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Everolimus Dual Effects of an *Area Vasculosa* Angiogenesis and Lymphangiogenesis

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Abstract. *Recently approved as treatment for astrocytoma, kidney and pancreatic cancer, everolimus acts on tumor cells by inhibiting tumor cell growth and proliferation, as well as by inhibition of angiogenic activity by both direct effects on vascular cell proliferation and indirect effects on growth factor production. The effects of everolimus on early stages of normal vasculogenesis, angiogenesis and lymphangiogenesis are not yet available. We found increased development of intravascular pillars by using area vasculosa of the chick chorioallantoic membrane treated with everolimus. An active lymphangiogenic response was highlighted by the expression of Prospero homeobox protein 1 (Prox1) and podoplanin, together with vascular endothelial growth factor receptor C (Vegf-C) and vascular endothelial growth factor receptor 3 (Vegfr-3) expression on day 4 in the treated group. These findings suggest a potential role of everolimus in the activation of lymphangiogenesis.*

The initiation and maturation of the vasculature is an essential process during embryonic development. The first blood vessels are extra-embryonic and derive from blood islands which become visible in the proximal region of the yolk sac – *area vasculosa* - at about stage 8 of chick embyro development. This process continues with the subsequent appearance of vessels in the *area pellucida* and in the embryo itself.

The *area vasculosa* represents a good experimental model for the study of vasculogenesis and angiogenesis (1). Early steps of extra embryonic vessels development and molecular mechanisms involved in this process have largely been studied by using chick *area vasculosa* model (2-4). Blood vessels' development from endothelial precursors are influenced by several microenvironmental conditions such as mechanical (5, 6), gravitational (7) and chemical factors (8). Many antiangiogenic and antivascular therapeutic agents have been tested in mature vessels chick embryo chorioallantoic membrane (9, 10), but few studies have reported the effects of such substances on the early steps of vessel development in the *area vasculosa* of the chick embryo (11-13).

Recently approved as treatment for advanced kidney cancer (14), subependymal giant cell astrocytoma associated with tuberous sclerosis not suitable for surgical intervention (15) and progressive or metastatic pancreatic neuroendocrine tumors not surgically removable (16), everolimus acts directly on tumor cells by inhibiting tumor cell growth and proliferation, as well as by inhibition of angiogenic activity by both direct effects on vascular cell proliferation and indirect effects on growth factor production. Few reports are available about the effects of everolimus on early steps of *in vitro* vasculogenesis and angiogenesis (17) and there are virtually no data about its effects on lymphangiogenesis nor on the *area vasculosa*.

Thus, the purpose of the present study was to evaluate the effects of everolimus on the *area vasculosa* in regard to early angiogenic and lymphangiogenic events.

Materials and Methods

Experimental design. The chick embryo chorioallantoic membrane model was chosen as the experimental model for the present study because it has the same vasculogenic and angiogenic mechanisms as in humans; moreover, the gene which encodes PROX1, the first and mandatory marker which characterize the differentiation of lymphatic endothelial cells from precursors or venous endothelial cells is highly conserved between chicken and humans, with overall

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homology of 94%. In the region of the homeodomain and the prospero domain, the genes are identical. In addition, the chick embryo choriallantoic membrane model is a rapid, reproducible and reliable model for the study of angiogenesis and lymphangiogenesis because of rapid development of the vascular network and the posibility by direct and dynamic monitoring of this developing structure by stereomicroscopy.

We allocated White Leghorn fertilized eggs to two groups of twenty eggs each, one control group and one everolimus-treated group. The embryos were incubated at 37˚C in a humid atmosphere. The experiment was performed on chicken embryos grown by the shell-free culture method. After three days of incubation, eggs were opened and their contents were carefully poured into a plastic Petri dish, 80 mm in diameter. The study started from the third day of incubation by applying 50 μl of diluted everolimus (saline solution, 5 ng/ml, 10 ng/ml, 20 ng/ml, Fluka, Buchs, Switzerland) twice per day. Everolimus concentrations and time points had been chosen according to the previously established protocols described in other studies which used everolimus or its analogues as treatment for implanted tumor tissues in rat and chick embryo chorioallantoic membrane models (18, 19). On experimental day 4, immediatelly before collecting the specimens, intravenous injections of 0.1 ml of 2.5% fluorescein isothiocyanate (FITC) (2000 kDa, Sigma–Aldrich Chemie Gmbh, München, Germany) was injected; the control group was treated with saline solution for the same period.

Pillar quantification. Vessels were visualized by fluorescence microscopy and microvascular patterns were monitored using an LE CCD Optronics video camera (Visitron System, Puchheim, Germany). Fluorescence microscopy was performed with a Polyvar-Reichert microscope using ×10 and ×25 objectives.

Primary processing. *Area vasculosa* specimens collected on day four of the experiment, were fixed in 10% buffered formalin and paraffin embedded. Three micrometers sections were obtained from each paraffin block and one slide from each specimen was stained by routine haematoxylin and eosin method for microscopic evaluation.

Immunohistochemistry. Additional sections from each embryo were immunostained by using a panel of antibodies for lymphatic endothelial cells, in order to enable a more complete immunohistochemical characterization of lymphangiogenesis starting from precursors. Antibody towards PROX1 antigen highlights *area vasculosa* cells which differentiate through the lymphatic lineage together with receptor 3 for vascular endothelial cell factor C (Vegfr 3). Ligand for Vegfr 3, vascular endothelial growth factor C (Vegf C) was immunohistochemically assessed. Antibody towards podoplanin a 36 kDa type I transmembrane mucoprotein with several O-glycosylation sites was also used. Podoplanin represents a target gene of the homeobox gene PROX1. It characterizes already differentiated lymphatic endothelial cells. All specific features of the antibodies mentioned, their clones, dilutions, manufacturers, antigen retrieval methods, immunohistochemical methods applied, chromogens and positive controls used in the present study are summarized in Table I.

All immunohistochemical procedures were performed in an automated manner, by using PT Link machine (Dako Cytomation, Carpinteria, California, USA) for automated dewax and antigen retrieval steps and Dako Autostainer (Dako Cytomation, USA) for next steps which complete the immunohistochemical procedure.

Results

First evidences for the effects of everolimus on immature vessels of *area vasculosa* were observed on *in vivo* angiogenesis assessement after FITC injection for the group treated with 5 ng/ml everolimus. Vessels were small, with particular morphology in the everolimus-treated group. All three parameters (vessel area, vessel density and pillar density) evaluated per 10^4 μm^2 *area vasculosa* were different in the control group as compared with the treated one. The vascular area increased from an average of 3434.25 μm^2 in the PBS-treated control group to 4018.5 μm^2 for everolimus-treated group. Complete and/or incomplete intravascular pillar projections observed in the everolimus-treated group produced a highly splitting appearance of the blood vessel lumen from FITC injected vessels (Figure 1) which can explain the higher vascular area found in the everolimus-treated group. By quantification of pillar density, the mean ratio of pillar in the treated compared to the control group was 2.4. Microvessel density was slighty increased in the treated group. We noticed no changes in microvessel density, vascular area and pillar number for groups treated with 10 and 20 ng/ml everolimus.

On the same specimens we performed immunohistochemical staining for lymphatic markers in order to determine if everolimus has any effects on early stages of lymphangiogenesis from the *area vasculosa*. Contrary to data from the literature that showed lymphatics in the chick embryo chorrioallantoic membrane on day 6 of incubation, our present finding supports the occurrence of early lymphangiogenic events in the *area vasculosa* from day 4 of gestation. We detected scattered positive nuclear signal (2 to 3 per microscopic field at ×200) in vascular islands from the *area vasculosa* of the control group using anti-Prox1 antibody immunostaining. An interesting finding was observed in the *area vasculosa* treated with 20 ng/ml everolimus. In this case, clusters of PROX1 positive cells mixed with vascular precursors were detected in vascular islands (Figure 2). Vegfr 3 and podoplanin were overexpressed in the 20 ng/ml everolimus-treated group in comparison with the control. In addition, several podoplanin-expressing cells were detected in the treated group for the same dilution. These cells were distributed in small groups and had numerous branches. Their cytoplasm had a high tendency to become vacuolated and to form lumen (Figure 3).

Discussion

The *area vasculosa* of the chick embryo chorioallantoic membrane is a potent angiogenic site in the early development of vascular network which mimics the first steps of angiogenesis and lymphangiogenesis described in the human embryo yolk sac very well. It can be considered

Table I. *Detailed characterization of antibodies and their working system features.*

Antibody name	Clone	Dilution	Incubation time	Expression pattern	Positive control
Prox1 (Acris Antibodies, Herford, Germany)	Polyclonal	1:200	1 hour	Nuclear	Developing nervous tissue
VEGFR3 (Neomarkers, Fremont, California, USA)	Polyclonal	Ready to use	30 minutes	Membranar and cytoplasmic	Endothelium of lymph vessels
VEGF C (Santa Cruz Biotechnology, Santa Cruz, California, USA)	Polyclonal	1:200	30 minutes	Cytoplasmic	Human colon cancer tissue
Podoplanin (Santa Cruz Biotechnology, Santa Cruz, California, USA)	Monoclonal, Gp 36	1:200	1 hour	Cytoplasmic	Type 1 alveolar cells from lung

as a good model for vascular development studies because it contains blood islands with endothelial precursors. Both intussusceptive and sprouting angiogenesis can be found here. These two angiogenic mechanisms have different molecular pathways, and are influenced by different inhibitors (20). No significant effects of everolimus were observed concerning microvessel density in the *area vasculosa* from control and treated groups but a significant increase of pillar density and vascular area was found in the group treated with 5 ng/ml.

Everolimus most likely suppresses the vascular sprouting and this is the reason for extensive intussusceptive angiogenesis. Everolimus-induced angiogenic switch from sprouting to intussusception was previously reported by Piguet *et al*. (19), in a syngeneic orthotopic model of angiogenesis in hepatocellular carcinoma after combined treatment of everolimus and sorafenib. For tumor blood vessels, the switch from sprouting to intussusceptive angiogenesis represents a tumor escape mechanism as a part of an angioadaptative mechanism that probably serves to repair antiangiogenic drug-damaged tumor vasculature. Like everolimus, other angiogenic inhibitors can induce development of intravascular pillar. Hlushchuck *et al*. (21) reported the same phenomenon of intussusceptive mechanism switch for mammary carcinoma allograft treated with PTK787/ZK222854, a specific inhibitor of both VEGF-receptor tyrosine kinases.

Everolimus, a rapamycin analog, is a macrolide with potent immunosuppressive and antiproliferative properties. Like rapamycin, everolimus binds the cyclophilin (FKBP-12), and this complex binds the serine-threonine kinase mammalian target of rapamycin (mTOR), when it is associated with raptor and (mLST8), to form a complex (mTORC1), which inhibits signaling downstream (22). The effects of everolimus is solely on the mTORC1 protein and not on the mTORC2 protein. As a reactive response to this mTORC1-selective inhibition, everolimus produces hyperactivation of the kinase (AKT) by not inhibiting the mTORC2-positive feedback loop. It has already been showed that (PI3K)/AKT cell signaling pathway activation is necessary for lymphatic reprogramming of Kaposi sarcoma endothelial cells through specification of PROX1 transcription factor induction on lymphatic endothelial cell (23). Previous reports sustain our immunohistochemical finding of increasing of PROX1 positive cells number in 20 ng/ml *area vasculosa* treated group compared with control group.

The increased number of PROX1 positive cells induced by this high dose of everolimus raises many questions regarding the involvement of local factors and intussusceptive angiogenesis in lymphangiogenesis. Higher blood pressure produced by intussusception induced development of intravascular pillars in a previous study (24) and in everolimus-treated *area vasculosa* was probably followed by more plasma extravasation in the extracellular matrix and increased interstitial pressure, which would lead to a greater need to drain the fluid. This hypothesis is sustained by previous studies regarding flow-guided lymphangiogenesis (25-27).

Although everolimus is already approved as therapy for a few cancer types, we were not able to find direct evidence of everolimus affecting tumor lymphangiogenesis. Among recent articles concerning everolimus use in patients with metastatic breast and kidney cancer or in experimental tumor models, only two suggested indirect evidence concerning potential activation of lymphangiogenesis by everolimus treatment. The most recent reported that patients with PTEN loss metastatic breast cancer and treated with everolimus had lower overall survival. It is well known that *PTEN* loss activates the AKT pathway, also involved in lymphangiogenesis and, probably, activation of lymph vessel development stimulates lymphatic metastasis (28). For a mouse model of thyroid cancer, it was shown that everolimus treatment did not prevent vascular invasion and lymphatic metastatic spread of tumor cells (29).

Our findings demonstrate by direct *in vivo* evidence the effects of everolimus on the early steps of angiogenesis in the *area vasculosa* of the chick embryo chorioallantoic membrane, and, to our knowledge, for the first time its role in lymphangiogenesis induction. For ethical reasons this study can not be transferred to human embryos *in vivo* and this could be

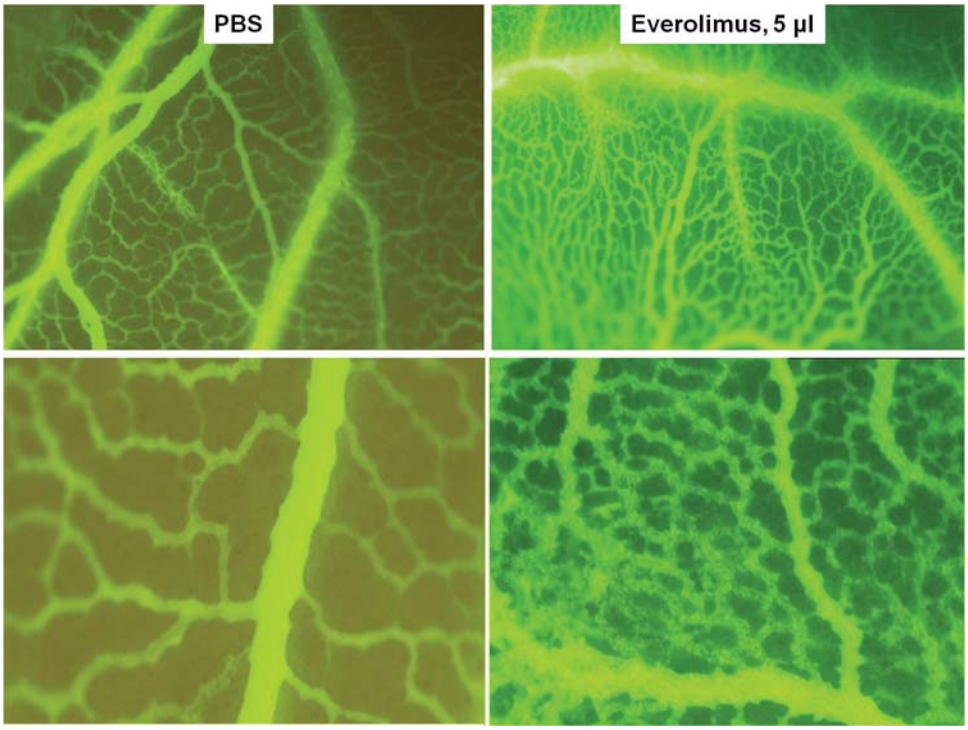

Figure 1. *Fluorescein isothiocyanate (FITC) injected vessel assessement from the control group and and that treated with 5 ng/ml everolimus. Large number of pillars can be seen in the treated group at ×200 and ×400 magnification (right) compared with the PBS control group (left).*

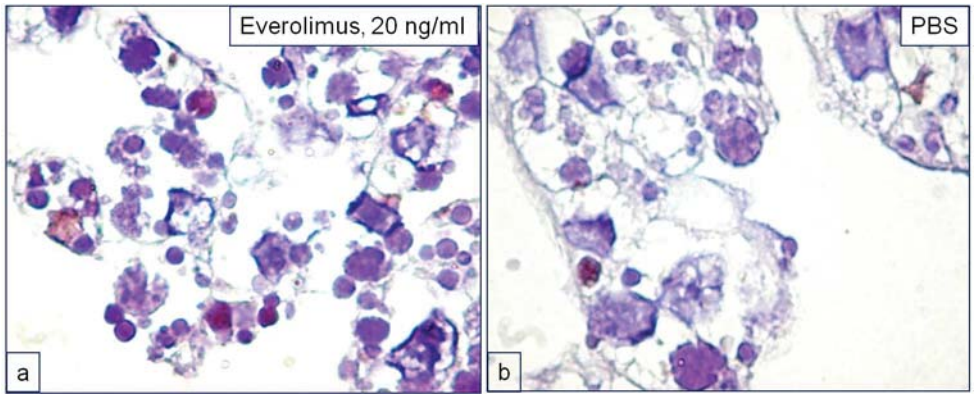

Figure 2. *Presence of Prospero homeobox protein 1 (PROX1)-positive lymphatic precursors on day 4 of area vasculosa development. Note the higher density of PROX1-positive nuclei in the group treated with 20 ng/ml everolimus (a) and few PROX1-positive cells in the control group (b) (×400).*

considered as a limitation of the present study. However, developmental similarities and over 90% homology of *PROX1* gene between chicken and humans. make this study reliable.

In conclusion, everolimus acts in a dose dependent manner on normal angiogenesis and lymphangiogenesis in the *area vasculosa*. It stimulates pillar development at a low dose and promotes lymphatic endothelial cells differentiation from precursor cells of blood islands at higher concentrations.

We presented here the first experimental data about the effects of everolimus in chick embryo *area vasculosa* normal blood vessels and lymphatic endothelial cells differentiation. Our findings could be useful for a better understanding of the different responses of normal and tumor blood or lymphatic vessels to everolimus treatment and might explain, in part, the controversies regarding the effects of everolimus treatment on lymphatic spread in recent clinical trials in humans.

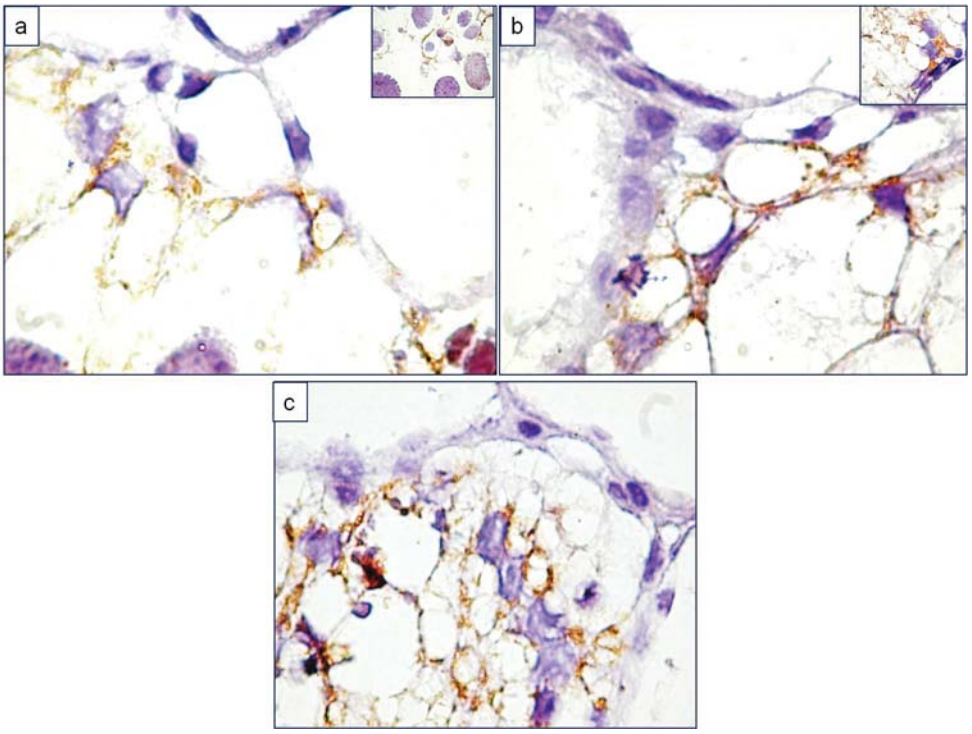

Figure 3. *Expression of lymphatic markers podoplanin, vascular endothelial growth factor receptor 3 (VEGFR3) and vascular endothelial growth factor C (VEGF C) in the group treated with 20 ng/ml everolimus. VEGFR3-positive cells, are apparent, some of them with high tendency to become vacuolated and to form lumen (a). VEGFR3-positive structure with luminal morphology and nucleated cell inside it (a, inset). Podoplanin-(b) and VEGF C-(c)-positive cells and luminal structures from the group treated with 20 ng/ml everolimus area vasculosa (×400).*

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