Control of leucocyte differentiation from embryonic stem cells upon vasculogenesis and confrontation with tumour tissue

Madeleine Hannig a, Hans-Reiner Figulla a, Heinrich Sauer b, Maria Wartenberg a

a Clinic of Internal Medicine I, Cardiology Division, Friedrich Schiller University, Jena, Germany
b Department of Physiology, Faculty of Medicine, Justus Liebig University, Giessen, Germany

Received: March 10, 2008; Accepted: June 20, 2008

Abstract

Embryonic stem (ES) cells spontaneously differentiate capillary-like structures as well as leucocytes such as monocytes/macrophages, neutrophils, natural killer (NK) cells and cytotoxic T lymphocytes. The interplay between vasculogenesis and leucocyte differentiation as well as the population of tumour tissues with ES cell-derived leucocytes and endothelial cells is, however, not sufficiently specified. In the present study, gene expression of the cell surface markers CD68 and CD14 (expressed on monocytes and macrophages), Mac-1 (CD11b) (expressed on granulocytes, monocytes and NK cells) and CD16 (expressed on neutrophils) was investigated in murine CGR8 ES cells in relation to the endothelial cell markers CD31 and vascular endothelial (VE)-cadherin. Expression of leucocyte markers increased from day 7–8 of cell culture on. Furthermore, addition of macrophage colony-stimulating factor to the cell culture medium resulted in a threefold increase in the number of CD68+ monocytes/macrophages. Treatment of embryoid bodies with lipopolysaccharide (LPS) up-regulated CD14 thus suggesting functionality of the CD14 LPS receptor. Differentiation of vascular structures positive for CD31 and VE-cadherin preceded leucocyte differentiation by 2 days (i.e. from day 5–6 on) suggesting that vasculogenesis may be a determinant of leucocyte differentiation. Consequently the Fik-1 antagonist SU5416 which inhibits vasculogenesis of ES cells significantly blunted leucocyte differentiation. Confrontation culture of embryoid bodies with multicellular breast tumour spheroids initiated significant increase of leucocyte cell numbers and invasion of leucocytes into the tumour tissue. In summary our data demonstrate that during ES cell differentiation vasculogenesis precedes leucocyte differentiation, and point towards the direction that leucocyte cell invasion into tumour tissue may initiate the pro-inflammatory microenvironment necessary for tumour vascularization.

Keywords: embryonic stem cells • tumour angiogenesis • haematopoiesis, leucocytes • confrontation culture

Introduction

It is commonly accepted that tumour angiogenesis is a prerequisite for tumour growth. Angiogenesis is stimulated by angiogenic factors released by tumour cells, though other cells, such as tumour-associated macrophages, neutrophils, natural killer (NK) cells and dendritic cells also contribute towards increasing the angiogenic process in cancer [1]. Current opinion holds that native immune cells recruited into tumours in turn stimulate the endothelium and are responsible for an indirect pathway of tumour vascularization [2], which is based on the observation that tumours are infiltrated by different leucocyte cell types. It is now becoming clear that the tumour microenvironment is largely orchestrated by inflammatory cells, which participate in the neoplastic process, fostering proliferation, survival and migration [3]. However, it is not known whether leucocyte cell types could arise from endothelial cells during their invasion into the tumour mass.

Differentiating embryonic stem (ES) cells are increasingly emerging as an important source of haematopoietic progenitors with a potential to be useful for both basic and clinical research applications. During recent years it has been shown that pluripotent ES cells are capable to differentiate into different haematopoietic lineages including leucocyte subtypes e.g. monocytes/macrophages [7], T lymphocytes [8], NK cells [9], neutrophil cells [10] and dendritic cells [11]. It is meanwhile generally accepted that the haematopoietic cell lineage and the endothelial cell lineage derive from a common precursor the haemangioblast [12]. In mice, cell aggregates consisting of mesodermal cells originating from the
Materials and methods

Materials

Lipopolysaccharide (LPS) from E. coli K-235 was provided by Sigma-Aldrich (Taufkirchen, Germany) and used in concentrations of 0.1, 0.1, 10 and 100 ng/ml. Recombinant murine macrophage colony-stimulating factor (M-CSF) was obtained from CellSystems (St. Katharinen, Germany) and used in concentration of 250 ng/ml.

ES cell culture

The ES cell line CGR8 was obtained from the European Collection of Cell Cultures (ECACC). Originally, the germ-line competent cell line CGR8 was established from the inner cell mass of a 3.5-day male pre-implantation mouse embryo (Mus musculus, strain 129). The CGR8 cell line was cultured on gelatine-coated cell culture dishes in Glasgow minimal essential medium (GMEM; Sigma) to keep the cells in stem cell stage for proliferation. Medium was supplemented with 9% heat-inactivated (56 °C, 30 min.) foetal bovine serum (FBS, Sigma), 2 mM L-glutamine (Biochrom, Berlin, Germany), 45 μM β-mercaptoethanol (Sigma) and 102 U/ml leukaemia inhibitory factor (LIF) (Chemicon, Hampshire, UK) in a humidified environment containing 5% CO2 at 37 °C and passed every 2–3 days. At day 0 of differentiation, adherent cells were enzymatically dissociated using 0.25% trypsin with 1 mM ethylenediaminetetraacetic acid (EDTA) in Hanks’ balanced salt solution (HBSS) (Invitrogen, Karlsruhe, Germany). A total of 1 × 104 cells were seeded in 250-ml siliconized spinner flasks (Integra Biosciences, Fernwald, Germany) containing 125 ml Iscove’s medium supplemented with 16% FBS (Sigma), 100 μM β-mercaptoethanol (Sigma), 2 mM L-glutamine (Biochrom) and 2 mM non-essential amino acids (Biochrom). After 24 hrs, 125 ml medium was added to give a final volume of 250 ml. The spinner flask medium was stirred at 22.5 r.p.m. using a stirrer system (Integra Biosciences) and 150 ml cell culture medium were exchanged every day.

Cultivation of multicellular tumour spheroids

The mouse mammary tumour cell line 4T1 was cultured in Dulbecco's modified Eagle's medium (DMEM, Invitrogen) supplemented with 8.7% FBS (Invitrogen), 2 mM L-glutamine, 100 μM β-mercaptoethanol (Sigma), 2 mM non-essential amino acids (Biochrom), 43 U/ml penicillin and 43 μg/ml streptomycin (both Biochrom). Cell monolayers were dissociated enzymatically with 0.25% trypsin with 1 mM EDTA in HBSS (Invitrogen) and seeded into 250-ml spinner flasks. For the experiments, tumour spheroids with an age of 5–7 days were used.

Generation of confrontation cultures

For the generation of confrontation cultures, multicellular tumour spheroids and embryoid bodies were removed from spinner flasks. To discriminate tumour spheroids grown in confrontation culture from embryoid bodies, tumour spheroids were labelled with the long-term cell tracker dye 5-chloromethylfluorescein diacetate (CMFDA) as described previously [17]. One embryoid body (10 days old) and one tumour spheroid (5–7 days old) were inoculated in a 35 μl drop of mixed culture medium (50% spheroid medium, 50% embryoid body medium) placed onto the lid of a 10-cm Petri dish, the lid was turned around and placed on the Petri dish, which was filled with 10 ml of sterile phosphate buffered saline (PBS). Within 24 hrs, embryoid bodies and tumour spheroids closely attached within the hanging drops and were then plated onto gelatine-coated glass slides in 24-well-plates.

Immunohistochemistry

Immunohistochemistry was performed with outgrown embryoid bodies plated on day 4 to gelatine-coated glass slides in 24-well-plates. For
primary antibodies, we used monoclonal antimouse platelet endothelial cell adhesion molecule (PECAM-1; CD31) (Chemicon), monoclonal antimouse CD45 (Chemicon), monoclonal antimouse CD68 (Serotec, Dusseldorf, Germany) and monoclonal antimouse Neutrophil antigen (Serotec). All antibodies were used in 1:100 dilution.

The tissues were fixed in ice-cold methanol/acetone (7:3) for 60 min. at –20°C, and washed with PBS containing 0.01% Triton X-100 (PBST; Sigma). Blocking against unspecific binding was performed for 60 min. with 10% fat-free milk powder (Heirler, Radolfzell, Germany) dissolved in PBST. The tissues were subsequently incubated overnight at 4°C with primary antibodies dissolved in PBST supplemented with 10% milk powder. The tissues were then washed three times with PBST and re-incubated with either a Cy2- or a Cy5-conjugated goat anti-rat IgG (Chemicon) at a dilution of 1:100 in PBST containing 10% milk powder. After washing three times with PBST the tissues were stored in PBST until inspection.

Confocal laser-scanning microscopy

Fluorescence recordings were performed by means of a confocal laser scanning setup (LSM 410, Zeiss, Jena, Germany) connected to an inverted microscope (Axiovert 135, Zeiss). The confocal setup was equipped with a 5 mW helium/neon laser, single excitation 633 nm (excitation of Cy5) and an argon laser, single excitation 488 nm (Cy2 and CMFDA). Emission was recorded using the band-pass filter BP 505–570 IR and long-pass filter sets LP 475 and LP 650, respectively. A 20×, numerical aperture (N.A.) 0.75 and a 25×, N.A. 0.8, water-corrected objective (Neofluar, Zeiss) were used.

Fluorescence recordings were performed by a confocal laser-scanning microscope (LSM 510, Zeiss) connected to an inverted microscope (Axiovert 135, Zeiss). For excitation of the Cy2-fluorochrome or CMFDA,
the 488-nm band of an argon laser setup and for the excitation of Cy5 fluorochrome, the 633-nm band of a helium/neon laser setup was used. Emission was recorded using a 505–570-nm band-pass or a 650-nm long-pass filter set. Fluorescence values of control samples stained only with secondary antibody were subtracted from samples stained with primary and secondary antibody. The pinhole settings of the confocal setup were adjusted to give a full-width half maximum of 6 μm. Fluorescence was determined from the top to a depth of 20 μm of the tissue, composed of five optical sections with an interval of 5 μm in z-direction and the fluorescence values in the respective optical section were evaluated by the image analysis software of the confocal setup.

**Real-time RT-PCR**

Total RNA from CGR8 embryoid bodies was prepared using Trizol (Invitrogen) according to manufacturer’s recommendations followed by genomic DNA digestion using DNaseI (Invitrogen). Total RNA concentration was determined by the optical density (260 nm) method. cDNA synthesis was performed with 2 μg RNA in a total volume of 40 μl with SuperScript RTase (Invitrogen). Primer sequences for quantitative real-time PCR were:

- **PECAM**: forw CAGGTGTGCGAATGCTCTCT
  rev ATGGGTGCAGTTCCATTTTC
- **CD45**: forw TCAACAAAAACGAGATCGTC
  rev GGTTTAGGGCCATTAGTTC
- **CD68**: forw TAAAGGGGGCTGGGGCCATA
  rev CTCGGGCTCTGATGATGTC
- **CD16**: forw GAGTGATTTCTGACTGGCTGC
  rev TGGAAGAATGAGATCCTGTCA
- **Mac-1**: forw ACCTCTAATGGTCCCTTGCTG
  rev TGGTTGTGTTGATGAGGTGA
- **VE-cadherin**: forw GTCAACATATAGGGAACCTGTCT
  rev TCATTCTTTTACAGATTGG
- **CD14**: forw GACCATGGAGCGTGTGCTT
  rev ACCAATCTGGCTTCGGATCT
- **Polymerase 2a**: forw: GACAAAACTGGCTCCTCTGC
  rev: GCTTGCCCTCTACATTCTGC

The primer concentration was 10 pM. Amplifications were performed in a Cycler Optical Module (Applied Biosystems 7500, Darmstadt, Germany) using SYBR®-Green (Qiagen). The following programs were used: 95°C for 15 min. hot start step/denaturation

---

**Fig. 3** mRNA expression of the monocyte/macrophage markers CD68 (A), CD14 (B), the granulocyte/monocyte/NK-cell marker Mac-1 (C) and the neutrophil marker CD16 (D) during the differentiation of CGR8 ES cells. Note that mRNA expression of the respective genes increased from day 8 of cell culture and remained constantly elevated until day 16. The mRNA expression levels are presented in relation to the expression of the housekeeping gene Polymerase 2a. In each data point n = 3 experiments are represented. *P < 0.05, statistically significant as compared to day 2 of cell culture.
94°C for 30 sec., AT 30 sec., 72°C 30 sec., 40 times
50°C for 10 min.
Annealing temperatures were:
61°C for PECAM, CD68, CD16, Mac-1, CD14
60°C for VE-cadherin
59°C for CD45
Fluorescence increase of SYBR Green was automatically measured after each extension step.
Amplified transcripts were loaded on a 2% agarose gel. CT values were automatically obtained. Relative expression values were obtained by normalizing CT values of the tested genes in comparison with CT values of the housekeeping genes using the ΔΔCT method.

**Statistical analysis**

Data are given as weighted mean value ± S.E.M., with n denoting the number of experiments unless otherwise indicated. In each experiment at least 10 embryoid bodies were analysed. Either one-way ANOVA or Student’s t-test for unpaired data was applied as appropriate. A value of P < 0.05 was considered significant.

**Results**

**Expression of vascular markers during differentiation of CGR8 ES cells**

The present study was undertaken to characterize the time sequence of ES cell-derived vasculogenesis and leucocyte differentiation and to investigate their interdependence. To achieve these aims cell culture time-dependent expression of the endothelial markers PECAM-1 and VE-cadherin was investigated by real time RT-PCR (Fig. 1) and compared to the expression of leucocyte-specific genes (Figs 2 and 3). As previously described [18], PECAM-1 expression was evident in undifferentiated ES cells but was down-regulated until day 4 of cell culture. From day 5 of cell culture PECAM-1 was again up-regulated with two peaks of expression occurring at days 8–9 and day 15–16, indicating an early-phase of vascular plexus formation and later phase of vascular remodelling. In contrast VE-cadherin was only marginally expressed during early stages of differentiation, i.e. from day 2 to day 5 of differentiation but followed a similar scheme of expression with two peaks at days 8–9 and day 15 comparably to the expression of PECAM-1.

**Expression of CD45, CD14, CD11b, CD68 and Mac-1 during differentiation of CGR8 ES cells**

To correlate the expression of endothelial cell markers and leucocyte cell markers mRNA expression of CD45, which is a pan leucocyte marker was investigated (Fig. 2A). In parallel immunohistochemical analysis of CD45+ cells was performed (Fig. 2B).
Induction of CD14 by LPS and stimulation of monocyte/macrophage differentiation by M-CSF

Expression of leucocyte-associated genes does not necessarily mean that functional cells are differentiated. To investigate cell function 8-day-old embryoid bodies were treated with LPS in increasing concentrations. Subsequently mRNA expression of the LPS receptor CD14 was assessed as a read out of leucocyte function (Fig. 4A, n = 3). It was evident that CD14 expression increased with the dose of LPS, indicating the occurrence of a physiological immunological inflammation reaction which is typically associated to an up-regulation of the CD14 LPS receptor following stimulation with LPS. To demonstrate cytokine-induced proliferation of macrophages embryoid bodies were treated with 250 ng/ml M-CSF from day 8 to day 10 of cell culture (Fig. 4B, n = 3). It was evident that this treatment increased the cell area positive for CD68 expression cells approximately threefold, indicating that the cell system of embryoid bodies responded towards agents that are known to induce macrophage differentiation and proliferation.

Vasculogenesis of ES cells precedes monocyte/macrophage differentiation

The analysis of mRNA expression of leucocyte and endothelial cell markers revealed that endothelial cell markers were expressed about 2 days earlier (day 5–6 of cell culture) as compared to leucocyte cell markers (day 7–8) (see Figs 1–3). To investigate whether this differential increase in gene expression was likewise reflected in the expression of structural proteins, double immunohistochemistry was performed by the use of the endothelial cell markers.

Fig. 5 Time sequence of blood vessel formation versus the expression of the monocyte/macrophage marker CD68. Double immunofluorescence images show PECAM-1+ capillaries (red) and CD68+ cells (green). It is apparent that PECAM-1+ cell structures are already visible on day 6 of cell culture, whereas CD68+ cells appear not earlier than on day 10 of differentiation. The bar represents 20 μm.
marker PECAM-1 in parallel to the monocyte/macrophage marker CD68 (Fig. 5, n = 3). PECAM-1 staining of cells was evident already on day 6 of cell culture with increasing cell areas positive for PECAM-1\(^{+}\) cells until day 8. Cells positive for CD68 were evident on day 10 of cell culture with increasing cell numbers until day 14, thus clearly indicating that vasculogenesis/angiogenesis preceded the differentiation of leucocytes.

**Inhibition of leucocyte differentiation by anti-angiogenic treatment with SU5416**

The data of the present study suggest that vasculogenesis/angiogenesis precedes leucocyte differentiation. If vasculogenesis/angiogenesis is a prerequisite for leucocyte differentiation it should be assumed that anti-angiogenic treatment would inhibit leucocyte differentiation. To validate this assumption, 6-day-old embryoid bodies that already displayed PECAM-1\(^{+}\) cell areas were treated until day 12 of cell culture with SU5416 which inhibits signalling via the Flk-1 receptor tyrosine kinase [19]. As shown in Fig. 6A–C (n = 4) treatment with SU5416 (4 \(\mu\)M) significantly reduced the cell number of cells positive for CD45, CD68 and neutrophil antigen. Cell numbers were assessed as the cell area positive for the respective antigen in relation to the total cell area of outgrown embryoid bodies. The images show representative immunostainings. The bar represents 20 \(\mu\)m. *P < 0.05, statistically significant as compared to the untreated control.

**Increase in leucocyte numbers upon confrontation culture of ES cells with tumour tissue**

Tumour growth is associated with angiogenesis and inflammation. It is known for several years that blood vessel and macrophage density increase simultaneously with pathological progression during tumour-induced angiogenesis [20]. It is, however, not yet known whether increased numbers of leucocytes within tumour tissues arise from recruitment of these cells to the tumour tissue or whether
leucocytes differentiate and proliferate from invading precursor cells. To assess whether a tumour microenvironment would stimulate leucocyte differentiation/proliferation confrontation cultures of embryoid bodies and 4T1 mouse mammary tumour spheroids were generated. Upon 4 days of confrontation culture capillary-like endothelial cell structures invaded the tumour tissue and tumour cells spread into the embryoid body (Fig. 7; n = 5). When CD45<sup>+</sup>, CD68<sup>+</sup> and neutrophil antigen-positive cells were assessed in confrontation cultures it became apparent that CD68<sup>+</sup> (n = 4) and CD45<sup>+</sup> (n = 4) cells readily invaded into the tumour tissue, whereas neutrophil antigen-positive cells formed a shell around the tumour cells without any penetration (n = 3) (see Fig. 8C). Interestingly confrontation culture was accompanied by a significant increase in CD68<sup>+</sup> (Fig. 8A, n = 3) and CD45<sup>+</sup> (Fig. 8B, n = 3) cell numbers as compared to the leucocyte cell numbers in embryoid bodies cultured alone, thus indicating that the tumour microenvironment stimulated differentiation and/or proliferation of leucocytes from precursor cells which may belong to the endothelial cell lineage.

**Discussion**

During embryogenesis haematopoietic cells have been demonstrated to originate from a common precursor which give rise to endothelial progenitors and haematopoietic progenitors, the latter subsequently generating primitive as well as definitive haematopoietic cell lineages [13, 21]. Subsequent studies have refined this concept by demonstrating that during ES cell differentiation specification of Flk-1<sup>+</sup> mesoderm into haematopoietic and
endothelial cell lineages occurs in sequential steps with a subpopulation of VE-cadherin-positive endothelial cells that have the capacity of differentiation of multiple definitive haematopoietic progenitors [22, 23]. Comparably it has been demonstrated in human ES cells that haematopoiesis and endothelial maturation occur exclusively from a subset of embryonic endothelium that possesses haemangioblastic properties [24].

In the present study the time sequence of endothelial cell differentiation and differentiation of leucocyte cell types was investigated. It was demonstrated that murine ES cells expressed the endothelial cell markers PECAM-1 and VE-cadherin and formed capillary-like cell structures. With a time delay of approximately 2 days endothelial cell differentiation was followed by the expression of the pan leucocyte marker CD45, the monocyte/macrophage markers CD68, CD14 and Mac-1 and the neutrophil marker CD16. Stimulation of embryoid bodies with LPS resulted in up-regulation of the LPS-receptor CD14. Furthermore, incubation of embryoid bodies with M-CSF increased the number of CD68+ cells suggesting that functional monocytes/macrophages were generated. The time delay between the differentiation of endothelial cells and leucocytes suggested that endothelial cell differentiation may be a prerequisite of leucocyte differentiation. This assumption was tested by incubating 6-day-old embryoid bodies with SU5416 which is an inhibitor of Flk-1 and additionally Fit-1 signalling by inhibiting tyrosine kinase activity [25]. SU5416 has been shown to inhibit endothelial cell migration and angiogenesis [25]. In the embryoid body model SU5416 was shown to inhibit vasculogenesis (data not shown). Notably 6-day-old embryoid bodies were already positive for endothelial cell markers whereas cells positive for CD45 were completely absent. Treatment with SU5416 significantly reduced cell areas covered with CD45+, CD68+ and neutrophil antigen-positive cells suggesting that either angiogenesis directly stimulates leucocyte cell differentiation from undifferentiated ES cells or that immature or mature endothelial cells give rise to a leucocyte progeny.

Recently it has been pointed out that a characteristic inflammatory infiltrate is associated with constant tissue remodelling in a growing tumour and with tumour angiogenesis [1]. The origin of these inflammatory cells is not yet defined. Previous studies have demonstrated that bone marrow derived cells are recruited into tumour vasculature in response to angiogenic signals, and some of the cells within the newly forming tumour vessels are HSCs in origin. It seems therefore conceivable that inflammatory leucocyte cell types arise from endothelial progenitor cells within the tumour vasculature, thus establishing the inflammatory microenvironment that may provide the suitable pro-angiogenic cytokine cocktail necessary for proper vascular growth within the tumour tissue. In the present study, we used our previously established confrontation culture system [26] comprising of ES cell-derived embryoid bodies and multicellular 4T1 breast tumour spheroids to demonstrate that within 4 days of confrontation culture capillary-like cell structures were detectable within the tumour tissue. Investigation of leucocyte cell markers within the tissue of confrontation cultures demonstrated that CD45+ and CD68+ cells readily invaded the tumour tissue, which may result in the pro-inflammatory state of increased reactive oxygen species production previously observed in confrontation cultures [27]. In contrast, neutrophil cells positive for neutrophil antigen formed a shell around the embryoid body tissue with no invasion into the tumour. Most notably it was observed that confrontation culture significantly increased tissue areas positive for CD45+ and CD68+ cells, which supports the assumption that during tumour angiogenesis the specific tumour microenvironment either stimulates differentiation of leucocytes presumably from a pre-existing endothelial cell population or promotes a rapid proliferation of these cells. Chronic inflammation that has been attributed to the initiation and progression of cancer as well as to vascular growth and blood vessel formation [28]. If indeed interdependence exists between tumour angiogenesis and leucocyte cell reactions any anti-angiogenic treatment that targets the tumour vasculature should in parallel abolish the inflammatory stage of solid tumours which may be stimulatory for vascular growth.

Acknowledgements

This work was supported by the excellence cluster ‘Cardiopulmonary System’ (ECCPS) of the German Research Foundation (DFG), the SFB 604 ‘Multifunctional Signalling Proteins’ and the SET Foundation (Frankfurt).

References

1. Noonan DM, De Lerma BA, Vannini N, et al. Inflammation, inflammatory cells and angiogenesis: decisions and indecisions. Cancer Metastasis Rev. 2008; 27: 31–40.
2. Allavena P, Sica A, Solinas G, et al. The inflammatory micro-environment in tumor progression: the role of tumor-associated macrophages. Crit Rev Oncol Hematol. 2008; 66: 1–9.
3. Coussens LM, Werb Z. Inflammation and cancer. Nature. 2002; 420: 860–7.
4. Vittet D, Prandini MH, Berthier R, et al. Embryonic stem cells differentiate in vitro to endothelial cells through successive maturation steps. Blood. 1996; 88: 3424–31.
5. Wartenberg M, Gunther J, Hescheler J, et al. The embryoid body as a novel in vitro assay system for antiangiogenic agents. Lab Invest. 1998; 78: 1301–14.
6. Wang R, Clark R, Bautch VL. Embryonic stem cell-derived cystic embryoid bodies form vascular channels: an in vitro model of blood vessel development. Development. 1992; 114: 303–16.
7. Moore KJ, Fabummi RP, Andersson LP, et al. In vitro-differentiated embryonic stem cell macrophages: a model system for studying atherosclerosis-associated macrophage functions. Arterioscler
8. de Pooter RF, Cho SK, Carlyle JR, et al. In vitro generation of T lymphocytes from embryonic stem cell-derived prehematopoietic progenitors. Blood. 2003; 102: 1649–53.

9. de Pooter RF, Cho SK, Zuniga-Pflucker JC. In vitro generation of lymphocytes from embryonic stem cells. Methods Mol Biol. 2005; 290: 135–47.

10. Keller G, Kennedy M, Papayannopoulou T, et al. Hematopoietic commitment during embryonic stem cell differentiation in culture. Mol Cell Biol. 1993; 13: 473–86.

11. Senju S, Hirata S, Matsuyoshi H, et al. Generation and genetic modification of dendritic cells derived from mouse embryonic stem cells. Blood. 2003; 101: 3501–8.

12. Sabin FR. Preliminary note on the differentiation of angioblasts and the method by which they produce blood-vessels, blood-plasma and red blood-cells as seen in the living chick. 1917. J Hematother Stem Cell Res. 2002; 11: 5–7.

13. Chung YS, Zhang WJ, Arentson E, et al. Lineage analysis of the hemangioblast as defined by FLK1 and SCL expression. Development. 2002; 129: 551–20.

14. Ema M, Faloon P, Zhang WJ, et al. Combinatorial effects of Flk1 and Tal1 on vascular and hematopoietic development in the mouse. Genes Dev. 2003; 17: 380–93.

15. Shalaby F, Rossant J, Yamaguchi TP, et al. Failure of blood-island formation and vasculogenesis in Flk-1-deficient mice. Nature. 1995; 376: 62–6.

16. Colmone A, Sipkins DA. Beyond angiogenesis: the role of endothelium in the bone marrow vascular niche. Transl Res. 2008; 151: 1–9.

17. Gunther S, Ruhe C, Derikito MG, et al. Polyphenols prevent cell shedding from mouse mammary cancer spheroids and inhibit cancer cell invasion in confrontation cultures derived from embryonic stem cells. Cancer Lett. 2007; 250: 25–35.

18. Redick SD, Bautch VL. Developmental platelet endothelial cell adhesion molecule expression suggests multiple roles for a vascular adhesion molecule. Am J Pathol. 1999; 154: 1137–47.

19. Fong TA, Shawver LK, Sun L, et al. SU5416 is a potent and selective inhibitor of the vascular endothelial growth factor receptor (Flk-1/KDR) that inhibits tyrosine kinase catalysis, tumor vascularization, and growth of multiple tumor types. Cancer Res. 1999; 59: 99–106.

20. Biswas SK, Sica A, Lewis CE. Plasticity of macrophage function during tumor progression: regulation by distinct molecular mechanisms. J Immunol. 2008; 180: 2011–7.

21. Kennedy M, Firpo M, Choi K, et al. A common precursor for primitive erythropoiesis and definitive hematopoiesis. Nature. 1997; 386: 488–93.

22. Fujimoto T, Ogawa M, Minegishi N, et al. Step-wise divergence of primitive and definitive haematopoietic and endothelial cell lineages during embryonic stem cell differentiation. Genes Cells. 2001; 6: 1113–27.

23. Fraser ST, Ogawa M, Yu RT, et al. Definitive hematopoietic commitment within the embryonic vascular endothelial-cadherin(+) population. Exp Hematol. 2002; 30: 1070–8.

24. Wang L, Li L, Shojaei F, et al. Endothelial and hematopoietic cell fate of human embryonic stem cells originates from primitive endothelium with hemangioblastic properties. Immunity. 2004; 21: 31–41.

25. Itokawa T, Nokihara H, Nishioka Y, et al. Antiangiogenic effect by SU5416 is partly attributable to inhibition of Flt-1 receptor signaling. Mol Cancer Ther. 2002; 1: 295–302.

26. Wartenberg M, Donmez F, Ling FC, et al. Tumor-induced angiogenesis studied in confrontation cultures of multicellular tumor spheroids and embryoid bodies grown from pluripotent embryonic stem cells. FASEB J. 2001; 15: 995–1005.

27. Wartenberg M, Budde P, De Mares M, et al. Inhibition of tumor-induced angiogenesis and matrix-metalloproteinase expression in confrontation cultures of embryoid bodies and tumor spheroids by plant ingredients used in traditional Chinese medicine. Lab Invest. 2003; 83: 87–98.

28. Porta C, Subhra KB, Larghi P, et al. Tumor promotion by tumor-associated macrophages. Adv Exp Med Biol. 2007; 604: 67–86.