Members of the VEGF family are key mediators of hem- and lymphangiogenesis and are expressed under physiologic as well as pathologic conditions. Intracellular downstream signaling is mediated by VEGF binding to specific tyrosine kinase VEGF receptors (VEGF-R), VEGF-R1 (also known as Fms-like tyrosine kinase-1), and VEGF-R2 (also known as kinase insert domain receptor/Flik-1), which are primarily expressed on the surface of vascular endothelial cells.

In numerous malignant tumor cells, VEGF-A was found to be overexpressed, thus stimulating the development and maintenance of sufficient tumor vasculature. This VEGF-driven tumor-associated angiogenesis is not only mandatory for primary tumor growth once exceeding the size sustainable by mere nutrient and oxygen diffusion (approximately 2- to 3-mm tumor diameter), but it is also regarded as the initial preparatory step for distant metastasis.

Elevated VEGF-A levels have also been detected in eyes of patients with uveal melanoma (UM). Increased VEGF-A aqueous concentrations were found to be associated with larger tumor dimensions. Sahin et al. reported that increased VEGF-A immunoreactivity in enucleated eyes of UM patients was associated with the presence of distant metastases. Also, higher VEGF-A serum concentrations were correlated with a raised metastatic frequency and poor survival in a murine UM model described by Crosby et al.

Of interest, it has recently been shown, for various nonocular cancer entities, that malignant cells, apart from abundant VEGF secretion, also express the corresponding receptors VEGF-R1 and VEGF-R2. This suggests that tumor-originated VEGF-A signaling might affect tumor cell behavior itself, besides its well-established pro-angiogenic features on vascular cells. In fact, autocrine VEGF-A tumor-cell stimulation, which can comprise increased mitogenic activity, tumor cell survival, increased invasiveness, resistance to anticancer drugs, or further amplification of VEGF-A expression, has been demonstrated for numerous malignancies, including cutaneous malignant melanoma, breast cancer, squamous cell carcinoma of the head and neck, bladder cancer, and acute myeloid leukemia.

Based on this knowledge, we were interested to analyze whether UM cells are influenced by VEGF-A in an autocrine stimulatory manner as well. This could have implications on developing adjuvant strategies in UM treatment, as anti-VEGF-A therapeutics such as bevacizumab or VEGF-R antagonists, primarily directed against tumor vasculature, would then address UM progression even in a 2-fold respect.

**PURPOSE.** Tumor-derived VEGF-A, apart from expediting sufficient vascularization, subsequent tumor growth, and metastatic spread, can act on malignant cells themselves provided that VEGF receptors 1 or 2 (VEGF-R1, -R2) are co-expressed. The study goal was to investigate whether such autocrine VEGF-A signaling exists in uveal melanoma (UM).

**METHODS.** Primary (MEL-270, OM-431) and metastatic (OMM-2.3, OMM-2.5) UM cell lines were analyzed for VEGF-A, VEGF-R1, and VEGF-R2 expression by RT-PCR, ELISA (VEGF-A protein), and immunocytochemistry (VEGF receptors). Proliferation of UM cells incubated with neutralizing anti-VEGF-A antibody bevacizumab (≤2.5 mg/mL), or VEGF-A (≤100 ng/mL) was assessed by bromodeoxyuridine (BrdU) ELISA. It was measured by real-time PCR, whether VEGF-A (100 ng/mL) modulated the expression ratio of VEGF-A itself and its antiangiogenic antagonist pigment epithelium-derived factor (PEDF).

**RESULTS.** All UM cells expressed VEGF-A, VEGF-R1, VEGF-R2 mRNA, and protein. In each cell line, the proliferation was stimulated by VEGF-A or inhibited by blocking VEGF-A, or both: bevacizumab significantly decreased the proliferation in MEL-270 (P = 0.005), OMM-2.3 (P = 0.001), and OMM-2.5 (P = 0.011). Increased VEGF-A signaling significantly raised the proliferation in MEL-270, OM-431 (P < 0.001, respectively), and OMM-2.3 (P = 0.043) in a dose-dependent manner but did not significantly change the VEGF-A/PEDF mRNA expression ratio.

**CONCLUSIONS.** Autocrine VEGF-A signaling seems to be present in UM, sustaining the proliferation of both primary and metastatic UM cells. Apparently, VEGF-A signaling in UM cells neither acts retroactively on VEGF-A expression, in the sense of a feedback loop, nor contributes to a pro-angiogenic shift of the VEGF-A/PEDF ratio.

**Keywords:** VEGF-A, bevacizumab, autocrine signaling, uveal melanoma, tumor growth, proliferation

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**Autocrine Impact of VEGF-A on Uveal Melanoma Cells**

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Malignant melanoma, breast cancer, squamous cell carcinoma of the head and neck, bladder cancer, and acute myeloid leukemia.
Therefore, we analyzed VEGF-A and VEGF-R1/R2 expression profiles of two primary and two metastatic human UM cell lines and investigated in vitro whether (1) the inhibition of autocrine VEGF-A signaling by the neutralizing anti–VEGF-A antibody bevacizumab, or (2) the increased VEGF-A levels by the addition of exogenous recombinant human VEGF-A modified the proliferative capacity of UM cells. Furthermore, we investigated whether VEGF-A signaling in UM cells influenced the expression of pro-angiogenic VEGFA (feedback loop) or of antiangiogenic pigment epithelium-derived factor (PEDF). An increase in the VEGF-A/PEDF ratio has been held co-responsible for the “angiogenic switch” leading from tumor dormancy to a more aggressive phenotype and metastatic disease in UM (Yang H, et al. IOVS 2004;45:ARVO E-Abstract 5198).21

**Materials and Methods**

**Cell Lines and Culturing Conditions**

The human primary UM cell line OM-431 was originally isolated by Daniel M. Albert (University of Wisconsin, Madison, WI, USA).22 The primary UM cell line, MEL-270, and the metastatic UM cell lines, OMM-2.3 and OMM-2.5, were isolated by Bruce R. Ksander (Schepens Eye Research Institute, Boston, MA, USA).23 All UM cell lines used for the present work were kindly provided by Bruce R. Ksander (Schepens Eye Research Institute, Boston, MA, USA).23 All UM cell lines used for the present work were kindly provided by Bruce R. Ksander (Schepens Eye Research Institute, Boston, MA, USA).25 All UM cell lines used for the present work were kindly provided by Bruce R. Ksander (Schepens Eye Research Institute, Boston, MA, USA).25 All UM cell lines used for the present work were kindly provided by Bruce R. Ksander (Schepens Eye Research Institute, Boston, MA, USA).25 All UM cell lines used for the present work were kindly provided by Bruce R. Ksander (Schepens Eye Research Institute, Boston, MA, USA).25 All UM cell lines used for the present work were kindly provided by Bruce R. Ksander (Schepens Eye Research Institute, Boston, MA, USA).25

**Analysis of VEGF-A, VEGF-R1, VEGF-R2, and PEDF mRNA Expression**

Uveal melanoma cells were grown to 90% confluence. Messenger RNA was isolated using RNasea Micro Kit (Qiagen, Valencia, CA, USA). Complementary DNA was synthesized with random hexamers using reverse transcriptase (SuperScript III; Invitrogen, Carlsbad, CA, USA). Primers (MWG Biotech, Ebersberg, Germany) were designed using Primer3 software and BLAST (Basic Local Alignment Search Tool, National Center for Biotechnology Information) as described previously.27 Polymerase chain reactions (25 μL) contained 50 ng cDNA, 0.4 μM of each forward and reverse primer, and master mix (SoFast EvaGreen Supermix; Bio-Rad, Hercules, CA, USA). Reverse transcriptase-PCR was performed under the following conditions: initial denaturation step at 95°C for 2 minutes, 40 cycles at 95°C for 5 seconds and at 60°C for 15 seconds, followed by an additional denaturation step at 95°C for 60 seconds. All RT-PCR products were analyzed by gel electrophoresis on a 2% agarose gel and were visualized by GelRed nucleic acid staining (Biotium, Inc., Hayward, CA, USA) using the ChemiDoc Imager (Bio-Rad, Munich, Germany). Real-time PCRs were performed under the above-mentioned conditions to quantify VEGF-A and PEDF mRNA expression, using HPRT1 as reference gene. A no-template control (NTC) was included in all experiments to exclude DNA contamination. Messenger RNA isolation, cDNA synthesis, and PCRs were performed twice. Primer sequences were as follows: HPRT1 forward 5'-CCTGCGTTCGTTATGAGTG-3', reverse 5'-GGCTCCCAT CTCCTTACAC-3'; VEGF-A forward 5'-ACAGGTACAGGGATGA GGACAC-3', reverse 5'-AAGCAGTTGAAGATGAGCGAG-3'; VEGF-R1 forward 5'-CTACACTCTCCTGAGACAGA-3', reverse 5'-GCTCCACTCTTACAGAACA-3'; VEGF-R2 forward 5'-ACC TCAGCCTTCTCCGTGATG-3', reverse 5'-GACGTATCTC CTTGCGTCGTGATTAGTG-3' and PEDF forward 5'-CATTCCTCTTCTCG GTGG-3', reverse 5'-ACGCGTCTCTTCTCTCAAC-3'.

**ELISA Analysis of UM Cell–Secreted VEGF-A Protein**

Uveal melanoma cells were seeded on a 24-well plate at a concentration of 1.5 x 10^4 cells/mL and were left to attach for 8 hours. Full medium was replaced by FCS-free RPMI medium. After 12 hours, supernatants were collected and then evaluated for VEGF-A concentrations using a human VEGF Duoset ELISA Kit (R&D Systems).

**Immunocytochemistry of VEGF-R1 and VEGF-R2 Expression**

Coverslips were placed into the wells of a 24-well plate and precoated with 0.1% gelatin. Uveal melanoma cells were then seeded into the wells (concentration, 1.5 x 10^4 cells/mL) and left to attach overnight. Full medium was replaced, and cells were fixed in 3% paraformaldehyde in PBS for 15 minutes. After blocking unspecific staining in a buffer containing 10% FCS and 0.2% Triton X-100 for 45 minutes, cells were incubated overnight at 4°C with either rabbit anti–human-VEGF-R1 antibody or rabbit anti-human-VEGF-R2 antibody (both from Zytomed Systems, Hamburg, Germany), both diluted 1:100 in PBS with 2% FCS. Primary antibody binding was detected using an AlexaFluor 550 goat anti-rabbit secondary antibody (Life Technologies). After washing in PBS, coverslips were carefully removed from the wells and mounted with the stained cells towards the microscope slide. Cell nuclei were counterstained with DAPI (Carl Roth, Karlsruhe, Germany), which was added to the fluorescence anti-fade mounting medium (Dako, Hamburg, Germany) to a final concentration of 1 μg/mL. Vascular endothelial growth factor–R1 and VEGF-R2 fluorescence staining was assessed using a Leica TCS SP8 confocal laser microscope (Leica, Wetzlar, Germany). Human placenta sections served as positive tissue control. The antibody diluent alone instead of the primary antibody was used as negative control.

**BrdU Cell Proliferation Assays**

For the proliferation assays, UM cells were seeded at a density of 4 x 10^3 on a 96-well plate in full medium and were left to attach for 24 hours. Thereafter, cells were incubated with different concentrations of either bevacizumab (Avastin; Roche Pharma, Grenzach, Germany) or human recombinant VEGF-A isoform 165 (Peprotech, Hamburg, Germany) as follows:

1. Influence of bevacizumab: Bevacizumab was added to the full medium resulting in final concentrations of 0.25 mg/mL or 2.5 mg/mL, respectively; and
2. Influence of recombinant human VEGF-A: The full medium was aspirated and replaced by FCS-free RPMI medium containing human recombinant VEGF-A isoform 165 at concentrations of 10 ng/mL or 100 ng/mL.

Twenty-four hours later, each well was supplemented with 10 μL of BrdU-labeling solution (Colorimetric BrdU Cell Proliferation ELISA; Roche, Mannheim, Germany). After 4 hours of incubation, cells were fixed and stained according to the manufacturer’s instructions. Colorimetric analysis of BrdU
incorporation was performed with an ELISA plate reader (Epoch Microplate Spectrophotometer; Biotek, Bad Friedrichshall, Germany), at 450 nm (reference wavelength, 690 nm). For each UM cell line, the mean absorbance of the control wells (medium without bevacizumab/VEGF-A) was calculated and defined as 100%. Mean absorbance values of the other experimental groups were related to this value. The assay was performed three times.

Cytotoxicity Assay

Bevacizumab concentrations used in the BrdU cell proliferation assay were analyzed for cytotoxic effects on UM cells using Trypan blue viable cell counting. Cells in full medium were seeded at a density of $1.5 \times 10^4$ per well in a 96-well plate and cultured until reaching confluence. Full medium was then removed and replaced by an equal volume (100 µL) of FCS-free RPMI medium containing bevacizumab concentrations of 0.25 mg/mL or 2.5 mg/mL. After incubation for 24 hours at 37°C, the medium was aspirated from each well. Wells were incubated with 30 µL Trypsin-EDTA (PromoCell, Heidelberg, Germany) for 2 minutes, followed by adding an equal volume of trypsin neutralizing solution (TNS; PromoCell) in each well. Cells were resuspended and mixed with an equal volume of 0.4% Trypan blue, immediately followed by counting unstained (viable) cells and stained (dead) cells in the Neubauer counting chamber. Every cell with a slight blue staining was classified as dead.

Statistical Analysis

Quantitative study data in the text are given as mean ± SD. Differences in the quantitative study data, including VEGF-A ELISA data, proliferation and viability assays, and real-time PCR data were tested for statistical significance at a significance level of $\alpha = 0.05$, using the Mann-Whitney U test. Spearman’s rho ($\rho$) correlation coefficient was used to measure the strength of association between bevacizumab/VEGF-A concentrations and the proliferation ratio. Statistical analysis was conducted using SPSS software (Version 19.0; IBM, Chicago, IL, USA).

RESULTS

Induction of Hypoxia in UM Cells

Hypoxia of UM cells was assured by HIF-1α ELISA 10 hours and 24 hours after addition of CoCl$_2$ to the culture medium. At both time points, CoCl$_2$ treatment was associated with a significant, at least 4-fold increase in HIF-1α protein expression in all cell lines ($P < 0.001$ in all cases, data not shown).

Expression of VEGF-A, VEGF-R1, VEGF-R2 mRNA, and Protein in UM Cell Lines

Reverse transcription–PCR analysis was performed in order to detect mRNA expression of the primary UM cell lines MEL-270, OM-431, and the metastatic UM cell lines OMM-2.3 and OMM-2.5. All four cell lines expressed VEGF-A mRNA (Fig. 1A). Vascular endothelial growth factor-A protein as assessed by sandwich ELISA was also expressed in all cell lines (Fig. 1B). Concentrations of secreted VEGF-A were $55.72 \pm 11.09$ pg/mL for MEL-270, $122.70 \pm 20.36$ pg/mL for OM-431, $55.70 \pm 22.92$ pg/mL for OMM-2.3, and $263.65 \pm 22.70$ pg/mL for OMM-2.5. The OMM-2.5 cells secreted significantly more VEGF-A than each of the other cell lines: MEL-270, OM-431, and OMM-2.3 ($P = 0.007$, $P = 0.023$, and $P = 0.012$, respectively).

Messenger RNA of both VEGF-A receptors VEGF-R1 and VEGF-R2 was expressed by the four cell lines MEL-270, OM-431, OMM-2.3, and OMM-2.5 (Fig. 2A). Expression of VEGF-R1 and VEGF-R2 at the protein level was assessed by immunocytochemistry and confocal laser microscopy (Fig. 2B). All four cell lines showed considerable staining for both VEGF-R1 and VEGF-R2, which was in line with mRNA expression profiles. Vascular endothelial growth factor–R1 and VEGF-R2 protein expression were detectable in the entire cytosol including the perinuclear region. Negative controls in all four cell lines did not show any staining, thus excluding unspecific binding of the detection system.

Proliferation Assays

Uveal melanoma cells were incubated with two different concentrations of the neutralizing anti–VEGF-A antibody bevacizumab. Proliferation was assessed by BrdU incorporation. Cell lines MEL-270, OMM-2.3, and OMM-2.5 showed a dose-dependent inhibition of proliferation by bevacizumab (Fig. 3A). Compared with untreated cells (proliferation = 100%) incubation with 2.5 mg/mL bevacizumab led to a significantly decreased proliferation ratio of $87\% \pm 18\%$ in MEL-270 cells ($P = 0.005$), $90\% \pm 4\%$ in OMM-2.3 cells ($P = 0.001$), and $91\% \pm 7\%$ in OMM-2.5 ($P = 0.011$). The two variables, bevacizumab concentration and proliferation ratio, were negatively correlated. Spearman’s rho ($\rho$), as a measure for the strength of this association, was $-0.22 (P = 0.011)$ for MEL-270 cells, $-0.49 (P = 0.005)$ for OM-431 cells, $-0.43 (P = 0.012)$ for OMM-2.3 cells, and $-0.52 (P = 0.007)$ for OMM-2.5 cells.
As opposed to the latter cell lines, proliferation of cell line OM-431 was not decreased under the influence of bevacizumab. None of the tested concentrations was associated with a significant change of BrdU incorporation in comparison with untreated cells: proliferation ratio 100% ± 7% for untreated cells, 99% ± 2% for bevacizumab 0.25 mg/mL, and 100% ± 2% for bevacizumab 2.5 mg/mL.

In addition, we tested whether exogenous VEGF-A affected UM cell proliferation. Cell lines MEL-270, OM-431, and OMM-2.3 showed a dose-dependent increase of BrdU incorporation when treated with VEGF-A (Fig. 3B). In MEL-270, compared with untreated cells (100% ± 35%) the proliferation ratio was significantly increased to 141% ± 27% at 10 ng/mL VEGF-A (P = 0.001), and to 165% ± 21% at 100 ng/mL VEGF-A (P < 0.001). In OM-431, compared with untreated cells (100% ± 18%) the proliferation ratio was significantly raised to 114% ± 19% (P = 0.005) at 10 ng/mL VEGF-A, and further increased to 122% ± 20% (P < 0.001) by 100 ng/mL VEGF-A. In OMM-2.3, the proliferation ratio was significantly increased by 100 ng/mL VEGF-A (118% ± 26%, P = 0.043) but remained unchanged under 10 ng/mL VEGF-A (98% ± 19%). The two variables VEGF-A concentration and proliferation ratio were positively correlated. Spearman's rho (\( \rho \)), as a measure for the strength of this association, was 0.70 (P < 0.001) for MEL-270, 0.42 (P < 0.001) for OM-431, and 0.28 (P = 0.049) for OMM-2.3. As opposed to that, no significant change in proliferation was detectable in cell line OMM-2.5, when incubated with the respective VEGF-A concentrations (P > 0.05, respectively): proliferation ratio 100% ± 14% for untreated cells, 95% ± 11% with 10 ng/mL VEGF-A, and 106% ± 19% with 100 ng/mL VEGF-A.

### Cytotoxicity Assay

Uveal melanoma cells were tested for cytotoxic effects of bevacizumab in concentrations of 0.25 mg/mL and 2.5 mg/mL, which had previously been used in the proliferation assay, by Trypan blue viable cell counting (Fig. 4). In none of the four cell lines, did incubation with bevacizumab significantly affect
the ratio of viable and dead cells in comparison with medium alone \((P > 0.05, \text{ respectively})\). In MEL-270, the VCR was 93\% \pm 1\% in the control group (medium alone), 95\% \pm 2\% under 0.25 mg/mL bevacizumab, and 92\% \pm 3\% under 2.5 mg/mL bevacizumab. In OM-431, the VCR was 91\% \pm 2\% in the control group, 90\% \pm 2\% under 0.25 mg/mL bevacizumab, and 90\% \pm 3\% under 2.5 mg/mL. In OMM-2.3, the VCR was 89\% \pm 2\% in the control group (medium alone), 89\% \pm 3\% under 0.25 mg/mL bevacizumab, 88\% \pm 2\% under 2.5 mg/mL. In OMM-2.5, the VCR was 89\% \pm 4\% in the control group (medium alone), 87\% \pm 4\% under 0.25 mg/mL bevacizumab, 89\% \pm 5\% under 2.5 mg/mL. A toxic effect of bevacizumab concentrations up to 2.5 mg/mL on UM cells was thus not detectable.

**Impact of VEGF-A on UM Cell Expression of Pro-Angiogenic (VEGF-A) and Antiangiogenic (PEDF) Growth Factors**

In order to analyze whether increased VEGF-A signaling modified the expression of VEGF-A itself or the expression of antiangiogenic PEDF, mRNA levels of these two growth factors were measured via real-time PCR in untreated UM cells and incubated with 100 ng/mL recombinant VEGF-A. In all four UM cell lines, the increased VEGF-A signal did not affect the expression level of either pro-angiogenic VEGF-A or antiangiogenic PEDF in a significant way. Compared with untreated cells, the relative VEGF-A mRNA expression under the influence of 100 ng/mL VEGF-A was 1.04 \pm 0.03 in MEL-270, 1.05 \pm 0.11 in OM-431, 0.91 \pm 0.07 in OMM-2.3, and 1.01 \pm 0.04 in OMM-2.5 \((P > 0.05 \text{ in all cases, Fig. 5A})\). Compared with untreated cells, the relative PEDF mRNA expression with 100 ng/mL VEGF-A was 1.06 \pm 0.16 in MEL-270, 0.94 \pm 0.12 in OM-431, 1.00 \pm 0.08 in OMM-2.3, and 1.49 \pm 0.34 in OMM-2.5. None of these differences were statistically significant (Fig. 5B). At most, there was a statistical trend \((P = 0.12)\) for increased PEDF expression in metastatic OMM-2.5 cells incubated with VEGF-A. Treatment with VEGF-A was thus not associated with an increase in the VEGF-A/PEDF expression ratio in any UM cell line.

**DISCUSSION**

As a prerequisite for potential autocrine VEGF-A effect on UM cells, we first investigated whether VEGF-A and its receptors,
VEGF-R1 and VEGF-R2, were expressed by UM cells. In both primary and both metastatic UM cell lines VEGF-A expression could be verified on the mRNA and the protein level. This finding is in accordance with previous publications, which also found VEGF-A expressed by several UM cell lines, including MEL-270, OM-431, OMM-2.3, and OMM-2.5. 28–31

With regard to VEGF receptor expression, RT-PCR analysis and immunocytochemistry showed that VEGF-R1 and VEGF-R2 were both expressed by all four cell lines, thus basically enabling VEGF-A to impact primary as well as metastatic UM cells in an autocrine manner. Both receptors, VEGF-R1 and VEGF-R2, have already been shown to mediate autocrine VEGF-A signaling in nonocular malignant tumor cell entities.15,32,33

We investigated whether inhibition of VEGF-A signaling by bevacizumab affected UM cell proliferation. In three of the four cell lines (MEL-270, OMM-2.3, and OMM-2.5), proliferation was significantly decreased when incubated with bevacizumab. Only OM-431 cell proliferation remained unaffected by bevacizumab. Recently, El Filali et al.54 performed proliferation assays on two human UM cell lines (including OMM-2.3) treated with bevacizumab and found a dose-dependent decrease of proliferation, which corresponds to our results. Yang et al.35 reported accordingly that bevacizumab, apart from downregulating the proliferation marker Ki-67 in UM cells (cell line MEL-290) in vitro, significantly decreased the growth of primary UM tumors in the in vivo setting of a murine UM model as well.

A cytotoxicity assay was performed in order to clarify whether the observed decrease in melanoma cell proliferation of MEL-270, OMM-2.3, OMM-2.5 cells was in fact attributable to VEGF-A blockade and not to potential toxicity of bevacizumab. The latter did not reveal any evidence for toxic effects of bevacizumab concentrations between 0.25 mg/mL and 2.5 mg/mL on any of the cell lines. In previous studies, such bevacizumab concentrations were not found to be toxic on a range of nonmalignant ocular cell entities either, including corneal keratinocytes, corneal fibroblasts, corneal endothelial cells,36 choroidal endothelial cells, retinal ganglion cells, and retinal pigment epithelial cells.57–59

Of interest, we observed increased melanoma cell proliferation when VEGF-A levels were further raised by adding exogenous VEGF-A. This effect was statistically significant for cell lines MEL-270, OM-431, and OMM-2.3. Only OMM-2.5 cells were not significantly stimulated by exogenous VEGF-A. In view of the fact that secreted VEGF-A protein levels in OMM-2.5 supernatants were significantly higher than in the three other cell lines, it is likely that endogenous VEGF-A expression in OMM-2.5 was already sufficient to induce the full extent of autocrine VEGF-A signaling.

In the case of OM-431, bevacizumab treatment did not show any antiproliferative effect, yet these cells did respond to exogenous VEGF-A with increasing proliferation. Reasons for this irresponsiveness to bevacizumab might be that the amount of endogenous VEGF-A expression was below the threshold, which might be necessary to significantly affect proliferation in these cells. However, VEGF-A protein expression in OM-431 was not significantly lower than in other cell lines. A different explanation could be a mainly intracellular interaction of VEGF-A and its receptors. In several nonocular tumor cells, including subsets of acute leukemia cells and breast cancer cells, VEGF-R1 and VEGF-R2 receptors were shown to be predominantly localized in the intracellular compartment.32,33 In these cases, autocrine tumor cell stimulation by VEGF-A exists, but it remains a primary intracrine process, which evades inhibition by extracellular neutralizing agents such as bevacizumab.49 Confocal laser microscopic assessment of VEGF receptor expression in our study revealed a distinct intracellular localization including the perinuclear region of both VEGF-R1 and VEGF-R2, which is a requirement for intracrine signaling, even though this finding was not exclusive for OM-431 cells.

Apart from proliferative effects, we evaluated whether autocrine VEGF-A, as in some nonocular malignant cell entities,12–17 could potentially impact its own expression level in terms of a positive or negative feedback loop, and/or the expression of the antiangiogenic antagonist PEDF. A rise of the VEGF-A/PEDF ratio was shown to further sustain tumor-associated angiogenesis (angiogenic switch) and consecutive tumor progression. However, under VEGF-A levels, which had been shown to stimulate UM cell proliferation, we were not able to detect a significant change in VEGF-A or PEDF expressions, thus not in the ratio of the two growth factors either.

Taken together, we could demonstrate (1) that UM cells express VEGF-A, (2) that primary as well as metastatic UM cell lines express both related VEGF-A receptors VEGF-R1 and VEGF-R2, and (3) that primary and metastatic UM cell proliferation is stimulated by VEGF-A, inhibited by blocking VEGF-A (via bevacizumab), or both. These findings provide evidence that UM cell properties are influenced by autocrine VEGF-A signaling, tumor-driven and tumor-driving at the same time. The presence of autocrine tumor-stimulating signaling loops makes VEGF-A an even more interesting target for future

**FIGURE 5.** Increasing the VEGF-A signal by incubation with 100 ng/mL exogenous VEGF-A did not significantly affect the pro-angiogenic VEGF-A (A) or the antiangiogenic PEDF (B) mRNA expression in any of the tested UM cell lines. Data shown as mean ± SD; n.s., not significant (P > 0.05).
adjunctive strategies in UM treatment beyond antiangiogenic aspects.

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