Hypoxia- and acidosis-driven aberrations of secreted microRNAs in endometrial cancer *in vitro*

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Abstract. Due to their post-transcriptional regulatory impact on gene expression, microRNAs (miRNA, miRs) influence decisively cellular processes of differentiation, proliferation and apoptosis. In oncogenic pathways various miRNAs exert either oncogenic or tumor suppressor activities in a stage-specific manner. Dysregulation of miRNA expression pattern has been associated with several human cancers including endometrial cancer (EC). In the present study, expression profile alterations of EC associated secreted miRNAs were determined under the microenvironmental stress situations hypoxia and acidosis occurring in tumor progression and metastasis. The potential influence of hypoxia and acidosis vs. control conditions on the expression levels of 24 EC-relevant miRNA types was quantitatively accessed via real-time PCR in three established EC *in vitro* models. Expression data were analyzed statistically. *In vitro* application of hypoxia resulted in downregulation of miR-15a, miR-20a, miR-20b and miR-128-1 in Ishikawa cells (type I EC) and upregulation of miR-21 in EFE-184 cells (type I EC). Acidosis triggered upregulation of tumor promoting miR-125b in AN3-CA cell (type II EC), whereas in Ishikawa cells (type I EC) miRNAs with tumor suppressive function were found altered in divergent directions, both up- (let-7a) and down- (miR-22) regulated. Our current findings emphasize the functional importance of secreted miRNAs in the immediate response of EC cells to exogenic stress situations such as the typical tumor epiphenomena hypoxia and acidosis. Focusing on the specific potential of secreted, thus circulating miRNA molecules, alterations in expression levels not only influence intracellular gene expression and signaling cascades, but also transfer the induction of (tumor)biological cellular changes to adjacent cells.

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Introduction

Due to the vast functional multiplicity, microRNAs (miRNA, miRs) shaped up as the most important class of small non-coding RNAs (ncRNAs) (1) playing a crucial role in post-transcriptional regulation of gene expression and signaling pathways. Mature miRNA is incorporated into the RNA-induced silencing complex (RISC) which usually leads to mRNA degradation and translational repression (2,3). Using this mechanism, miRNAs influence cellular processes of differentiation, proliferation and apoptosis. Due to their ability to participate in modulation of oncogenic pathways miRNAs are important key regulators of tumor suppressor genes and oncogenes (4-6). A widespread dysregulation of miRNAs is observed in a variety of human cancers (7-9).

miRNA molecules can be localized intracellularly or, after secretion, circulate in the extracellular compartment (10). Release of miRNA in the extracellular compartment can take place as passive leakage from cells or can include active export by apoptotic bodies, high-density lipoproteins, microvesicles, exosomes and protein complexes (11-13). Argonaute complexes, which are regular complexes of the RNA silencing machinery, and Nucleosphosmin-1 play a predominant role in protein-complex dependent secretion of miRNAs (14). In this context, miRNA packaging and extracellular export is mediated by Nucleophosmin-1, which also acts as a protector from RNA degradation (11,15). For exosomal miRNA secretion, Zhang *et al* reported trafficking and sorting mechanisms which play an important role in cell-cell communication processes (16).

Endometrial cancer (EC) is the most common gynecologic malignancy, accounting for 7% of all cancers in women in the United States (17). Incidence as well as death rate of EC has increased during recent years (18). EC can be subclassified into two categories: type I cancers are usually well to moderately differentiated, estrogen-related endometrial carcinomas (EEC) with good prognosis, which are associated with endometrial hyperplasia, whereas type II cancers are non-estrogenic high

grade serous or clear cell carcinomas with poor prognosis, which are associated with atrophic endometrium. Molecular characteristics of EC cells do not always correlate with the traditional classification systems which have been based on clinical features or on histopathological findings (19-21).

MiRNAs have been shown to participate in regulation of endometrial growth and differentiation (22-26) and carcinogenesis of the endometrium has been associated with alterations in miRNA expression pattern. Furthermore, distinct miRNA signatures characterizing EC subtypes have been described (24,27-30). Since miRNAs have been shown to be stable molecules which are well preserved in serum and other body fluids (21,31,32) their use as promising non-invasive biomarkers in EC is under investigation (21). Jia *et al* reported in 2013 the identification of four serum-based miRNA types as potential non-invasive biomarkers for endometrioid endometrial cancer (32).

Alterations in miRNA expression patterns were also found in tumor cells exposed to stressors such as extracellular hypoxia and acidosis (33,34). However, there are no reports on miRNA expression under hypoxic or acidic conditions in EC cells. To our knowledge, to date there are only few studies investigating miRNA expression aberrations under the influence of hypoxia in endometriosis (35-37).

Hypoxia is an important regulatory factor in tumor growth and induces transcriptional cascades that promote a more aggressive tumor phenotype. In order to survive and proliferate in a hypoxic microenvironment tumor cells undergo genetic and adaptive changes and release substances that affect the microenvironment to promote tumor angiogenesis (38,39). These processes contribute to a malignant phenotype and aggressive tumor behavior as well as metastasis (38).

Kulshreshtha *et al* suggested that hypoxia may represent a key contributing stress factor for microRNA alterations in cancer. In colon and breast cancer cell lines, expression of specific miRNAs was altered under hypoxia (40). Hua *et al* described a miRNA directed regulation of VEGF and other angiogenetic factors under hypoxia in cells from a human nasopharyngeal carcinoma cell line (41).

Acidosis of the extracellular microenvironment is a consequence of increased glycolysis due to the adaption of tumor cells to prolonged periods of hypoxia. Cancer cells suffering oxygen deprivation use the glycolytic pathways to maintain their ATP level for survival and proliferation. Acidification of the peritumoral microenvironment induces necrosis and apoptosis of normal cells, thus creating space into which the tumor cells may proliferate (42). Acidosis promotes tumor invasion by degradation of the extracellular matrix through the discharge of proteolytic enzymes (43). Lowered pH levels also inhibit natural killer cell activity and the cytolytic activity of cytotoxic T-lymphocytes, therefore causing a diminished immune response to tumor antigens (44). This way, acidosis facilitates tumor progression and the development of invasive phenotypes. Furthermore, acidosis and hypoxia have been associated with resistance to therapeutic strategies such as multi-drug resistance and poor prognosis (45).

The present study investigated the quantitative expression of 24 different miRNAs secreted by EC cells *in vitro* in response to the exogenic stimuli hypoxia and acidosis. The panel of 24 specific miRNA types (let-7a, let-7b, let-7d, let-7i, miR-10a, -10b, -15a, -15b, -17, -19b, -20a, -20b, -21, -22, -26.1, 27a, -29c, -30b, -92a, -125b, -128-1, -135b, -200c, -222) was collocated based on a targeted pre-selection process focusing on EC-associated circulating miRNAs with tumor biological, diagnostic and therapeutic relevance. An initial screening expression analysis attested that all 24 secreted miRNAs were stably detectable in the supernatant of the selected three EC *in vitro* models (Ishikawa, EFE 184, AN3-CA; see Materials and methods for details). The current data on the tumor biological relevance of the selected miRNA types is summarized in Table I (1,25,26,28-30,41,46-75).

Materials and methods

Cell culture conditions and treatments. Established endometrial cancer cell lines Ishikawa i) (type I, well differentiated, ER and PR positive) (76). AN3-CA ii) (type II, poorly differentiated, ER negative) and EFE-184 iii) (type I) were incubated in humidified atmosphere at 37˚C and 5% CO_2 in i) RPMI-1640 or (ii and iii) DMEM/F12 medium supplemented with 10% newborn calf serum (Gibco™, Thermo Fisher Scientific, Karlsruhe, Germany), 1% HEPES buffer (Gibco) and 100 U/ml penicillin/streptomycin (Sigma-Aldrich®, Taufkirchen, Germany).

For the induction of stress conditions hypoxia and acidosis cells were subcultured in 25 cm^2 cell culture flasks and grown for 18 h. Hypoxia was induced by incubating cells in hypoxic chamber (<3% CO_2) for a period of 18 h overnight. For acidosis exposure, cells were treated with 0.2% lactic acid in cell culture media and incubated for 48 h at 37˚C. Cells incubated under standard conditions in parallel served as controls. Following treatment, 2 ml of culture media (supernatant) were harvested and immediately processed. All experiments were performed in triplicates.

Total RNA isolation. Total RNAs from supernatant of cultured cells were isolated by using the innuPREP Micro RNA Kit (Analytic Jena AG, Jena, Germany) according to the manufacturer's protocol. Assessment of RNA quantity was done by UV-spectrometry (NanoDrop ND1000 (PEQLAB, Erlangen, Germany). Until further processing RNA samples were stored at -80˚C.

Reverse transcription (RT) and quantitative PCR. The reaction mixture of RT consists of 4 μl Maxima RT-buffer (Thermo), 1 μl 5 μM poly(T) adaptor primer (Biomers, Konstanz, Germany), 1 μl 5 mM dNTPs (Jena Bioscience, Jena, Germany), 0.25 μl Maxima reverse transcriptase (Thermo), 0.25 μl SUPERase in RNase inhibitor (Thermo) and 500 ng of the total RNA sample. The reaction was carried out at 42˚C for 30 min and by 85˚C for 10 min in a Nexus Thermal Cycler (Eppendorf, Hamburg, Germany). Until further analysis, processed cDNA was stored at -20˚C.

Relative expression levels of specific microRNAs were assessed by quantitative PCR applying a SYBR Green assay in a duplicate analysis. cDNA (1 μl) with concentration of 5 ng/μl was mixed up with 9 μl master mix containing 1 μl 10 Xq PCR buffer, 0.5 μl 5 mM dNTPs (Jena Bioscience), 0.5 μl miRNA specific qPCR primer (Biomers), 0.5 μl SYBR Green (Roche Diagnostics GmbH, Mannheim Germany), 0.05 μl

Table I. Tumor-biological relevance of pre-selected miRNA types.

Pre-selected miR	Putative target	Reported function	References
Let-7	BAX	Enhanced survival and proliferation of cancer cells (promoting EC)	Zhang *et al* (70)
Let-7a	Aurora B	Inhibition of EC growth	Liu *et al* (57)
Let-7b	High mobility group AT-hook2	Inhibition of aggressive phenotypes	Romero-Perez *et al* (64)
Let-7d	LIN28, C-MYC, K-RAS, HMGA2 and IMP-1	Tumor suppressor or oncogene	Kolenda *et al* (54)
Let-7i	RAS, HMGA2, c-Myc, CDC25A, CDK6 and cyclin D2	Tumor suppressor	Yang *et al* (94)
miR-10a	USF2, HOXA1, HOXD10, HOXB1, HOXB3, RB1CC1	Invasion, metastasis (promoting EC)	Dai *et al* (50)
miR-10b	TBX5m DYRK1A, PTEN	Oncomir, Migration, invasion, proliferation	Kim *et al* (53)
miR-15a	Bck, MCL1, CCND1, WNT3	Tumor suppressor	Aqeilan *et al*, Bonci *et al* (46,47)
miR-15b	VEGF, CCND1, CCNE1	Tumor suppressor, antiangiogenic	Zhao *et al* (71)
miR-17	VEGF-A, NOR-1, GALNT3	Antiangiogenic	Doebele *et al*, Hua *et al*, Lu *et al*, Ramon *et al* (41,51,63,95)
miR-19b	PTEN, TGFβ	Oncogene component of mir-17-92-Cluster	Doebele *et al*, Lu *et al*, Ramon *et al*, Fuziwara *et al* (51,63,73,95)
miR-20a	VEGF-A	Reduces angiogenesis in EC	Doebele *et al*, Lu *et al*, Ramon *et al* (51,63,95)
miR-20b	MMP-2	Tumor suppressor	Park *et al* (59)
miR-21	Maspin, Pdcd4, PTEN	Tumorigenesis Cell proliferation (promoting EC)	Qin *et al*, Torres *et al* (30,62)
miR-22	Cyclin D1, MMP2, MM9 Erα	Tumor suppressor	Li *et al*, Wang *et al* (56,69)
miR-26a-1	CCNE1, ERα, FGF9, MTDH, EZH2, MCL-1	Antiproliferation, proapoptotic, metastasis inhibition, growth inhibition	Chen *et al* (49)
miR-27a	FOXO1 BAX	Myometrial invasion Inhibition of apoptosis, increased survival of cancer cells (promoting EC)	Zhang *et al*, Mozos *et al*, Myatt *et al* (70,22,23)
miR-29c	SPARCS (in EC) PI3K, CD42	Tumor suppressor	Castilla *et al*, Park *et al* (24,25)
miR-30b	SIX1	Inhibits migration and invasion	Zhao *et al* (72)
miR-92a	PTEN/PI3K/Akt/mTOR	Member of miR-17-92 cluster, promotes carcinogenesis in endometrial epithelium, oncogene	Torres *et al* (67)
miR-125b	TP53INP1	Proliferation and migration of cancer cells (promoting EC)	Jiang *et al*, Shang *et al* (28,29)
	ERBB2	Inhibition of cancer cell invasion (protecting against EC)	
miR-128-1	BMI1, E2F3	Tumor suppressor	Shan *et al* (65)
miR-135b	VEGF A, HIF1 A, HIFAN	Tumor promoting Upregulated in EC	Tsukamoto *et al* (68)

Table I. Continued.

Pre-selected miR	Putative target	Reported function	References
miR-200c	TIMP2 BRD7 ZEBs, VEGF-A, FLT1, IKKβ, KLF9, FBLN5	Promoting EC: Metastasis of cancer cells Induction of cell survival and proliferation Reduction of apoptosis Transition into cancerous states	Li *et al*, Park *et al*, Snowdon *et al* (32,33,34)
	FN1, MSN, NTRK2, LEPR, ARHGAP19	Protective: Downregulation promotes EMT phenotype and aggressive behavior of cells	
miR-222	c-kit CD117, PBX3	Antiangiogenic activity	Ramon *et al,* Poliseno *et al*, Yanokura *et al* (14,35,36)

Table II. Molecular classification of investigated endometrial cancer cell lines.

Cell line	Classification	Immunoprofile	Primary tumor	Origin
Ishikawa	Type I	ER^+, PR^+,	Endometrial adenocarcinoma	Uterus
EFE-184	Type I	ER^+, PR^-	Endometrium carcinoma	Ascitic fluid
AN3-CA	Type II	ER^-, PR^+	Endometrial adenocarcinoma	Lymph node metastasis

ER, estrogen receptor; PR, progesterone receptor.

HotStart Taq (Jena Bioscience) and 6.54 μl nuclease-free water (Analytic Jena). Primers consisted of a specific primer pair for each miRNA type and for the housekeeping genes.

Quantitative PCRs were performed on a LightCycler96 (Roche Diagnostics GmbH) at 95˚C for 2 min, followed by 40 cycles at 95˚C, for 5 sec and for 30 sec at 60˚C.

Data were analyzed in Microsoft Excel using ΔCt method based on reference value (geometric mean of housekeeping genes, RNU48, miR-26b, miR-16 and miR-103 defined via 'BestKeeper' software tool (77) to determine single microRNA expression levels.

Pre-selection of miRNA specimen. A comprehensive survey was performed applying Pubmed interface for original research (http://www.ncbi.nlm.nih.gov/) and the miRTarBase platform (http://mirtarbase.mbc.nctu.edu.tw/) (78), specific miRNA information registry, to determine potential study-relevant miRNA types. These sources were screened for relevant specimen in regard to 'endometrial cancer', typical endometrial cancer target genes and pathways as well as literature. A methodological pre-screening of >40 miRNA types was performed, which was based on the acquired information on potential miRNA candidates, to evaluate the experimental *in vitro* setting focusing on secreted miRNA specimen in cell culture media. The pre-set of miRNAs was filtered to collocate the final set of miRNAs which were reliably detectable in culture media. The hereby identified miRNA types and their functional implications are summarized in Table I.

Statistical analysis. Observed miRNA expression levels were graphically visualized in box plots, showing median and quartiles in cell lines Ishikawa, EFE-184 and AN3-CA under hypoxia, acidosis and normoxic conditions. The influence of treatment on miRNA expression levels of the different BC cell lines was investigated using a linear model with factor treatment (hypoxia and acidosis compared to normoxic conditions) and cell line, including an interaction term.

Results

In the present study we investigated the regulatory effect of hypoxia and acidosis on the expression levels of 24 different EC associated microRNA types (let-7a, let-7b, let-7d, let-7i, miR-10a, -10b, -15a, -15b, -17, -19b, -20a, -20b, -21, -22, -26-1, 27a, -29c, -30b, -92a, -125b, -128-1, -135b, -200c, -222) that are actively secreted into the tissue culture supernatant by EC cells. The characteristics of the investigated EC cell lines Ishikawa, EFE-184 and AN3-CA are summarized in Table II.

All 24 examined microRNAs were stably detectable in the supernatants of all three *in vitro* models. In order to examine potential regulatory effects triggered by typical microenvironmental alterations occurring in solid tumors during tumor growth, progression and metastasis on miRNA expression (79-82) hypoxia and acidosis were induced in the three cell lines. Expression levels of all EC associated miRNA types were determined under normoxic, hypoxic and acidotic conditions as mean ΔCt values of the distinct miRNA type

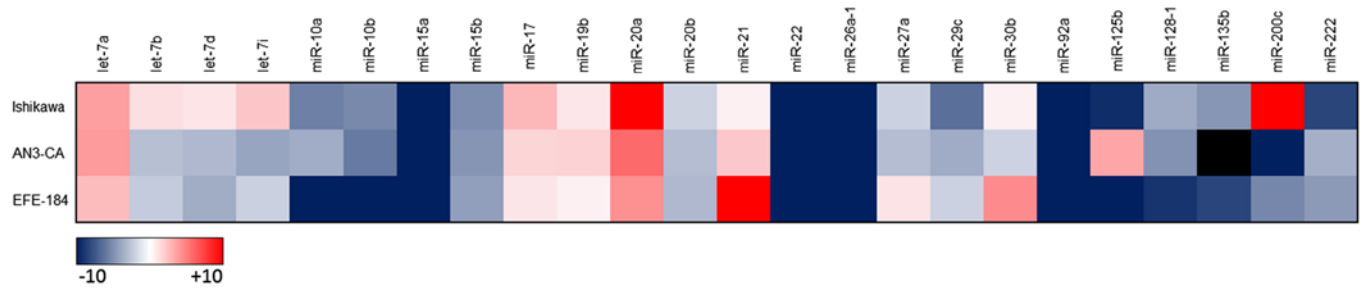

Figure 1. Expression profiles of secreted miRNAs in Ishikawa, EFE-184 and AN3-CA cells under normoxic (control) conditions. miRNA-expressions were determined in triplicates by real-time quantitative PCR and calculated using ΔCt method based on reference value (geometric mean of housekeeping genes, RNU48, miR-26b, miR-16 and miR-103) to determine single microRNA's expression levels.

Table III. Two-way analysis of variance for levels of secreted miR-15a under control, acidotic and hypoxic conditions in three different endometrial cancer cell lines.

	Estimate	Standard error	95% CI	p-value
Intercept	0.047	0.007	0.034, 0.060	0.000
Control				
EFE-184	-0.033	0.009	-0.052, -0.015	0.002
Ishikawa	0.023	0.009	-0.005, 0.032	0.174
Acidosis				
AN3-CA	-0.007	0.009	-0.025, 0.012	0.489
EFE-184	0.000	0.009	-0.018, 0.018	1.000
Ishikawa	-0.007	0.009	-0.025, 0.012	0.489
Hypoxia				
AN3-CA	0.000	0.009	-0.018, 0.018	1.000
EFE-184	0.003	0.009	-0.015, 0.022	0.728
Ishikawa	-0.020	0.009	-0.038, -0.002	0.048

Intercept is calculated on basis of AN3-CA control.

Table IV. Two-way analysis of variance for levels of secreted miR-20a under control, acidotic and hypoxic conditions in three different endometrial cancer cell lines.

	Estimate	Standard error	95% CI	p-value
Intercept	6.573	1.113	4.391, 8.756	0.000
Control				
EFE-184	-1.657	1.575	-4.743, 1.429	0.307
Ishikawa	5.797	1.575	2.711, 8.883	0.002
Acidosis				
AN3-CA	-1.453	1.575	-4.539, 1.633	0.368
EFE-184	-0.660	1.575	-3.746, 2.426	0.680
Ishikawa	-1.993	1.575	-5.079, 1.093	0.222
Hypoxia				
AN3-CA	-0.177	1.575	-3.263, 2.909	0.912
EFE-184	0.007	1.575	-3.079, 3.093	0.997
Ishikawa	-3.577	1.575	-6.663, -0.491	0.036

Intercept is calculated on basis of AN3-CA control.

normalized against the geometric mean of the four housekeepers RNU48, miR-16, miR-26b and miR-103.

Under standard culture conditions each of the EC cell lines displayed a unique miRNA expression profile (Fig. 1). In response to hypoxia and acidosis, singular marked alterations in secreted miRNA expression levels were identified predominantly in Ishikawa cells.

Hypoxia triggered regulatory effects on the expression levels of miR-15a, miR-20a, miR-20b, miR-21 and miR-128-1. In Ishikawa cells, multivariable regression analysis identified a significant hypoxia-dependent downregulation of miR-15a, miR-20a, miR-20b and miR-128-1 expression. In detail, the expression level of miR-15a was decreased by a mean value of 0.020 (CI: -0.038 - -0.002; p=0.048) (Table III, Fig. 2), of miR-20a by 3.577 (CI: -6.663 - -0.491; p=0.036) (Table IV, Fig. 2), miR-20b by 0.340 (-0.544 - -0.136; p=0.004) (Table V, Fig. 2) and of miR-128-1 by 0.073 (-0.136 - -0.011; p=0.034) (Table VI, Fig. 2). In EFE-184 and AN3-CA cells a comparable hypoxia-driven downregulatory effect on miRNA expression could not be detected. In EFE-184 cells a hypoxia-induced upregulation of miR-21 expression was observed. The expression levels of secreted miR-21 levels in hypoxia-conditioned EFE-184 cells were increased by a mean value of 2.103 (CI: 0.300 - 3.907; p=0.035) (Table VII, Fig. 2). A hypoxia-dependent upregulatory effect on miRNA expression of AN3-CA and Ishikawa cells was not observed.

In response to acidosis, single marked alterations in secreted expression for let-7a, miR-22 and miR-125b were observed. In Ishikawa cells, ΔCt expression levels of let-7a were increased by a mean value of 1.980 (-0.442 - 3.518; p=0.021) (Table VIII, Fig. 3) and ΔCt expression levels of miR-22 were decreased by a mean value of 0.023 (-0.044 - -0.003; p=0.040) (Table IX, Fig. 3). In AN3-CA cells a specific acidosis-dependent upregulation was observed for miR-125b. In detail, a mean increase of secreted miR-125b ΔCt level by 1.007 (CI: 0.512 - 1.501; p=0.001) became evident (Table X, Fig. 3). No significant acidosis-dependent miRNA expression level alterations were detected in EFE-184 cells.

No marked hypoxia- or acidosis-driven alterations in expression levels of let-7b, let-7d, let-7i, miR-10a, -10b, -15b, -17, -19b, -26-1, 27a, -29c, -30b, -92a, -135b, -200c and 222 were observed in the investigated EC cell lines (data not shown).

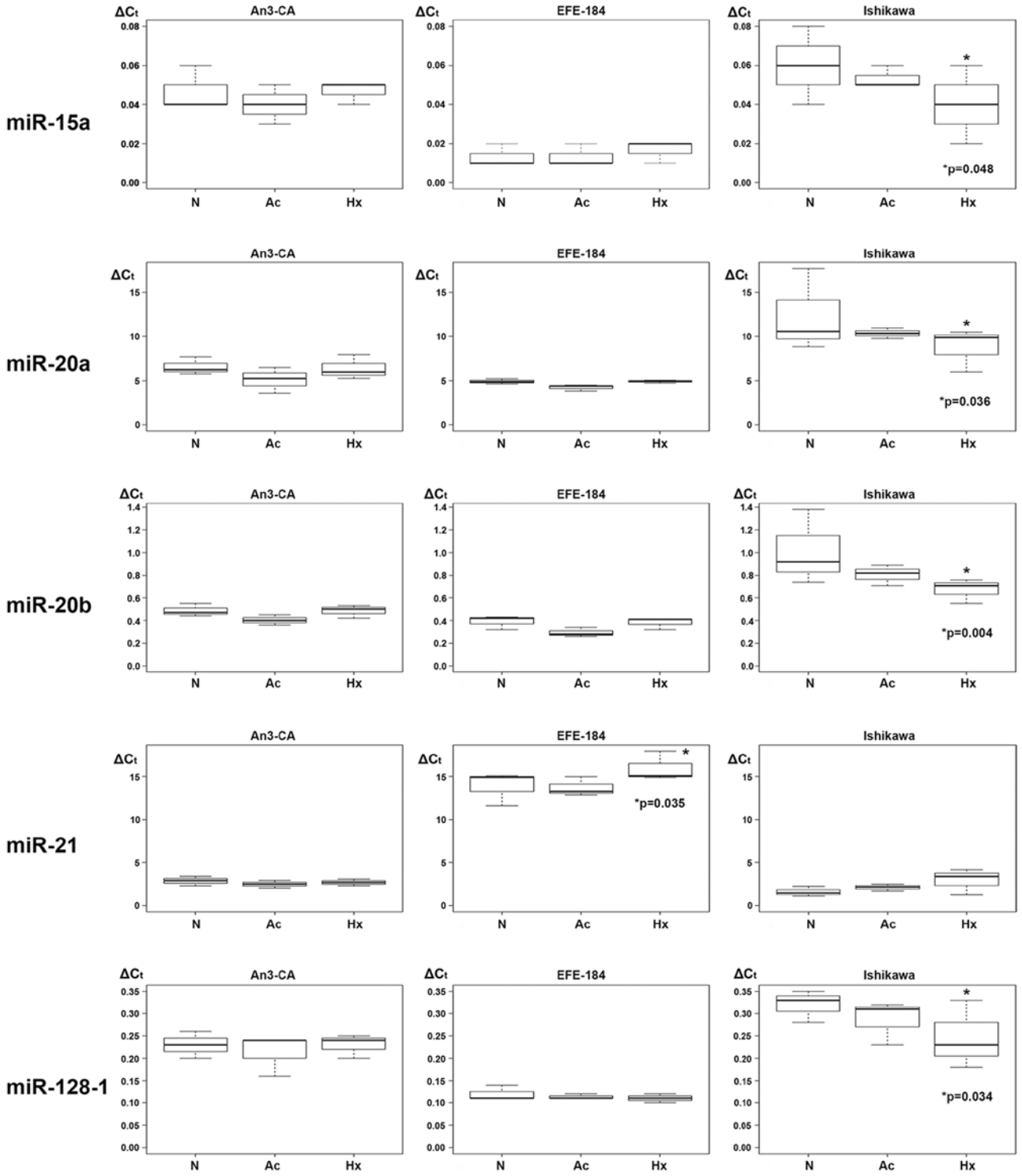

Figure 2. Box plot diagram of expression levels of significantly altered secreted miRNAs in response to hypoxia. Expression levels of secreted miR-15a, miR20a, miR-20b, miR-21 and miR-128-1 in endometrial cancer cell lines are displayed under varying treatment conditions. Levels of secreted miRNA expression were determined in AN3-CA, EFE-184 and Ishikawa cells under control conditions (N), extracellular acidosis (Ac) and hypoxia (Hx). Box plots demonstrate median (thick black line), lower and upper quantile range (box lines), and standard deviation range (dashed lines bounded by horizontal lines). Significant expression level alterations ($^{*}p \leq 0.05$) of control conditions versus treatment options. Based on triplicate experiments, real-time quantitative PCR.

Discussion

Recent and ongoing studies account for a continually growing number of miRNA types with regulatory influence on EC tumorigenesis. While some of these miRNAs play an important role as tumor suppressors, expression of others is associated with promotion of tumor growth, invasion and metastasis (oncogenic miRNAs, oncomiRs). In the present study, we analyzed the expression of a panel of secreted EC associated microRNAs with impact on angiogenesis, proliferation, invasion, migration and metastasis including let-7a, let-7b, let-7d, let-7i, miR-10a, -10b, -15a, -15b, -17, -19b, -20a, -20b, -21, -22,

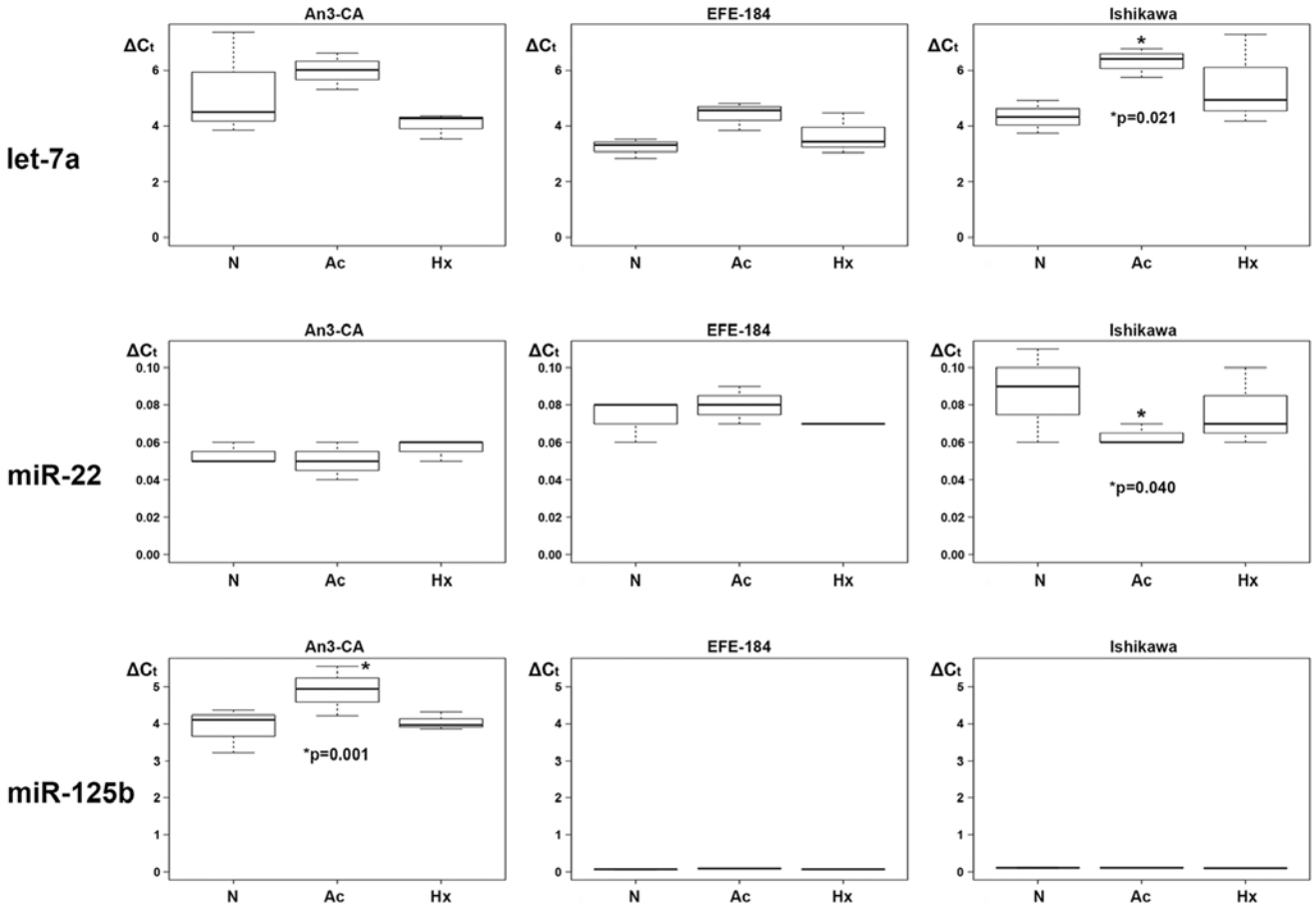

Figure 3. Box plot diagram of expression levels of significantly altered secreted miRNAs in response to acidosis. Expression levels of secreted let-7a, miR22 and miR-125b in endometrial cancer cell lines are displayed under varying treatment conditions. Levels of secreted miRNA expression were determined in AN3-CA, EFE-184 and Ishikawa cells under control conditions (N), extracellular acidosis (Ac) and hypoxia (Hx). Box plots demonstrate median (thick black line), lower and upper quantile range (box lines), and standard deviation range (dashed lines bounded by horizontal lines). Significant expression level alterations ($^{*}p \leq 0.05$) of control conditions versus treatment options. Based on triplicate experiments, real-time quantitative PCR.

-26.1, 27a, -29c, -30b, -92a, -125b, -128-1, -135b, -200c, -222. We were able to reliably detect all 24 secreted miRNA types in the supernatants of the three analyzed EC cell lines. Based on these EC *in vitro* models a quantitative analysis of secreted miRNA expression levels was employed to detect potential alterations under the typical tumor biological epiphenomena hypoxia and acidosis.

Our data clearly account for a hypoxia- and acidosis-dependent regulatory effect in the expression levels of eight specific secreted miRNAs (let-7a, miR-15a, miR-20a, miR-20b, miR-21, miR-22, miR-125b and miR-128-1) among the investigated miRNA types. The observed alterations in miRNA expression levels were not consistent in all three EC cell lines. Significant miRNA deregulation due to hypoxia or acidosis was predominantly observed in Ishikawa cells. In EFE-184 and AN3-CA cells a regulatory effect was observed solely for one specific miRNA type. Hypoxia resulted in downregulation of miR-15a, miR-20a, miR-20b and miR-128-1 in Ishikawa cells and upregulation of miR-21 in EFE-184 cells.

miR-15a is considered to trigger tumor suppressive effects by inhibiting several target oncogenes, like BCL2, MCL1, CCND1 and WNT3A (46,47). Thus, cells react with diminished proliferation, an increase in apoptosis rates and suppressed tumorigenicity both *in vitro* and *in vivo*. In chronic lymphocytic lymphoma (CLL), prostate carcinomas and pituitary adenomas, downregulation of miR-15a expression has been observed (46). In the present study, hypoxic conditions resulted in decreased miR15a expression in Ishikawa cells. This finding accounts for a reduced inhibitory signaling function on angiogenesis in type I EC cells under hypoxic stress and is supported by the results of Hua *et al* reported that in a tumor cell model VEGF expression is repressed, besides others, by miRNAs (miR15b, miR16) from the miR15-16 clustered family (41).

The miR-17-92 cluster, which contains six mature miRNAs (miR-17, -18a, -19a, -19b, -20a and -92a), conducts a complex role in angiogenetic signaling cascades (65) In the present analysis, we evaluated four specific miRNAs of this cluster: miR-17, miR-19b, miR-20a and miR-92a. Anti-angiogenetic activity of miR-20a by targeting VEGF-A has been reported (41,65). Inconsistent results have been reported for the role of miR-17 so far. While some investigatory approaches hypothesize an anti-angiogenetic activity (41,51), other studies account for either pro-angiogenetic activity (83,84) or no association with VEGF-A expression (65). Our data identified a significant hypoxia-driven expression reduction of miR20a in Ishikawa cells. For miR-17, miR-19b and miR-92a no significant changes in expression levels were observed. Our results are in line with the findings of Ramon *et al* and support the proposition that miR-20a, but not miR-17 and miR-19b,

Table V. Two-way analysis of variance for levels of secreted miR-20b under control, acidotic and hypoxic conditions in three different endometrial cancer cell lines.

	Estimate	Standard error	95% CI	p-value
Intercept	0.487	0.073	0.343, 0.631	0.000
Control				
EFE-184	-0.097	0.104	-0.300, 0.107	0.365
Ishikawa	0.527	0.104	0.323, 0.730	0.000
Acidosis				
AN3-CA	-0.083	0.104	-0.287, 0.120	0.433
EFE-184	-0.097	0.104	-0.300, 0.107	0.365
Ishikawa	-0.207	0.104	-0.410, -0.003	0.062
Hypoxia				
AN3-CA	-0.003	0.104	-0.207, 0.200	0.975
EFE-184	-0.010	0.104	-0.214, 0.194	0.924
Ishikawa	-0.340	0.104	-0.544, -0.136	0.004

Intercept is calculated on basis of AN3-CA control.

Table VI. Two-way analysis of variance for levels of secreted miR-128-1 under control, acidotic and hypoxic conditions in three different endometrial cancer cell lines.

	Estimate	Standard error	95% CI	p-value
Intercept	0.230	0.023	0.186, 0.274	0.000
Control				
EFE-184	-0.110	0.032	-0.173, -0.047	0.003
Ishikawa	0.090	0.032	0.027, 0.153	0.011
Acidosis				
AN3-CA	-0.017	0.032	-0.079, 0.046	0.608
EFE-184	-0.007	0.032	-0.069, 0.056	0.837
Ishikawa	-0.033	0.032	-0.096, 0.029	0.310
Hypoxia				
AN3-CA	0.000	0.032	-0.063, 0.063	1.000
EFE-184	-0.010	0.032	-0.073, 0.053	0.757
Ishikawa	-0.073	0.032	-0.136, -0.011	0.034

Intercept is calculated on basis of AN3-CA control.

Table VII. Two-way analysis of variance for levels of secreted miR-21 under control, acidotic and hypoxic conditions in three different endometrial cancer cell lines.

	Estimate	Standard error	95% CI	p-value
Intercept	2.853	0.651	1.578 4.128	0.000
Control				
EFE-184	11.013	0.920	9.210 12.817	0.000
Ishikawa	-1.253	0.920	-3.057 0.550	0.190
Acidosis				
AN3-CA	-0.377	0.920	-2.180 1.427	0.687
EFE-184	-0.167	0.920	-1.970 1.637	0.858
Ishikawa	0.510	0.920	-1.293 2.313	0.586
Hypoxia				
AN3-CA	-0.177	0.920	-1.980 1.627	0.850
EFE-184	2.103	0.920	0.300 3.907	0.035
Ishikawa	1.330	0.920	-0.473 3.133	0.165

Intercept is calculated on basis of AN3-CA control.

Table VIII. Two-way analysis of variance for levels of secreted let7a under control, acidotic and hypoxic conditions in three different endometrial cancer cell lines.

	Estimate	Standard error	95% CI	p-value
Intercept	5.243	0.555	4.156, 6.331	0.000
Control				
EFE-184	-2.020	0.785	-3.558, -0.482	0.019
Ishikawa	-0.917	0.785	-2.454, 0.621	0.258
Acidosis				
AN3-CA	-0.737	0.785	-0.801, 2.274	0.360
EFE-184	1.180	0.785	-0.358, 2.718	0.150
Ishikawa	1.980	0.785	0.442, 3.518	0.021
Hypoxia				
AN3-CA	-1.183	0.785	-2.721, 0.354	0.149
EFE-184	0.427	0.785	-1.111, 1.964	0.593
Ishikawa	1.140	0.785	-0.398, 2.678	0.163

Intercept is calculated on basis of AN3-CA control.

displays an anti-angiogenetic activity, which is reduced under hypoxic stress conditions in EC tumor cells (65).

MiR-20b has a tumor suppressive function by impeding MMP-2 expression leading to cell cycle arrest (61) as well as regulative function on oxygen balance of cells. Lei *et al* described that tumor cells are able to adapt to alterations of oxygen concentration by miR-20b triggered regulation of HIF-1α and VEGF. In their study inhibition of miR-20b increased protein levels of VEGF and HIF-1α in normoxic tumor cells, an increase of miR-20b expression in hypoxic tumor cells resulted in reduced protein levels of VEGF and HIF-1α (85). Park *et al* showed that miR-20b expression was downregulated in bladder cancer and an upregulation of this miRNA type lead to inhibition of proliferation, migration and invasion (61). In gastric cancer, downregulated miR-20b expression was associated with hypoxia-induced chemoresistance. In the present study we observed reduced miR-20b expression levels in Ishikawa cells under hypoxic conditions. These data are in line with aforementioned reports of Lei *et al* as well as of Danza *et al* in gastric cancer (85,86).

Under hypoxic conditions expression levels of miR-128-1 were also found reduced in Ishikawa cells. miR-128-1 is supposed to act as a tumor suppressor inhibiting tumor cell proliferation, invasion and self-renewal by direct targeting

Table IX. Two-way analysis of variance for levels of secreted miR-22 under control, acidotic and hypoxic conditions in three different endometrial cancer cell lines.

	Estimate	Standard error	95% CI	p-value
Intercept	0.053	0.007	0.039, 0.068	0.000
Control				
EFE-184	0.020	0.011	-0.001, 0.041	0.074
Ishikawa	0.033	0.011	0.013, 0.054	0.005
Acidosis				
AN3-CA	-0.003	0.011	-0.024, 0.017	0.755
EFE-184	0.007	0.011	-0.014, 0.027	0.535
Ishikawa	-0.023	0.011	-0.044, -0.003	0.040
Hypoxia				
AN3-CA	0.003	0.011	-0.017, 0.024	0.755
EFE-184	-0.003	0.011	-0.024, 0.017	0.755
Ishikawa	-0.010	0.011	-0.031, 0.011	0.355

Intercept is calculated on basis of AN3-CA control.

Table X. Two-way analysis of variance for levels of secreted miR-125b under control, acidotic and hypoxic conditions in three different endometrial cancer cell lines.

	Estimate	Standard error	95% CI	p-value
Intercept	3.893	0.178	3.544, 4.243	0.000
Control				
EFE-184	-3.830	0.252	-4.324, -3.336	0.000
Ishikawa	-3.783	0.252	-4.278, -3.289	0.000
Acidosis				
AN3-CA	1.007	0.252	0.512, 1.501	0.001
EFE-184	0.027	0.252	-0.468, 0.521	0.917
Ishikawa	0.000	0.252	-0.494, 0.494	1.000
Hypoxia				
AN3-CA	0.153	0.252	-0.341, 0.648	0.551
EFE-184	0.003	0.252	-0.491, 0.498	0.990
Ishikawa	-0.010	0.252	-0.504, 0.484	0.969

Intercept is calculated on basis of AN3-CA control.

of BMI1 and E2F3 in glioblastomas (67). Furthermore, it has been associated with inhibition of the mTOR signaling pathway in glioblastomas. Decreased miR-128-1 expression levels in glioblastomas could be strongly associated with poor survival (87). The observed reduced expression levels of miR-128-1 in Ishikawa cells under hypoxic conditions may also account for a regulative function of miR-128-1 in EC.

MiR-21 expression levels were increased in EFE-184 cells under oxygen deprivation. This observation is in line with previous studies showing that miR-21 is an active participant in EEC malignant transformation by abrogating the regulatory function of PTEN, which has been found to be upregulated in EEC (24,64). Furthermore, a regulating role of miR-21 on the expression of the tumor suppressor gene Pdcd4 (programmed cell death-4) and Maspin, which is strongly associated with angiogenesis in EC, has been indicated (88). Whether Maspin acts as tumor suppressor or tumor promoter, seems to be complex and dependent on the tissue specific environment (89). In EC high Maspin expression has been associated with higher FIGO stage, lymph node involvement and the depth of myometrial invasion as well as poor prognosis (90). Additionally a correlation between high miR-21 expression and increased Maspin expression has been reported in EC (89).

Acidosis resulted in divergent miRNA expression patterns in the analyzed EC cell lines. While let-7a expression was found to be upregulated, miR-22 expression levels were downregulated under acidotic conditions in Ishikawa cells. Let-7a acts as tumor suppressor due to inhibition of EC growth by targeting and downregulating Aurora B (1, 21, 24). In EC let-7a expression levels are usually downregulated (58). miR-22 exerts tumor suppressive functions by influencing Cyclin D1, MMP2, PTEN and others (71). Li *et al* reported an inhibitory function of miR-22 expression on proliferation and invasion in estrogen receptor α-positive endometrial endometrioid carcinoma cells (57).

In AN3-CA cells miR-125b was significantly upregulated under acidotic conditions. These data are in line with previous findings showing an upregulated miR-125b expression in type II EC cells. Proliferation and migration of type II EC cancer cells was promoted by miR-125b in this study by targeting the tumor suppressor gene TP53INP1 (53).

In summary, our data clearly identify a hypoxia-dependent downregulation of secreted miRNAs with tumor suppressive and anti-angiogenetic function (miR-15a, miR-20a, miR-20b, miR-128-1) as well as upregulation of secreted miRNAs with tumor and angiogenesis promoting function (miR-21) in type I EC cell lines (Ishikawa, EFE-184). In contrast, in the analyzed type II EC cell line (AN3-CA) hypoxia did not show any significant impact on the expression pattern of the analyzed EC related miRNAs.

Acidosis triggered upregulation of the tumor promoting miRNA miR-125b in AN3-CA cells (type II EC). In Ishikawa cells (type I EC) the tumor promoting or tumor suppressive impact of acidosis is not clear yet since miRNAs with tumor suppressive function were found altered in divergent directions, both up- (let-7a) and down- (miR-22) regulated.

In conclusion, our current findings emphasize the functional importance of secreted miRNAs in the immediate response of EC cells to exogenic stress situations such as the typical tumor epiphenomena hypoxia and acidosis. Focusing on the specific potential of secreted, thus circulating miRNA molecules, that also conduct an inter-cellular (hormone-like) signaling function, alterations in expression levels not only influence intracellular gene expression and signaling cascades, but also transfer the induction of (tumor)biological cellular changes to adjacent cells (91,92) Thus, the fate of whole tissue areas may undergo transformation in respect to metabolic pathways, cell cycle control and neo-angiogenesis, that support malignant progression. To rule out the possibility of *in vitro*/*in vivo* differences of the analyzed secreted miRNA expression levels as well as to support the transferability of the observed results to *in vivo* settings, the data of the present *in vitro* study

on EC cancer cell lines should be confirmed by corresponding *in vivo* analyses based on tumor tissue specimen and/or body fluids (e.g. blood, urine) of EC patients.

Continuous elucidation of miRNA functions augments the potential of these regulatory nucleic acid elements to either be implemented as useful biomarkers in disease/malignancy diagnosis, prognosis and therapy monitoring, as well as novel therapeutic targets ('AntagomiRs') in clinical cancer management (1,21,93). The potential applicability of circulating miRNAs as non-invasive biomarkers for diagnosis (32) or as therapeutic targets (94,95) in EC is currently subject of various studies. In this context this *in vitro* model may serve as initial step to elucidate the influence of microenvironmental changes on expression profiles of circulating microRNAs in EC.

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