REVIEW

The chick embryo chorioallantoic membrane in the study of tumor angiogenesis

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Abstract

The chick embryo chorioallantoic membrane (CAM) is commonly used as an experimental *in vivo* assay to study both angiogenesis and antiangiogenesis in response to tissues, cells or soluble factors. This article summarizes literature data about the use of the CAM in the study of tumor angiogenesis and particularly our experimental data concerning the study of angiogenesis in multiple myeloma and in neuroblastoma.

***Keywords*:** angiogenesis, chorioallantoic membrane, multiple myeloma, neuroblastoma, tumor growth.

The crucial role of angiogenesis in tumor growth

Angiogenesis and the production of angiogenic factors are fundamental for tumor progression in the form of growth, invasion and metastasis [1]. New vessels promote growth by conveying oxygen and nutrients and removing catabolities [2]. These requirements vary, however, among tumor types, and change over the course of tumor progression [3]. Endothelial cells secrete growth factors for tumor cells and a variety of matrix-degrading proteinases that facilitate tumor invasion [4]. An expanding endothelial surface also gives tumor cells more opportunities to enter the circulation and metastasize [5].

Solid tumor growth occurs by means of an avascular phase followed by a vascular phase. Assuming that such growth is dependent on angiogenesis and that this depends on the release of angiogenic factors, the acquisition of angiogenic ability can be seen as an expression of progression from neoplastic transformation to tumor growth and metastasis [1]. Practically all solid tumors, including those of the colon, lung, breast, cervix, bladder, prostate and pancreas, progress through these two phases. The role of angiogenesis in the growth and survival of leukemias and other hematological malignancies has only become evident since 1994 thanks to a series of studies demonstrating that progression in several forms is clearly related to their degree of angiogenesis [6].

Tumor angiogenesis is linked to a switch in the balance between positive and negative regulators, and mainly depends on the release by neoplastic cells of specific growth factors for endothelial cells, that stimulate the growth of the host's blood vessels or the down-regulation of natural angiogenesis inhibitors. In normal tissues, vascular quiescence is maintained by the dominant influence of endogenous angiogenesis inhibitors over angiogenic stimuli. The switch depends on increased production of one or more positive regulators of angiogenesis, such as vascular endothelial growth factor (VEGF), fibroblast growth factor-2 (FGF-2), interleukin-8 (IL-8), placental growth factor (PlGF), transforming growth factor-β (TGF-β), platelet-derived growth factor (PDGF), pleiotrophins and others. These can be exported from tumor cells, mobilized from the extracellullar matrix, or released from host cells recruited to the tumor. The switch clearly involves more than a simple up-regulation of angiogenic activity and has thus been regarded as the result of the net balance between positive and negative regulators.

Assays for studying angiogenesis *in vivo*

The classical assays for studying angiogenesis *in vivo* include the hamster cheek pouch, rabbit ear chamber, dorsal skin and air sac, the chick embryo chorioallantoic membrane (CAM) and iris and avascular corneal of rodent eye. Several new models have been introduced including subcutaneous implantation of various three-dimensional substrates including polyester sponge, polyvinyl-alcohol foam disc covered on both sides with a Millipore filter (the disc angiogenesis system), and Matrigel, a basement membrane rich extracellular matrix. The three most widely used assays are the CAM, the rabbit corneal micropocket and the subcutaneous implants.

The CAM assay facilitates the testing of multiple samples and the generation of dose-dilution curves. It has been used to identify almost all of the known angiogenic factors [7]. The allantois is an extra-embryonic membrane, derived from the mesoderm in which primitive blood vessels begin to take shape on day 3 of incubation. On day 4, the allantois fuses with the chorion and forms the chorioallantois. Until day 8 of incubation, primitive vessels continue to proliferate and

to differentiate into an arteriovenous system and thus originate a network of capillaries that migrate to occupy an area beneath the chorion and mediate gas exchanges with the outer environment. The CAM vessels grow rapidly up to day 11, after which the endothelial cell mitotic index decreases just as rapidly, and the vascular system attains its final setup on day 18 of incubation, just before hatching.

The CAM in the study of tumor angiogenesis

The first evidence of the tumor-induced angiogenesis *in vivo* by using the CAM assay dated 1913 [8]. The CAM has long been a favored system for the study of tumor angiogenesis and metastasis [9–11], because at this stage the chick's immunocompetent system is not fully developed and the conditions for rejection have not been established [12]. As other vertebrates, chickens are protected by a dual immune system composed of B- and T-cells, controlling the antibody and cell mediated immunity, respectively. The B-cells are differentiated in the bursa of Fabricius, the organ equivalent to the bone marrow in mammals, whereas T-cells are differentiated in the thymus [13, 14]. Until day 10, the chick embryo immune system is not completely developed. The presence of T-cells can be first detected at day 11 and of B-cells at day 12 [15]. After day 15, the B-cell repertory begins to diversify and by day 18 chicken embryos become immunocompetent.

All studies of mammalian neoplasms in the CAM have utilized bioptic tumor specimens and cell suspensions derived from tumors (Tables 1 and 2).

Table 1 – ***Angiogenic response induced by tumors implanted onto the CAM***

Tumor	Authors
Adenocarcinoma of the endometrium	Palczak R and Splawinski J, 1989 [16]
B-cell non-Hodgkin's lymphoma	Ribatti D *et al.*, 1990 [17]
Glioblastoma	Kalgsbrun M *et al.*, 1976 [18]
Head and neck squamous cell carcinoma	Petruzzelli GJ *et al.*, 1993 [19]
Hepatocellular carcinoma	Marzullo A *et al.*, 1998 [20]
Lipoma	Lucarelli E *et al.*, 1999 [21]
Melanoma	Auerbach R *et al.*, 1976 [10]
Meningioma	Kalgsbrun M *et al.*, 1976 [18]
Neuroblastoma	Ribatti D *et al.*, 2002 [22]
Walker 256 carcinoma	Kalgsbrun M *et al.*, 1976 [18]

Table 2 – ***Angiogenic response induced by tumor cells implanted onto the CAM***

Tumor cells	Authors
B-16 melanoma cells	Takigawa M *et al.*, 1990 [23]
Endothelial cells isolated from patients with multiple myeloma	Vacca A *et al.*, 2003 [24]
Lymphoblastoid cells	Vacca A *et al.*, 1998 [25]
Mammary tumor cells transfected with VEGF	Ribatti D *et al.*, 2001a [26]
Neuroblastoma	Ribatti D *et al.*, 2002 [22]
Plasma cells isolated from patients with multiple myeloma	Ribatti D *et al.*, 2003 [27]
Walker carcinoma 256 cells	Kalgsbrun M *et al.*, 1976 [18]

Compared with mammals' models, where tumor growth often takes between three and six weeks, assays using chick embryos are faster. Between 2 and 5 days after tumor cell inoculation, the tumor xenografts become visible and are supplied with vessels of CAM origin. Tumors grafted onto the CAM remain non-vascularized for a couple of days, after which they can be penetrated by new blood vessels and begin a phase of rapid growth. Tumor cells can be identified in the CAM, as well as in the internal organs of the embryo, such as lungs, liver and brain [28, 29].

Walker 256 carcinoma specimens implanted on the CAM do not exceed a mean diameter of 0.93 ± 0.29 mm during the prevascular phase (approximately 72 hours). Rapid growth begins 24 hours after vascularization and tumors reach a mean diameter of 8.0 ± 2.5 mm by 7 days [30]. When tumor grafts of increasing size (from 1 to 4 mm) are implanted on the 9-day CAM, grafts larger than 1 mm undergo necrosis and autolysis during the 72-hour prevascular phase. They shrink rapidly until the onset of vascularization, when rapid growth resumes [30].

Ausprunk DH *et al.* (1975) compared the behavior of tumor grafts to grafts of normal adult and embryo tissues [31]. In tumor tissue, pre-existing blood vessels in the tumor graft disintegrated within 24 hours after implantation, and revascularization occurred by penetration of proliferating host vessels into the tumor tissue. By contrast, pre-existing vessels did not disintegrate in the embryo graft and anastomosed to the host vessels with almost no neovascularization. In adult tissues, pre-existing graft vessels disintegrated (although this process was slower than in tumor vessels) and did not stimulate capillary proliferation in the host. Lastly, tumor vessels did not reattach to those of the host.

Hagedorn M *et al.* (2005) have developed a glioblastoma multiforme tumor progression model on the CAM [32]. They demonstrated that avascular tumors formed within 2 days, than progressed through VEGFR-2-dependent angiogenesis, associated with hemorrhage, necrosis, and peritumoral edema. Blocking of VEGFR-2 and PDGFR signaling pathways by using small-molecule receptor tyrosine kinase inhibitors abrogated tumor development. Moreover, gene regulation during the angiogenic switch was analyzed by oligonucleotide microarrays, permitting identify-cation of regulated genes whose functions are associated mainly with tumor vascularization and growth.

The CAM in the study of angiogenesis and antiangiogenesis in multiple myeloma

In patients with monoclonal gammopathy of undetermined significance (MGUS) and multiple myeloma (MM), angiogenesis correlates with plasma cell growth (S-phase fraction) [33]. Moreover, angiogenesis is paralleled by an increased angiogenic ability of bone marrow plasma cell conditioned medium of patients with active MM as compared with those with non-active MM and MGUS, and partly dependent FGF-2 production [34].

Plasma cell conditioned media were tested to their ability to induce angiogenesis in the CAM [34]. The conditioned medium of 77% active MM patients induced an angiogenic response; by contrast, only 33% and 20% of conditioned medium from non-active MM and MGUS patients, respectively, induced the response. Anti-FGF-2 antibody partly inhibited conditioned medium angiogenic response. In another work, we have attempted a fine characterization of the angiogenic response induced by plasma cells obtained from patients with active-MM, as compared to those obtained from patients with non-active MM and respectively MGUS, in the CAM assay. To this purpose, we have investigated about the time-course of the angiogenic response induced by gelatin sponges soaked with the cell suspensions and implanted on the CAM surface from day 8 to day 12 of incubation by evaluating the number of vessels, of the vessel bifurcation and the intervascular distance at 24, 48, 72 and 96 hours after the implants [27]. Results demonstrated that that plasma cell suspensions obtained from patients with active MM induce a vasoproliferative response, significantly higher as compared to that induced by cell suspensions obtained from patients with non-active MM and, respectively, with MGUS. These responses are a function of the day of implantation. In fact, implants made from day 8 to day 10 are strongly angiogenic, while those made from day 11 to day 12 do not. This finding might depend on the fact that CAM endothelium exhibits an intrinsically high mitotic rate until day 10 [35]. Thereafter, the endothelial mitotic index declines rapidly, and the vascular system attains its final arrangement on day 18, just before hatching [35]. Consequently, cell suspensions implanted on the CAM of successively older embryos, are not able to induce a vasoproliferative response in parallel with the reduced rates of growth of CAM's endothelial cells.

More recently, we have isolated endothelial cells (EC) from bone marrow of patients with MM (MMEC) [24]. They show intrinsic angiogenic ability, because they rapidly form a capillary network *in vitro*, and extrinsic ability, because they generate numerous new vessels *in vivo* in the CAM assay [24]. We have attempted a fine characterization of the angiogenic response induced by MMEC, as compared with MGUSEC in the CAM assay, by investigating the effects of MMEC and MGUSEC on the expression of endogenous levels in the CAM of VEGF, FGF-2, angiopoietin-1 (Ang-1), hypoxia-inducible factor-1α (HIF-1α) and endostatin by reverse transcriptase-polymerase chain reaction (RT-PCR) [36, 37]. RT-PCR demonstrated that the expression of endostatin mRNA detected in MM treated CAM was significantly lower respect to control CAM. These data suggest that angiogenic switch in MM may involve loss of an endogenous angiogenesis inhibitor, such as endostatin.

Recently, we have demonstrated that zoledronic acid exerted an antiangiogenic activity in MMEC mediated by abrogation of their VEGF/VEGFR-2 autocrine loop and gene modulation of other angiogenic targets [38]. Moreover, in the CAM assay, zoledronic acid 10 and 30 μM was added to the MMEC conditioned medium, a significant reduction of the angiogenic response was found. These data suggest that zoledronic acid has an antiangiogenic effect in MM, which may be involved in an indirect antitumor activity.

The CAM in the study of angiogenesis and antiangiogenesis in human neuroblastoma

Neuroblastoma is the most frequently occurring solid tumor in children. Several recent studies implicate angiogenesis in the regulation of neuroblastoma growth and inhibition of angiogenesis is a promising approach in the treatment of this tumor [39].

In a first paper published in 1998, we investigated two human neuroblastoma cell lines, LAN-5 and GI-LI-N, for their capacity to induce angiogenesis in the CAM assay [40] and demonstrated that conditioned medium from both cells lines, LAN-5 cells more than GI-LI-N ones, induced angiogenesis.

The role that the oncogene MYCN plays in the regulation of angiogenesis in neuroblastoma remains controversial. With the aim to better elucidate this matter, we tested fresh biopsy samples from patients with MYCN amplified and with MYCN nonamplified tumors for their angiogenic capacity by using the CAM assay [22]. Moreover, conditioned medium obtained from five different human neuroblastoma cell lines MYCN amplified and non-amplified and bioptic fragments obtained from xenografts derived from four neuroblastoma cell lines injected in nude mice, were assayed for their angiogenic potential. Results clearly demonstrated that MYCN amplification parallel angiogenesis in neuroblastoma. When fresh biopsy samples from patients, conditioned medium derived from neuroblastoma cells lines and bioptic fragments derived from xenografts of the same cell lines injected in nude mice were tested, the response was univocal: the angiogenic response was significantly higher in the MYCN amplified specimens as compared to nonamplified ones.

In 2001, we studied the effects of the synthetic retinoid fenretinide (HPR) *in vivo* by using the CAM assay [41]. Results showed that HPR inhibited VEGF- and FGF-2-induced angiogenesis in the CAM assay. A significant antiangiogenic potential of HPR has been observed also in neuroblastoma biopsies induced angiogenesis *in vivo* in the CAM assay. Finally, immunohistochemistry experiments performed in the CAM assay demonstrated that endothelial staining of both VEGFR-2 and FGF2R-2 was reduced after implantation of HPR-loaded sponges, as compared to controls. These data suggest that HPR exerts its antiangiogenic activity through both a direct effect on endothelial cell proliferative activity and an inhibitory effect on the responsivity of the endothelial cells to the proliferative stimuli mediated by angiogenic growth factors.

We have investigated the antiangiogenic activity of interferon gamma (IFN-γ) by using an experimental model in which IFN-γ gene transfer clampens the tumorigenic and angiogenic activity of ACN

neuroblastoma cell line in immunodeficient mice [42]. We demonstrated that ACN/ IFN-γ xeongrafts had less *in vivo* angiogenic potential than the vector-transfected ACN/neo, when grafted onto the CAM.

In another study, we evaluated the synergistic antiangiogenic effect of low-dose of vinblastine and rapamycin in neuroblastoma [43, 44]. The angiogenic responses induced by neuroblastoma cell derived-conditioned medium, neuroblastoma tumor xenografts and human neuroblastoma biopsy specimens were inhibited in the CAM assay by each drug and more significantly by their combination. The observation that these well-known drugs display synergistic effects as antiangiogenics when administered frequently at very low dose may be of significance in the designing of new ways of treating neuroblastoma.

Bortezomib is a selective and reversible inhibitor of the 26S proteasome that shows potent antitumor activity *in vitro* and *in vivo* against several human cancers of adulthood. No data are available on bortezomib activity against human neuroblastoma and we demonstrated that bortezomib inhibited angiogenesis in CAM stimulated by conditioned medium from neuroblastoma cell lines, by neuroblastoma xenografts, and by primary neuroblastoma biopsy specimens [45].

The therapeutic efficacy of cancer active-targeting using doxorubicin (DOX)-loaded immunoliposomes was evaluated with the CAM model [46]. The DOX-loaded liposomes were coupled either to monoclonal antibodies targeting tumor cells (anti-GD2) or to NGR peptides that target tumor vessels. The antiangiogenic effects of these formulations were tested on xenografts derived from neuroblastoma cell lines grown on the CAM surface. When anti-GD2 or NGR liposomes were administered separately, 50–60% of vessel growth inhibition was achieved, whereas administering a combination of both types of liposomes increased vessel growth inhibition to 90%. The higher efficiency of the combined treatment was further validated in tumor-bearing mice.

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References

[1] RIBATTI D., VACCA A., DAMMACCO F., *The role of the vascular phase in solid tumor growth: a historical review*, Neoplasia, 1999, 1(4):293–302.

[2] PAPETTI M., HERMAN I. M., *Mechanisms of normal and tumor-derived angiogenesis*, Am J Physiol Cell Physiol, 2002, 282(5):C947–C970.

[3] HLATKY L., HAHNFELDT P., FOLKMAN J., *Clinical application of antiangiogenic therapy: microvessel density, what it does and doesn't tell us*, J Natl Cancer Inst, 2002, 94(12):883–893.

[4] MIGNATTI P., RIFKIN D. B., *Biology and biochemistry of proteinases in tumor invasion*, Physiol Rev, 1993, 73(1):161–195.

[5] AZNAVOORIAN S., MURPHY A. N., STETLER-STEVENSON W. G., LIOTTA L. A., *Molecular aspects of tumor cell invasion and metastasis*, Cancer, 1993, 71(4):1368–1383.

[6] VACCA A., RIBATTI D., *Bone marrow angiogenesis in multiple myeloma*, Leukemia, 2006, 20(2):193–199.

[7] RIBATTI D., NICO B., VACCA A., PRESTA M., *The gelatin sponge-chorioallantoic membrane assay*, Nat Protoc, 2006a, 1(1):85–91.

[8] RIBATTI D., *The first evidence of the tumor-induced angiogenesis in vivo by using the chorioallantoic membrane assay dated 1913*, Leukemia, 2004, 18(8):1350–1351.

[9] DAGG C. P., KARNOFSKY D. A., RODDY J., *Growth of transplantable human tumors in the chick embryo and hatched chick*, Cancer Res, 1956, 16(7):589–594.

[10] AUERBACH R., KUBAI L., SIDKY Y., *Angiogenesis induction by tumors, embryonic tissues, and lymphocytes*, Cancer Res, 1976, 36(9 Pt 2):3435–3440.

[11] QUIGLEY J. P., ARMSTRONG P. B., *Tumor cell intravasation alu-citated: the chick embryo opens the window*, Cell, 1998, 94(3):281–284.

[12] LEENE W., DUYZINGS M. J. M., VAN STEEG C., *Lymphoid stem cell identification in the developing thymus and bursa of Fabricius of the chick*, Z Zellforsch Mikrosk Anat, 1973, 136(4):521–533.

[13] FUNK P. E., THOMPSON C. B., *Current concepts in chicken B cell development*, Curr Top Microbiol Immunol, 1996, 212:17–28.

[14] DAVISON T. F., *The immunologists' debt to the chicken*, Br Poultr Sci, 2003, 44(1):6–21.

[15] JANSE E. M., JEURISSEN S. H., *Ontogeny and function of two non-lymphoid cell populations in the chicken embryo*, Immunobiology, 1991, 182(5):472–481.

[16] PALCZAK R., SPLAWINSKI J., *Angiogenic activity and neovascularization in adenocarcinoma of the endometrium*, Int J Gynaecol Obstet, 1989, 29(4):343–357.

[17] RIBATTI D., VACCA A., BERTOSSI M., DE BENEDICTIS G., RONCALI L., DAMMACCO F., *Angiogenesis induced by B-cell non-Hodgkin's lymphomas. Lack of correlation with tumor malignancy and immunologic phenotype*, Anticancer Res, 1990, 10(2A):401–406.

[18] KALGSBRUN M., KNIGHTON D., FOLKMAN J., *Tumor angiogenesis activity in cells grown in tissue culture*, Cancer Res, 1976, 36(1):110–114.

[19] PETRUZZELLI G. J., SNYDERMAN C. H., JOHNSON J. T., MYERS E. N., *Angiogenesis induced by head and neck squamous cell carcinoma xenografts in the chick embryo chorioallantoic membrane model*, Ann Otol Rhinol Laryngol, 1993, 102(3 Pt 1):215–221.

[20] MARZULLO A., VACCA A., RONCALI L., POLLICE L., RIBATTI D., *Angiogenesis in hepatocellular carcinoma: an experimental study in the chick embryo chorioallantoic membrane*, Int J Oncol, 1998, 13(1):17–21.

[21] LUCARELLI E., SANGIORGI L., BENASSI S., DONATI D., GOBBI G. A., PICCI P., VACCA A., RIBATTI D., *Angiogenesis in lipoma: An experimental study in the chick embryo chorioallantoic membrane*, Int J Mol Med, 1999, 4(6):593–596.

[22] RIBATTI D., RAFFAGHELLO L., PASTORINO F., NICO B., BRIGNOLE C., VACCA A., PONZONI M., *In vivo angiogenic activity of neuroblastoma cells correlates with MYCN oncogene overexpression*, Int J Cancer, 2002, 102(4):351–354.

[23] TAKIGAWA M., ENOMOTO M., NISHIDA Y., PAN H. O., KINOSHITA A., SUZUKI F., *Tumor angiogenesis and polyamines: α-difluoromethylornithine, an irreversible inhibitor of ornithine decarboxylase, inhibit B16 melanoma-induced angiogenesis in ovo and the proliferation of vascular endothelial cells in vitro*, Cancer Res, 1990, 50(13):4131–4138.

[24] VACCA A., RIA R., SEMERARO F., MERCHIONNE F., COLUCCIA M., BOCCARELLI A., SCAVELLI C., NICO B., GERNONE A., BATELLI F., TABILIO A., GUIDOLIN D., PETRUCCI M. T., RIBATTI D., DAMMACCO F., *Endothelial cells in the bone marrow of patients with multiple myeloma*, Blood, 2003, 102(9):3340–3348.

[25] VACCA A., RIBATTI D., IURLARO M., ALBINI A., MINISCHETTI M., BUSSOLINO F., PELLEGRINO A., RIA R., RUSNATI M., PRESTA M., VINCENTI V., PERSICO M. G., DAMMACCO F., *Human lymphoblastoid cells produce extracellular matrix-degrading enzymes and induce endothelial cell proliferation, migration, morphogenesis, and angiogenesis*, Int J Clin Lab Res, 1998, 28(1):55–68.

[26] RIBATTI D., NICO B., MORBIDELLI L., DONNINI S., ZICHE M., VACCA A., RONCALI L., PRESTA M., *Cell-mediated delivery of fibroblast growth factor-2 and vascular endothelial growth factor onto the chick chorioallantoic membrane: endothelial fenestrations and angiogenesis*, J Vasc Res, 2001a, 38(4):389–397.

[27] RIBATTI D., DE FALCO G., NICO B., RIA R., CRIVELLATO E., VACCA A., *In vivo time-course of the angiogenic response induced by multiple myeloma plasma cells in the chick embryo chorioallantoic membrane*, J Anat, 2003, 203(3):323–328.

[28] GORDON J. R., QUIGLEY J. P., *Early spontaneous metastasis in the human epidermoid carcinoma Hep3/chick embryo model: contribution of incidental colonization*, Int J Cancer, 1986, 38(3):437–444.

[29] BOBEK V., PLACHY J., PINTEROVA D., KOLOSTOVA K., BOUBELIK M., JIANG P., YANG M., HOFFMAN R. M., *Development of a green fluorescent protein metastatic-cancer chick-embryo drug-screen model*, Clin Exp Metastasis, 2004, 21(4):347–352.

[30] KNIGHTON D., AUSPRUNK D., TAPPER D., FOLKMAN J., *Avascular and vascular phases of tumour growth in the chick embryo*, Br J Cancer, 1977, 35(3):347–356.

[31] AUSPRUNK D. H., KNIGHTON D. R., FOLKMAN J., *Vascularization of normal and neoplastic tissues grafted to the chick chorioallantois. Role of host and preexisting graft blood vessels*, Am J Pathol, 1975, 79(3):597–618.

[32] HAGEDORN M., JAVERZAT S., GILGES D., MEYRE A., DE LAFARGE B., EICHMANN A., BIKFALVI A., *Accessing key steps of human tumor progression in vivo by using an avian embryo model*, Proc Natl Acad Sci USA, 2005, 102(5):1643–1648.

[33] VACCA A., RIBATTI D., RONCALI L., RANIERI G., SERIO G., SILVESTRIS F., DAMMACCO F., *Bone marrow angiogenesis and progression in multiple myeloma*, Br J Haematol, 1994, 87(3):503–508.

[34] VACCA A., RIBATTI D., PRESTA M., MINISCHETTI M., IURLARO M., RIA R., ALBINI A., BUSSOLINO F., DAMMACCO F., *Bone marrow neovascularization*, *plasma cell angiogenic potential*, *and matrix metalloproteinase-2 secretion parallel progression of human multiple myeloma*, Blood, 1999, 93(9):3064–3073.

[35] AUSPRUNK D. H., KNIGHTON D. R., FOLKMAN J., *Differentiation of the vascular endothelium in the chick chorioallantois: a structural and autoradiographic study*, Dev Biol, 1974, 38(2):237–248.

[36] MANGIERI D., NICO B., BENAGIANO V., DE GIORGIS M., VACCA A., RIBATTI D., *Angiogenic activity of multiple myeloma endothelial cells in vivo in the chick embryo chorioallantoic membrane assay is associated to a down-regulation in the expression of endogenous endostatin*, J Cell Mol Med, 2007 Nov 20 [Epub ahead of print].

[37] HOLASH J., MAISONPIERRE P. C., COMPTON D., BOLAND P., ALEXANDER C. R., ZAGZAG D., YANCOPOULOS G. D., WIEGAND S. J., *Vessel cooption, regression and growth in tumors mediated by angiopoietins and VEGF*, Science, 1999, 284(5422):1994–1998.

[38] SCAVELLI C., DI PIETRO G., CIRULLI T., COLUCCIA M., BOCCARELLI A., GIANNINI T., MANGIALARDI R., BERTIERI R., COLUCCIA A. M., RIBATTI D., DAMMACCO F., VACCA A., *Zoledronic acid affects over-angiogenesis phenotype of endothelial cells in patients with multiple myeloma*, Mol Cancer Ther, 2007, 6(12 Pt 1):3256–3262.

[39] RIBATTI D., PONZONI M., Angiogenesis in malignant and non-malignant pediatric tumors. In: TEICHER B. A., ELLIS L. M. (eds), *Antiangiogenic agents an cancer therapy*, Humana Press, Totowa, NJ, 2008, 475–486.

[40] RIBATTI D., ALESSANDRI G., VACCA A., IURLARO M., PONZONI M., *Human neuroblastoma cells produce extracellular matrix-degrading enzymes, induce endothelial cell proliferation and are angiogenic in vivo*, Int J Cancer, 1998, 77(3):449–454.

[41] RIBATTI D., ALESSANDRI G., BARONIO M., RAFFAGHELLO L., COSIMO E., MARIMPIETRI D., MONTALDO P. G., DE FALCO G., CARUSO A., VACCA A., PONZONI M., *Inhibition of neuroblastoma-induced angiogenesis by fenretinide*, Int J Cancer, 2001b, 94(3):314–321.

[42] RIBATTI D., NICO B., PEZZOLO A., VACCA A., MEAZZA R., CINTI R., CARLINI B., PARODI F., PISTOIA V., CORRIAS M. V., *Angiogenesis in a human neuroblastoma xenograft model: mechanisms and inhibition by tumour-derived interferon-gamma*, Br J Cancer, 2006b, 94(12):1845–1852.

[43] MARIMPIETRI D., NICO B., VACCA A., MANGIERI D., CATARSI M. P., PONZONI M., RIBATTI D., *Synergistic inhibition of human neuroblastoma-related angiogenesis by vinblastine and rapamycin*, Oncogene, 2005, 24(45):6785–6795.

[44] MARIMPIETRI D., BRIGNOLE C., NICO B., PASTORINO F., PEZZOLO A., PICCARDI F., CILLI M., DI PAOLO D., PAGNAN G., LONGO L., PERRI P., RIBATTI D., PONZONI M., *Combined therapeutic effects of vinblastine and rapamycin on human neuroblastoma growth, apoptosis, and angiogenesis*, Clin Cancer Res, 2007, 13(13):3977–3988.

[45] BRIGNOLE C., MARIMPIETRI D., PASTORINO F., NICO B., DI PAOLO D., CIONI M., PICCARDI F., CILLI M., PEZZOLO A., CORRIAS M. V., PISTOIA V., RIBATTI D., PAGNAN G., PONZONI M., *Effect of bortezomib on human neuroblastoma cell growth, apoptosis, and angiogenesis*, J Natl Cancer Inst, 2006, 98(16):1142–1157.

[46] PASTORINO F., BRIGNOLE C., DI PAOLO D., NICO B., PEZZOLO A., MARIMPIETRI D., PAGNAN G., PICCARDI F., CILLI M., LONGHI R., RIBATTI D., CORTI A., ALLEN T. M., PONZONI M., *Targeting liposomal chemotherapy via both tumor cell-specific and tumor-vasculature-specific ligands potentiates therapeutic efficacy*, Cancer Res, 2006, 66(20):10073–10082.

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