Cell. Mol. Biol. 57 (supp): OL1456-OL1461
Published on January 1, 2011; DOI 10.1170/166

HYPOXIA FACILITATES CANCER ASSOCIATED CELL MARKER EXPRESSION IN STEM CELLS

L. XU[1], H. XIAO[2], M. XU[1]✉, L. YI[1] AND H. LIANG[1]

1 Department of Neurosurgery, Daping Hospital, Third Military Medical University, Chongqing, China
2 Department of Pathology, Daping Hospital, Third Military Medical University, Chongqing, China

Abstract

How normal body cells differentiate to cancer cells is not clear. The present study aimed to investigate the mechanism by which hypoxia drives the expression of cancer associated cell markers in stem cells. In this study, mouse bone marrow mononuclear cells were prepared and cultured under hypoxic environment. Rate of caner associated cell markers on stem cells was determined by flow cytometry. Inflammatory cytokine levels in culture supernatant were determined by enzyme-linked immunoassay. The results showed that after cultured under hypoxic environment for 48 h, the cancer associated cell markers increased significantly in stem cells. IL-1beta levels increased markedly after cultured in hypoxia. Macrophages were identified as the major source of IL-1beta. Blocking IL-1beta abolished the differentiation of cancer associated cell markers in stem cells. We conclude that hypoxia can increase aberrant expression of IL-1beta in macrophages that further facilitates the expression of cancer associated cell markers in stem cells.

Key words: Cancer; Stem cell; Hypoxia; Interleukin-1; macrophage.

Article information's

Received on May 31, 2010
Accepted on December 4, 2010

Corresponding author

Dr Minhui. Xu, Department of Neurosurgery, Daping Hospital, Third Military Medical University, 10 Changjiang Zhilu, Chongqing, 400042,China
Fax: 023-68757977
E-mail: minhuixu6@163.com

Abbreviations: **Mφ**: macrophages; **ELISA**: Enzyme-linked immuno-assay; VWF: Von Willebrand Factor; P-gp: P-glycoprotein; **TIMP-1**: Tissue inhibitor of metalloproteinases-1; **GM-CSF**: Granulocyte macrophage colony-stimulating factor.

INTRODUCTION

Cancer is one of the major diseases to threaten human life and compromise the life quality. The treatment of cancer is not satisfactory currently. Thus, it is urgent to have new ideas to understand the pathogenesis of cancer and develop novel therapeutic remedies for the treatment of cancer.

So far the mechanism by which naïve stem cells transform into cancer stem cells has not been fully understood. A small subpopulation of cell is designated as side population that features weakly staining and expresses CD133. Among the cancer-associated cell markers, CD133 (also named as prominin-1) is one of the most important and well studied cell markers. It is a 120 kDa, 5-transmembrane-domain glycoprotein, with 2 cytoplasmic loops, 2 glycosylated extracellular domains, and a cytoplasmic C-terminal domain (5). Cumulative reports indicate many body cells express CD133; however, its

physiological function is unknown. $CD133^+$ cancer cells are more radiation therapy resistant (2). This subpopulation cell has the tendency to differentiate to cancer stem cells (10, 23). However, little is known how naïve stem cells become $CD133^+$ stem cells.

Because of its essential role in many biological processes, oxygen level is tightly regulated at the tissue and cellular levels. Hypoxia can result in the production of reactive oxygen species in local tissue, which may cause genotoxic effects or cell death. Hypoxia is commonly present in tumor tissue because of the chaotic vascular architecture and regions of necrosis (14). Recent experimental evidence has expanded on the role of hypoxia in cancer by demonstrating differential responses to hypoxia between heterogenic sub-populations within the tumor. One of these populations, termed cancer stem cells, possess many phenotypic similarities to normal stem cells, such as having the ability to upregulate DNA repair kinases to evade radiation-induced genomic damage (1). However, how these cancer stem cells develop in tumor tissue remains to be further understood.

Tumor-associated macrophages (Mφ) have emerged as a critical component of the inflammatory microenvironment in tumors linked with tumor progression (17). Mφ number is increased in tumor tissue (12). It is believed that Mφ plays a critical role in tumor growth and progression (13). Inflammatory cells and inflammatory cytokines in local tissue also play roles in tumor differentiation and tumor growth (5). However, the precise mechanisms that maintain the tumor-associated Mφ are poorly understood. Here, we hypothesized that hypoxic environment modifies Mφ's properties that further facilitate the cancer associated cell marker expression.

MATERIALS AND METHODS

Reagents

Antibodies against CD133 (N-17), IL-1β (C-20), P-gp (K-16), TIMP-1 (MM-2), Ki67 (M-19), F4/80 (BM8) were purchased from Santa Cruz Biotec (Santa Cruz, CA). ELISA kits IL-1β (sensitivity: 1 pg/ml), IL-4 (sensitivity: 10 pg/ml), IL-6 (sensitivity: 0.7 pg/ml), IFN-γ (sensitivity: 8 pg/ml), TNF (sensitivity: 0.4 pg/ml) and neutralizing anti-IL-1β were purchased from R&D systems (Shanghai, China). Histopaque, insulin and epithelial growth factor were purchased from Sigma Aldrich (Shanghai, China).

Mice and bone marrow-derived mononuclear (BmMo) cell culture

Balb/c mice (male, 6-8 weeks old) were purchased from Beijing Animal Institute. The bone marrow was collected from mice. The procedures were approved by the animal research ethic committee at the Third Military Medical University. Single-cell suspensions of bone marrow cells were obtained by flushing the femurs of mice. BmMo were isolated by gradient density centrifugation. Cells were cultured in RPMI1640 media containing 10% fetal cow serum, 2 mM L-glutamine, 1 mM sodium pyruvate, 50 μM 2-ME, penicillin, and streptomycin. After the fifth week of culture, cells were treated under hypoxia (1% O_2) or normoxia for 48 h.

Spheres Culture

Single BmMo cells were plated at 100 cells/well on 96-well plates with an ultralow attachment surface (Fisher Scientific Co.). Cells were grown in serum-free Dulbecco's Modified Eagle's Medium supplemented with 4 μg/mL insulin and 20 ng/mL epithelial growth factor. Spheres were collected after 7 to 14 days. Before using for flow cytometry, spheres were treated with peptizing (sucking in and blowing out) to be single cell suspension and filtered with a cell strainer (0.1 mm).

Hypoxia-treated stem cell transplantation

$CD34^+$ Stro-1^+ $CD133^+$ cells treated with hypoxia or BmMo treated with normoxia (in which the $CD34^+$ Stro-1^+ $CD133^+$ cells were too few and unable to be isolated) were inoculated subcutaneously into the flanks of nude mice (5000 cells per mouse). Mice were monitored for tumor growth everyday by palpation on the sites. After sacrifice, tumor like tissue or tissue from transplantation sites was processed for H&E staining and observed by an anatomical pathologist.

Flow cytometry

Cells were stained with fluorescence labeled primary antibodies (1:100~200 in dilution) on ice for 30 min and analyzed by flow cytometry (FACSArray, BD Bioscience). For cells labeled with multiple antibodies, gating technique was applied following published procedures (25). Isotype IgG control staining was applied for each staining.

Enzyme-linked immunoassay (ELISA)

Cytokine levels in culture supernatant were determined by ELISA with commercial reagent kits and followed the manufacturer's instruction.

Statistics

Data were expressed as means ± SD. The values were analyzed using the two-tailed unpaired Student's t-test when data consisted of two groups or by ANOVA when three or more groups were compared. $P<0.05$ was set as the statistically significant criteria.

RESULTS

Hypoxia drives bone marrow mesenchymal stem cells to express cancer associated cell markers

To test the hypothesis that hypoxia might favor some specific cell marker expression

(that are expressed highly in some malignant tumors such as glioma), we cultured BmMo-derived sphere cells under the hypoxic environment for 48 h and examined the specific cell markers by flow cytometry. The results showed that marked more CD133 was observed in hypoxia-treated stem cells ($CD34^+$ $Stro\text{-}1^+$ cells) (Fig.1A) than those treated by normoxia (Fig.1B). In addition, several other specific cell markers (8), including Von Willebrand Factor (VWF), P-glycoprotein (P-gp), tissue inhibitor of metalloproteinases-1 (TIMP-1) and Ki67 were also observed in $CD133^+$ cells from hypoxia treated stem cells, but not in those treated with normoxia (Fig.1). The data suggest that hypoxic environment can facilitate the expression of several specific cell markers in stem cells that are highly expressed in cancer cells.

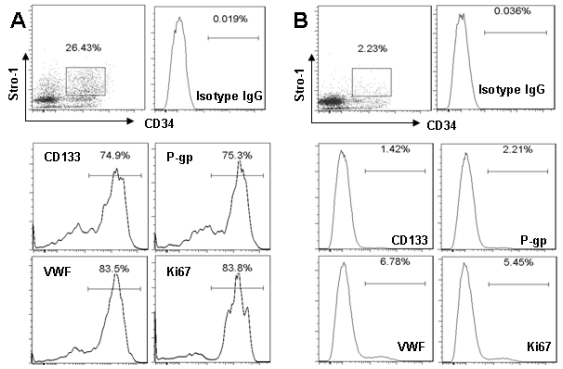

Figure 1. Hypoxia facilitates the expression of specific cell markers in stem cells. Mouse bone marrow mononuclear cells (BmMo) were prepared and cultured under hypoxia (A) or normoxia (B) for 48 h. Cell markers in the cells were examined by flow cytometry. The dot plots in A and B show $CD34^+$ $Stro\text{-}1^+$ stem cells (the gated cell population). The histograms in A and B indicate the gated stem cells also express several specific cell markers (annotated in each histogram). The panel of isotype IgG shows negative staining control. Experiments were repeated 3 times.

Hypoxia-treated stem cells have the tumorigenicity

Next, we subcutaneously injected isolated hypoxia-treated $CD34^+$ $Stro\text{-}1^+$ $CD133^+$ cells or normoxia-treated BmMo cells to nude mice. The formation of tumor was observed in all 6 mice injected with $CD34^+$ $Stro\text{-}1^+$ $CD133^+$ cells (Fig.2A-B) whereas none of the mice injected with normoxia-treated BmMo formed tumor. The neoplastic cells also expressed the cell markers as checked before transplantation (Fig.2C). To see if the neoplastic cells from mice still have tumorigenicity, we collected the neoplastic mass from mice, cultured for one week and re-transplanted to nude mice. Indeed, neoplasm grew in all the 6 recipient mice (Fig.2B).

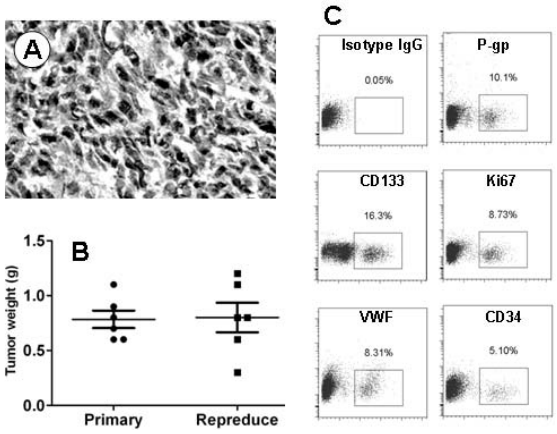

Figure 2. Hypoxia-treated stem cells form tumor in mice. Hypoxia-treated stem cells were prepared as detailed in figure 1. The cells were s.c. injected into 6 nude mice. Tumor growth was monitored until 4 months. After sacrifice, the tumor was removed, weighed and examined by histology and flow cytometry. A, histological image shows tissue structure of transplanted tumor. Magnification: ×200. B, scatter dot plots show the tumor weight from individual mouse. Primary: Neoplasm was collected from mice received hypoxia-generated $CD133^+$ cell transplantation. Reproduce: Neoplasm was collected from mice received primary mice-derived $CD133^+$ cells. C, flow cytometry dot plots show specific cell markers in transplanted tumor cells.

Macrophage (Mφ) is involved in the expression of CD133 in stem cells

Published data indicate that Mφ plays a critical role in tumor growth (9). Indeed, as shown by flow cytometry, close to 5% cell population was $F4/80^+$ Mφ in cultured BmMo cells (Fig.3A-B). We then predicted that these Mφs might be responsible for the expression of cancer associated cell markers such as CD133 in stem cells. To prove the hypothesis, we depleted $F4/80^+$ Mφs from isolated BmMo cells by the addition of anti-F4/80 antibody (500 ng/ml) and cultured under hypoxia for 48 h (the F4/80 cells were completely depleted at the end of culture as examined by flow cytometry; data not shown). The data implicate that Mφ plays a crucial role in the expression of cancer associated cell markers in hypoxia-treated stem cells.

Mφ-derived IL-1β facilitates the expression of CD133 in stem cells

NFκB is a master regulator for a series of inflammatory cytokines that can be

upregulated by hypoxia (20), some inflammatory cytokines have a close relation with the pathogenesis of cancer, such as IL-1 (4) and IL-6 (15). We next observed that high level of IL-1β was detected in culture supernatant of BmMo cells treated with hypoxia that was significantly higher than those treated with normoxia (Fig.4A). Further analysis revealed that F4/80$^+$ Mφ had high levels of IL-1β expression (Fig.4B). Based on the results, we considered that IL-1β might play a critical role in facilitating the expression of CD133 in stem cells. In separate experiments, a batch of BmMo cells was treated with hypoxia as well as neutralizing anti-IL-1β antibody (2 μg/ml) for 48 h. Indeed, very few CD133$^+$ cells were induced (Fig.4C). The results indicate that hypoxia initiates the expression of IL-1β in Mφ and the latter triggers naïve stem cells to express CD133 to become cancer stem cells.

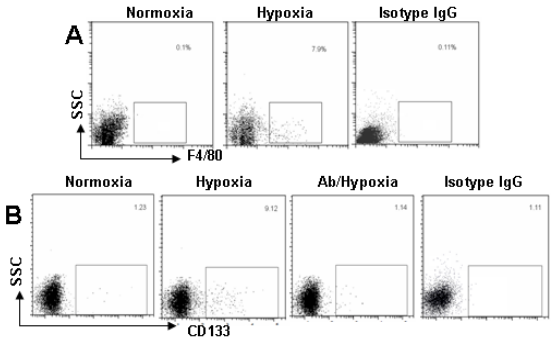

Figure 3. Mφ plays a role in hypoxia-induced CD133 in stem cells. BmMo cells were prepared and treated with normoxia (A1) or hypoxia (A2) as described in figure 1. A, flow cytometry dot plots show F4/80$^+$ cells (the gated cell population). B, BmMo cells were cultured under normoxia or hypoxia in the presence or absence of anti-F4/80 antibody (500 ng/ml) for 48 h. The gated cells are CD133$^+$ cells. The treatments were annotated above each plot. Data represent 3 separate experiments.

DISCUSSION

How normal cells become cancer cells is unknown. The present study provides a set of novel data that hypoxic environment drives Mφ to produce IL-1β; the latter triggers naïve stem cells to express a series of specific cell markers, such as CD133, which have been reported in several malignant tumors (2, 10, 23).

Stem cells are cells found in all multi cellular organisms. They are characterized by the ability to renew themselves through mitotic cell division and differentiate into a diverse range of specialized cell types (24). The bone marrow is the largest reservoir of adult stem cells in the body. Bone marrow-derived stem cells can differentiate to different cell types under certain environment (24), such as exposure to granulocyte macrophage colony-stimulating factor (GM-CSF), the stem cells may differentiate to dendritic cells in culture (6). The present study has added novel information that stem cell may be transformed to the cell that has tumorigenecity manifesting as expressing a series of specific markers that are expressed in tumors (18).

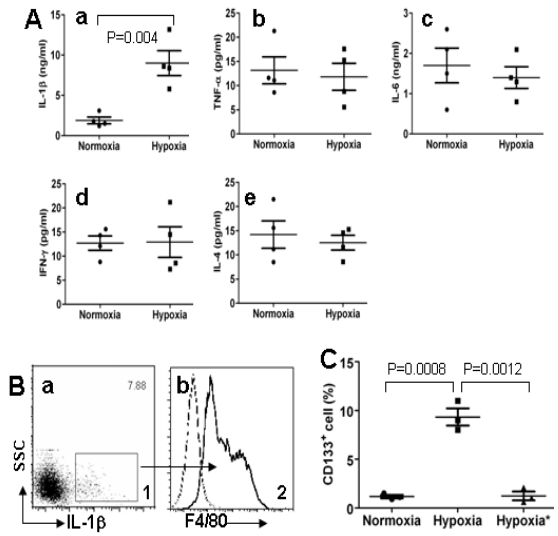

Figure 4. Hypoxia induces Mφ to produce IL-1β. BmMo cells were prepared and treated with hypoxia or normoxia as described in figure 1. A, scatter dot plots indicate the levels of several cytokines in supernatant that were determined by ELISA. Each dot represents individual datum. B, the cells were also analyzed by flow cytometry. Ba indicates IL-1β$^+$ cell population (the gated cells). Bb, the gated cells in B1 were further analyzed for F4/80 expression. Histograms show F4/80$^+$ cells (right histogram). The left histogram show isotype IgG staining control. C, scatter dot plots show CD133+ cells in BmMo cells after cultured under normoxia or hypoxia in the presence (*) or absence of anti- IL-1β antibody (2 μg/ml).

Epidemiological studies indicate that patients with chronic inflammation predispose to different cancers via mechanisms of promoting all stages of tumor development through multiple mechanisms which include enhanced proliferation and resistance to apoptosis of initiated cells, induction of DNA mutations, promotion of angiogenesis, invasion and metastasis. (21); administration with non-steroid anti-inflammatory agents has anti-tumor effect (16). Mφ is one of the major

inflammatory cells. It is proposed that cancer cells can recruit Mφ to cancer tissue to support tumor growth. Hypoxic environment has the chemotactic effect on recruiting Mφ from the peripheral blood stream (3). The present study provides further evidence that in hypoxic environment, some of the stem cells can become Mφ; the latter further facilitate the differentiation of cancer stem cells.

Our results demonstrate that naïve stem cells may transform into cancer stem cells under certain environment. BmMo were employed in our experimental system, some of the stem cells became cancer stem cells under the hypoxic environment. The fact implicates that at least a part of the cancer stem cells in tumor tissue are differentiated from metechymal stem cells because (i) the environment in tumor tissue meets the requirement of "hypoxia"; (ii) metechymal stem cells can reach tumor tissue via blood stream. Indeed, further evidence of this study demonstrates that the induced cancer stem cells have the capacity to form tumor in mice.

Now that we have revealed that Mφ has the capability to drive naïve stem cells to become $CD133^+$ cells in the present study. Logically, we need to answer the question what confers Mφ the capability to trigger the expression of $CD133^+$ in naïve stem cells. Hypoxia is assumed being critical in the process. The postulation is supported by the data that hypoxic environment increases the expression of IL-1β in Mφ. IL-1β is a proinflammatory cytokine; it is usually produced by Th1 cells and plays a critical role in immune inflammation such as inflammatory bowel disease (22). Over-expression of inflammatory cytokine is proposed to be one of the factors contributing to the pathogenesis of cancer (7). Others have noted that IL-1β is abundant in tumor sites (19). Although the inflammatory cytokines found in tumor sites can be different explanations including (i) it is an active action of body fighting against tumor and (ii) inflammatory cytokines facilitate tumor cell differentiation. Previous studies indicate that IL-1 has a close relationship with lung cancer (11). Our data support the notion of inflammatory cytokines facilitate tumor cell differentiation by providing evidence that IL-1β plays a critical role in cancer stem cell differentiation from naïve stem cells.

In summary, the present study provides the evidence that hypoxic environment induces naïve stem cells to differentiate to $CD133^+$ cancer stem cells, in which Mφ-derived IL-1β plays a critical role.

REFERENCES

1. Bao, S., Wu, Q., McLendon, R.E., Hao, Y., Shi, Q., Hjelmeland, A.B., Dewhirst, M.W., Bigner, D.D. and Rich, J.N. Glioma stem cells promote radioresistance by preferential activation of the DNA damage response. *Nature* 2006, **444**:756-60.

2. Blazek, E.R., Foutch, J.L. and Maki, G. Daoy medulloblastoma cells that express CD133 are radioresistant relative to CD133- cells, and the CD133+ sector is enlarged by hypoxia. *Int. J. Radiat. Oncol. Biol. Phys*. 2007, **67**:1-5.

3. Bosco, M.C., Puppo, M., Blengio, F., Fraone, T., Cappello, P., Giovarelli, M. and Varesio, L. Monocytes and dendritic cells in a hypoxic environment: Spotlights on chemotaxis and migration. *Immunobiology* 2008, **213**:733-49.

4. Christensen, K., Aaberg-Jessen, C., Andersen, C., Goplen, D., Bjerkvig, R. and Kristensen, B.W. Immunohistochemical expression of stem cell, endothelial cell, and chemosensitivity markers in primary glioma spheroids cultured in serum-containing and serum-free medium. *Neurosurgery* 2010, **66**:933-47.

5. Corbeil, D., Röper, K., Hellwig, A., Tavian, M., Miraglia, S., Watt, SM., Simmons, P.J., Peault, B., Buck, D.W., Huttner, W.B. The human AC133 hematopoietic stem cell antigen is also expressed in epithelial cells and targeted to plasma membrane protrusions. *J. Biol. Chem*. 2000; **275**, 5512-20.

6. Feng, B.S., Zheng, P.Y., Chen, X., Liao, X.Q. and Yang, P.C. Investigation of the role of cholera toxin in assisting the initiation of the antigen-specific Th2 response. *Immunol. Invest*. 2008, **37**:782-97.

7. Keita, M., Bessette, P., Pelmus, M., Ainmelk, Y. and Aris, A. Expression of interleukin-1 (IL-1) ligands system in the most common endometriosis-associated ovarian cancer subtypes. *J. Ovarian Res*. 2010, **3**:3.

8. Kurokawa, T., Miyamoto, M., Kato, K., Cho, Y., Kawarada, Y., Hida, Y., Shinohara, T., Itoh, T., Okushiba, S., Kondo, S. and Katoh, H. Overexpression of hypoxia-inducible-factor 1alpha(HIF-1alpha) in oesophageal squamous cell carcinoma correlates with lymph node metastasis and pathologic stage. *Br. J. Cancer* 2003, **89**:1042-7.

9. Le, Q.T., Denko, N.C., Giaccia, A.J. Hypoxic gene expression and metastasis. *Cancer Metastasis Rev*. 2004, **23**:293-310.

10. Liang, Y., Zhong, Z., Huang, Y., Deng, W., Cao, J., Tsao, G., Liu, Q., Pei, D., Kang, T. and Zeng, Y.X. Stem-like cancer cells are inducible by increasing genomic instability in cancer cells. *J. Biol. Chem*. 2010, **285**:4931-40.

11. Lind, H., Zienolddiny, S., Ryberg, D., Skaug, V., Phillips, D.H. and Haugen, A. Interleukin 1 receptor antagonist gene polymorphism and risk of lung cancer: a possible interaction with polymorphisms in the interleukin 1 beta gene. *Lung Cancer* 2005, **50**:285-90.

12. Mancino, A. and Lawrence, T. Nuclear factor-kappaB and tumor-associated macrophages. *Clin. Cancer Res*. 2010, **16**:784-9.

13. Mantovani, A., Schioppa, T., Porta, C., Allavena, P. and Sica, A. Role of tumor-associated macrophages in tumor progression and invasion. *Cancer Metastasis Rev*. 2006, **25**:315-22.
14. Mazumdar, J., Dondeti, V. and Simon, M.C. Hypoxia-inducible factors in stem cells and cancer. *J. Cell Mol. Med*. 2009, **13**:4319-28.
15. Ojalvo, L.S., King, W., Cox, D. and Pollard, J.W. High-density gene expression analysis of tumor-associated macrophages from mouse mammary tumors. *Am. J. Pathol*. 2009, **174**:1048-64.
16. Owegi, H.O., Egot-Lemaire, S., Waite, L.R. and Waite, G.N. Macrophage activity in response to steady-state oxygen and hydrogen peroxide concentration - biomed 2010. *Biomed. Sci. Instrum*. 2010, **46**:57-62.
17. Pollard, J.W. Tumour-educated macrophages promote tumour progression and metastasis Nat. Rev. *Cancer* 2004, **4**:71-8.
18. Prestegarden, L., Svendsen, A., Wang, J., Sleire, L., Skaftnesmo, K.O., Bjerkvig, R., Yan, T., Askland, L., Persson, A., Sakariassen, P.Ø. and Enger, P.Ø. Glioma cell populations grouped by different cell type markers drive brain tumor growth. *Cancer Res*. 2010, **70**:4274-9.
19. Puchalski, T., Prabhakar, U., Jiao, Q., Berns, B. and Davis, H.M. Pharmacokinetic and Pharmacodynamic Modeling of an Anti-Interleukin-6 Chimeric Monoclonal Antibody (Siltuximab) in Patients with Metastatic Renal Cell Carcinoma. *Clin. Cancer Res*. 2010, 16: 1652-61.
20. Seidel, S., Garvalov, B.K., Wirta, V., von Stechow, L., Schänzer, A., Meletis, K., Wolter, M., Sommerlad, D., Henze, A.T., Nistér, M., Reifenberger, G., Lundeberg, J., Frisén, J. and Acker, T. A hypoxic niche regulates glioblastoma stem cells through hypoxia inducible factor 2 alpha. *Brain* 2010, **133**:983-95.
21. Sgambato, A. and Cittadini, A. Inflammation and cancer: a multifaceted link. *Eur. Rev. Med. Pharmacol. Sci.* 2010,14:263-8.
22. Siegmund, B. Interleukin-1beta converting enzyme (caspase-1) in intestinal inflammation. *Biochem. Pharmacol*. 2002, **64**:1-8.
23. Tabu, K., Kimura, T., Sasai, K., Wang, L., Bizen, N., Nishihara, H., Taga, T. and Tanaka, S. Analysis of an alternative human CD133 promoter reveals the implication of Ras/ERK pathway in tumor stem-like hallmarks. *Mol. Cancer* 2010, **9**:39.
24. Tárnok, A., Ulrich, H. and Bocsi, J. Phenotypes of stem cells from diverse origin. *Cytometry A*. 2010, **77**:6-10.
25. Zhao, C.Q., Li, T.L., He, S.H., Chen, X., An, Y.F., Wu, W.K., Zhou, X.H., Li, P. and Yang, P.C. Specific immunotherapy suppresses Th2 responses via modulating TIM1/TIM4 interaction on dendritic cells. *Allergy* 2009, **65**:986-95.

