute leukaemia (Table I) were analysed. All of these patients were untreated and positive for BCR-ABL as tested in peripheral blood cells using nested RT-PCR.

**Growth characteristics.** With the exception of patient 3, MSCs could be cultivated from all patients and showed a

dense growth after a median of 7 days. Normally, cells started to grow out of highly proliferative centres and spread radiantly throughout the culture (Figure 1). These centres were only seen before the first passage and disappeared afterwards (Figure 2). The MSCs could easily be expanded and were analysed after the third passage. Upon FACS analysis, cells were found to be highly positive for CD73, CD90 and CD105, and negative for CD34 and CD45 (Figure 3). Two samples showed a small CD45 positive cell population (Table I).

**Cytogenetic and FISH results.** Cultured cells showed no cytogenetic aberrations when analysed with conventional chromosomal staining and no BCR-ABL translocation when analysed with FISH.

**Detection of BCR/ABL in MSCs.** The BCR-ABL gene could only be detected in 2 patients with RT-PCR. Both of these patients showed a significant CD45-positive population in the FACS analysis. The remaining 5 patients showed no BCR-ABL signal.

**Functional characterization of MSCs.** The MSCs were capable of differentiating into adipocytes and osteoblasts under suitable conditions after a median culture time of 20 days. The adipocytes could be clearly discriminated morphologically and were positive for adipocyte-specific genes (PPAR-gamma1 and PPAR-gamma2). The osteoblasts were identified through the detection of osteoblast-specific genes (core-binding factor alpha-1 (CBFA-1) and collagen, type 1) with PCR. Calcification of osteoblasts and their surroundings was verified with a positive von Kossa staining reaction.



Table I. Patient characteristics.

Patient	Disease	Phase	BCR-ABL PB (PCR)	BCR-ABL MSCs (FISH)	BCR-ABL MSCs (PCR)	BCR-ABL MSCs (CG)	MSC CG
1	CML	CP	pos	neg.	pos.	neg.	norm.
2	CML	CP	pos	neg.	neg.	neg.	norm.
3	CML	CP	pos	n.a.	n.a.	n.a.	n.a.
4	CML	CP	pos	neg.	neg.	neg.	norm.
5	CML	CP	pos	neg.	neg.	neg.	norm.
6	BPL	-	pos	neg.	neg.	neg.	norm.
7	BPL	-	pos	neg.	pos.	neg.	norm.
8	BPL	-	pos	neg.	neg.	neg.	norm.

PB: peripheral blood, PCR: polymerase chain reaction, MSCs: mesenchymal stem cells, FISH: fluorescence *in situ* hybridisation, CG: conventional cytogenetic analysis, CML: chronic myelogenous leukaemia, BPL: bi-phenotypic acute leukaemia, CP: chronic phase, pos: positive, neg: negative, norm: normal, n.a.: not available.

## Discussion

Recent studies suggested that MSCs can give rise to cells from the haematopoietic system (4) as well as tumour stem cells (3). Based on these data, we hypothesized that MSCs might also give rise to leukaemic stem cells and therefore set out to evaluate the relationship of MSCs from patients with either CML or Ph+ bi-phenotypic acute leukaemia to the leukaemic clone.

Our results suggest that cultured MSCs in patients with *BCR-ABL*-positive CML do not harbour this specific translocation. This finding is in accordance with the results of previously published studies (10, 11). Only two samples were positive for *BCR-ABL* when analysed with a highly sensitive RT-PCR method typically used for detecting minimal residual disease CML patients. FACS analysis of these samples showed a small CD45-positive cell population which suggested a contamination of the MSC population with haematopoietic cells, most likely macrophages. This is in line with the observations by Bhatia *et al.* who described *BCR-ABL*-positive macrophages contaminating *in vitro* cultures of stromal cells from BM of CML patients.(18) Based on these observations, this group suggested that the abnormal stromal function in CML might in fact be due to the presence of *BCR-ABL*-positive macrophages in the marrow microenvironment which may contribute to the selective expansion of leukaemic HSCs (18). Interestingly, there was no sign of clonal cells bearing the translocation t(9;22) when analysed with conventional cytogenetics or FISH, which confirms the hypothesis of contamination with only a few macrophages. This result may be due to the higher *BCR-ABL* detection threshold of these methods.

In addition to the lack of the *BCR-ABL* gene, we did not find evidence for other cytogenetic abnormalities. Therefore, it is highly unlikely that MSCs are directly related to the leukaemic cells or play a role in the genesis

of CML. One possible explanation for our results could be the primitive nature of MSCs along with the hypothesis that genetic changes occur in more mature progenitor cells. In order to investigate this hypothesis, we have analysed MSCs from patients with *BCR-ABL*-positive bi-phenotypic leukaemias. This rare type of leukaemia is an acute leukaemia with the morphological, cytochemical and immunophenotypical features of the proliferating blasts lacking sufficient evidence to be classified as either of myeloid or lymphoid origin (19). The cell of origin is thought to be an immature multipotent progenitor stem cell. Bi-phenotypic acute leukaemias present with a high percentage of cytogenetic abnormalities and about one third of the cases have the Philadelphia chromosome (19). This latter feature makes leukaemic cells easily distinguishable from normal cells. We have hypothesized that the leukaemic stem cell clone of bi-phenotypic leukaemia might be more immature than the CML clone and might therefore be more closely related to the MSC. However, we also found no evidence that the MSCs from three patients with bi-phenotypic leukaemia might be related to the *BCR-ABL*-positive leukaemic cells.

In view of our results, one may conclude that leukaemic stem cells are not directly related to MSCs in patients with CML or bi-phenotypic leukaemia. As all patients analysed were newly diagnosed and had not undergone treatment, the disappearance of *BCR-ABL* as a treatment effect can effectively be excluded in all patients studied.

Several groups have demonstrated the persistence of host MSCs in patients after allogeneic stem cell transplantation (20, 21). This suggests that the conditioning regimens used for stem cell transplantation do not eradicate MSCs. Since high-dose chemotherapy followed by allogeneic stem cell transplantation is currently the only curative treatment approach to CML, this is also consistent with the hypothesis that MSCs do not give rise to leukaemic cells.

This finding could be of potential interest since MSCs are currently investigated as immunomodulating cells in the stem cell transplantation setting and as a therapeutic strategy to treat graft *versus* host disease (22). From our findings and similar recent observations (10, 11), it may be suggested that autologous MSCs can be used in this setting without running the risk of re-transplantation of leukaemic stem cells.

In the present study, we have analysed merely the relation of MSCs to leukaemic cells in terms of cellular origin. A possible functional interaction between these two cells, however, cannot be ruled out and thus remains to be determined in future studies.

## Acknowledgements

This investigation was supported by the Austrian Ministry for Education, Science and Culture (GENAU GZ200.136/1-VI/1/2005).

## References

- Pittenger MF, Mackay AM, Beck SC, Jaiswal RK, Douglas R, Mosca JD, Moorman MA, Simonetti DW, Craig S and Marshak DR: Multilineage potential of adult human mesenchymal stem cells. *Science* 284(5411): 143-147, 1999.
- Majumdar MK, Thiede MA, Mosca JD, Moorman M and Gerson SL: Phenotypic and functional comparison of cultures of marrow-derived mesenchymal stem cells (MSCs) and stromal cells. *J Cell Physiol* 176(1): 57-66, 1998.
- Houghton J, Stoicov C, Nomura S, Rogers AB, Carlson J, Li H, Cai X, Fox JG, Goldenring JR and Wang TC: Gastric cancer originating from bone marrow-derived cells. *Science* 306(5701): 1568-1571, 2004.
- Jiang Y, Jahagirdar BN, Reinhardt RL, Schwartz RE, Keene CD, Ortiz-Gonzalez XR, Reyes M, Lenvik T, Lund T, Blackstad M, Du J, Aldrich S, Lisberg A, Low WC, Largaespada DA and Verfaillie CM: Pluripotency of mesenchymal stem cells derived from adult marrow. *Nature* 418(6893): 41-49, 2002.
- Deininger MW, Goldman JM and Melo JV: The molecular biology of chronic myeloid leukemia. *Blood* 96(10): 3343-3356, 2000.
- Kabarowski JH and Witte ON: Consequences of BCR-ABL expression within the hematopoietic stem cell in chronic myeloid leukemia. *Stem Cells* 18(6): 399-408, 2000.
- Nowell PC and Hungerford DA: Chromosome studies on normal and leukemic human leukocytes. *J Natl Cancer Inst* 25: 85-109, 1960.
- Gunsilius E, Duba HC, Petzer AL, Kahler CM, Grunewald K, Stockhammer G, Gabl C, Dirnhofer S, Clausen J and Gastl G: Evidence from a leukaemia model for maintenance of vascular endothelium by bone-marrow-derived endothelial cells. *Lancet* 355(9216): 1688-1691, 2000.
- Fang B, Zheng C, Liao L, Han Q, Sun Z, Jiang X and Zhao RC: Identification of human chronic myelogenous leukemia progenitor cells with hemangioblastic characteristics. *Blood* 105(7): 2733-2740, 2005.
- Carrara RC, Orellana MD, Fontes AM, Palma PV, Kashima S, Mendes MR, Coutinho MA, Voltarelli JC and Covas DT: Mesenchymal stem cells from patients with chronic myeloid leukemia do not express BCR-ABL and have absence of chimerism after allogeneic bone marrow transplant. *Braz J Med Biol Res* 40(1): 57-67, 2007.
- Jootar S, Pornprasertsud N, Petvises S, Rerkamnuaychoke B, Disthabanchong S, Pakakasama S, Ungkanont A and Hongeng S: Bone marrow derived mesenchymal stem cells from chronic myeloid leukemia t(9;22) patients are devoid of Philadelphia chromosome and support cord blood stem cell expansion. *Leuk Res* 30(12): 1493-1498, 2006.
- Fonatsch C and Streubel B: Classical and molecular cytogenetics. Berlin: Springer; pp. 113-141, 1998.
- Sinclair PB, Nacheva EP, Leversha M, Telford N, Chang J, Reid A, Bench A, Champion K, Huntly B and Green AR: Large deletions at the t(9;22) breakpoint are common and may identify a poor-prognosis subgroup of patients with chronic myeloid leukemia. *Blood* 95(3): 738-743, 2000.
- Maurer J, Janssen JW, Thiel E, van Denderen J, Ludwig WD, Aydemir U, Heinze B, Fonatsch C, Harbott J, Reiter A *et al*: Detection of chimeric BCR-ABL genes in acute lymphoblastic leukaemia by the polymerase chain reaction. *Lancet* 337(8749): 1055-1058, 1991.
- Mitterbauer G, Fodinger M, Scherrer R, Knobl P, Jager U, Laczika K, Schwarzingier I, Gaiger A, Geissler K, Greinix H *et al*: PCR-monitoring of minimal residual leukaemia after conventional chemotherapy and bone marrow transplantation in BCR-ABL-positive acute lymphoblastic leukaemia. *Br J Haematol* 89(4): 937-941, 1995.
- Justesen J, Stenderup K, Eriksen EF and Kassem M: Maintenance of osteoblastic and adipocytic differentiation potential with age and osteoporosis in human marrow stromal cell cultures. *Calcif Tissue Int* 71(1): 36-44, 2002.
- Sheehan D and Hrapchak B: Theory and Practice of Histotechnology. 2nd ed. Ohil: Battelle Press; pp. 226-227, 1980.
- Bhatia R, McGlave PB, Dewald GW, Blazar BR and Verfaillie CM: Abnormal function of the bone marrow microenvironment in chronic myelogenous leukemia: role of malignant stromal macrophages. *Blood* 85(12): 3636-3645, 1995.
- Brunning R, Matutes E, Borowitz M, Flandrin G, Head D, Vardiman J and Bennett J: Acute leukaemias of ambiguous lineage. Lyon: IARC Press; pp. 106-107, 2001.
- Simmons PJ, Przepiorka D, Thomas ED and Torok-Storb B: Host origin of marrow stromal cells following allogeneic bone marrow transplantation. *Nature* 328(6129): 429-432, 1987.
- Hongeng S, Petvises S, Rerkamnuaychoke B, Worapongpaiboon S, Tardtong P, Apibal S and Ungkanont A: Host origin of marrow mesenchymal stem cells following allogeneic cord-blood stem-cell transplantation. *Int J Hematol* 74(2): 235-236, 2001.
- Le Blanc K: Mesenchymal stromal cells: tissue repair and immune modulation. *Cytotherapy* 8(6): 559-561, 2006.

Received May 11, 2007

Revised October 17, 2007

Accepted October 22, 2007