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Activity of the GHRH Antagonist MIA-602 and its Underlying Action Mechanisms in Endometriosis and Breast Cancer

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Table of contents

Abbreviations

APS	ammonium persulfate
ADH	anti-diuretic hormone
cAMP	cyclic adenosine monophosphate
CCR	clinical cure rate
cDNA	complementary DNA
CREB	cAMP response element binding protein
CBP	CREB binding protein
CRH	corticotropin-releasing hormone
DAG	diacylglycerol
DMSO	dimethyl sulfoxide
DMEM	Dulbecco minimal essential medium
DTT	dithiothreitol
EDTA	ethylenediamine tetraacetate
EESCs	eutopic endometial stromal cells
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
ERK	extracellular signal regulated kinase
ESC	endometriotic stromal cell
FCS	fetal calf serum
GH	growth hormone
GHRH	growth hormone-releasing hormone
GHRHR	growth hormone-releasing hormone receptor
GLP-1	glucagon-like peptide 1
GnRH	gonadotropin-releasing hormone
GIP	gastric inhibitory polypeptide
GPCR	G-protein-coupled receptor
HCl	hydrogen chloride
HESCs	human endometrial stromal cells
h	hour

IP3	inositol triphosphate
IGF-I	Insulin-like growth factor I
L	liter
MAPK	mitogen-activated protein kinase
min	minute
ml	milliliter
µl	microliter
mRNA	messenger RNA
MTT	thiazolyl blue tetrazolium bromide
NaCl	sodium chloride
NaOH	sodium hydroxide
PACAP	pituitary adenylate cyclaseactivating peptide
p-Akt	phospho-Akt
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
pit-GHRHR	pituitary-type GHRHR
PLC	phospholipase C
PKA	cAMP-dependent kinase
PKC	protein kinase C
PFA	paraformaldehyde
PIH	prolactin-inhibiting hormone
pit-GHRHR	pituitary type GHRH receptor
qRT-PCR	quantitative reverse transcriptase PCR
rpm	revolutions per minute
RT	reverse transcriptase
SCLC	Small Cell Lung Cancer
SR I	serum replacement I
SDS	sodium dodecyl sulfate
s	second
SST	somatostatin

SV1	splice variant 1 of the GHRHR
TBS	Tris buffered saline
TEMED	N, N, N ', N'-tetramethylenediamine
TNBC	Triple negative breast cancer
TKIs	small molecule EGFR inhibitors
TRH	thyrotropin-releasing hormone
VEGF	vascular endothelial growth factor
VIP	vasoactive intestinal peptide

1 Introduction

1.1 Hypothalamic hormones

The hypothalamus is a very small, but extremely important part of the diencephalon that plays a significant role in the mediation of endocrine, autonomic and behavioral functions [1]. It responds to a variety of signals from the internal and external environment and secretes substances known as neurohormones that adjust the secretion of pituitary hormones [1].Primary hormones secreted by the hypothalamus include: growth hormone-releasing hormone (GHRH), somatostatin (SST), corticotropin-releasing hormone (CRH), thyrotropin-releasing hormone (TRH), gonadotropin-releasing hormone (GnRH), anti-diuretic hormone (ADH), prolactin-inhibiting hormone (PIH), and oxytocin. Hypothalamic hormones influence various bodily functions such as growth, reproduction, lactation, metabolism, and gastrointestinal function, as well as pathological processes such as tumorigenesis through the anterior pituitary hormones and their target glands [1, 2].

1.2 GHRH and its receptors

Growth hormone-releasing hormone (GHRH) was first suggested by Reichlin in 1961, but the breakthrough for the identification of this hormone was provided by Frohman and Szabo, who demonstrated of the ectopic GHRH released by carcinoid and pancreatic tumors [3, 4]. The principal action of the hypothalamic neuropeptide GHRH is considered to be the stimulation of the synthesis and release of growth hormone (GH) from the pituitary. The GH in turn stimulates the secretion of insulin-like growth factor-I (IGF-I) in the liver which plays an important role in malignant transformation, metastasis and tumorigenesis of various cancers. Recently, much evidence appeared that besides its hypophyseal action, GHRH is not only an endocrine regulator of several important physiological processes but also has been involved in the pathogenesis and growth of diverse malignancies by paracrine and/or autocrine mechanisms [5-10].

The human GHRH gene has been localized to chromosome 20. It includes five exons and spans about 10-18 kilobases of genomic DNA [5, 11]. The precursor protein for the

human GHRH contains 108 amino acids, which can be processed into the 44-amino acids GHRH peptide with the structure:

Tyr-Ala-Asp-Ala-Ile-Phe-Thr-Asn-Ser-Tyr-Arg-Lys-Val-Leu-Gly-Gln-Leu-Ser-Ala-Arg-Lys-Leu-Leu-Gln-Asp-Ile-Met-Ser-Arg-Gln-Gln-Gly-Glu-Ser-Asn-Gln-Gly-Arg-Gly-Ala-Arg-Ala-Arg-Leu-NH2 [5].

The N-terminal 29 amino acid fragment (GHRH1-29NH2) comprises the inherent biological activity. GHRH is mainly synthesized in the arcuate and ventromedial nuclei of the hypothalamus, and then releases into the hypothalamic-pituitary portal vascular system [5, 12]. At present, the expression of GHRH was also found in various normal extra-hypothalamic-pituitary tissues, such as the ovary, placenta, testis, pancreas, gastrointestinal tract, prostate and immune cells, and subsequently detected in many human cancer cell lines and tumors, such as breast, endometrial, lung and prostate cancers [5, 12-14]. These observations suggest that GHRH plays its role in a spectrum of physiological processes as well as the pathogenesis of cancer. Recent studies on GHRH support the hypothesis that this hormone is further involved in the metabolism of reactive oxygen and nitrogen species, in the proliferation and survival of pancreatic islets as well as in the proliferation and survival of cardiomyocytes *in vivo* after acute myocardial infarction and moreover in the malignant transformation, metastasis and tumorigenesis in various cancers [5, 12, 15-17]. GHRH has been shown to stimulate the proliferation and act as a potent mitogen in many of human cancers such as pancreatic, prostate, breast, colorectal, lung, endometrial and ovarian cancer [1, 4, 5, 12, 18]. Knocking down GHRH expression resulted in dramatically decreased cell proliferation in several cancer cell lines [5, 19].

The action of GHRH is initiated by binding to its receptor on target cells. The GHRH receptor (GHRHR) expressed in the pituitary is the normally processed type called the pituitary-type GHRHR (pit-GHRHR). In tumor cells splice variants (SVs) of the GHRHR are more abundant. Among the functionally active splice variants of the GHRHR, SV1 is the most prevalent receptor which differs from pit-GHRHR at the first 334 nucleotides in the N-terminal extracellular domain, where a 25-amino-acid sequence coded by intronic sequences replaces the first 89 amino acids of pit-GHRHR, and is probably the main splice variant mediating the effects of GHRH and its analogues in

peripheral tissues [20, 21]. The pit-GHRHR as well as the splice variant SV1 are seven-transmembrane receptors, belonging to the family of G-protein coupled receptors that also include secretin, vasoactive intestinal peptide (VIP), glucagon, glucagon-like peptide 1 (GLP-1), pituitary adenylate cyclase-activating polypeptide (PACAP), and gastric inhibitory polypeptide (GIP) receptors. Upon binding of GHRH to its receptor in the pituitary, the active subunit of the G-protein complex stimulates adenylyl cyclase resulting in an increase of intracellular cyclic adenosine monophosphate (cAMP) levels. cAMP activates the protein kinase A, which induces the phosphorylation of intracellular and membrane-associated proteins [20, 21]. Pit-GHRHR is expressed predominantly in the anterior pituitary gland, and with lower expression in several other tissues and cancer. Contrarily, SV1 is commonly expressed in some normal extra-pituitary tissues as well as in various tumors. In genetic engineering experiments, the transformation of endogenously GHRH receptor negative cells with the gene of the pit-GHRH and SV1 receptor could endow their responsiveness to exogenously given GHRH; especially a transfection of the gene for SV1 could promote proliferation even in the absence of exogenous GHRH [22, 23]. Thus, GHRH receptors can be considered as potential targets for anticancer therapy.

1.3 GHRH antagonists and its possible underlying action mechanisms

GHRH antagonists were first advocated in 1994 because somatostatin analogs did not adequately suppress GH and IGF-I levels in patients with neoplasms potentially dependent on IGF-I. The first GHRH antagonist (Ac-[Tyr1, D-Arg2] GHRH (1-29) NH2) was used as a standard for the subsequent development of more potent antagonistic analogs of GHRH being developed for the treatment of various cancers [20, 24, 25]. Subsequently, more-potent GHRH antagonists were synthesized by the group of Andrew V. Schally who was making numerous findings in the area of hypothalamic hormones during the past 60 years. This group further evaluated the inhibitory effects of GHRH antagonists both *in vivo* and *in vitro* of various human cancers, including prostate and colorectal cancers, small cell lung cancer (SCLC) and non-SCLC, breast carcinomas, ovarian and endometrial carcinomas [6, 12, 13, 16, 26, 27].

Up to now, four series of GHRH antagonists had been synthesized. The first potent antagonists (MZ-series) contained hydrophobic moieties at the N-terminus, the enzyme resistant agmatine at the C-terminus, and other substitutions such as 4-chloro-phenylalanine, 2-aminobutyric acid, and norleucine that increased the receptor-binding affinity and enhanced the stability, such as MZ-4-71 and MZ-5-156 [28]. Subsequently, more-potent antagonists JV-series including JV-1-36 and JV-1-38 were prepared, containing arginine, d-arginine or homoarginine residues in positions 9, 28, and 29. JV-1-63, JV-1-65 and JV-1-6 were later synthesized antagonists containing other substitutions at positions 8, 9, and 10. Antagonists JV-1-63 and JV-1-36 exhibited potent endocrine activity [28]. Acylation of JV-peptides by fatty acids at the N-terminus (either monocarboxylic or dicarboxylic, containing six to sixteen carbon atoms) led to development of MZ-J series of antagonists, for example MZ-J-7-110 and MZ-J-7-114 contained fatty acyl moieties at their amino terminus [28]. The binding affinity of these lipopeptide antagonists to GHRH receptors was

Table 1: Structure of the most commonly studied GHRH antagonists. From Siejka *et al.* 2012 [28]**.**

Standard antagonist	[Ac-Tyr^{1}, D-Arg^{2}] hGHRH(1-29)NH_2
MZ-4-71	[Ibu-Tyr^{1}, D-Arg^{2}, Phe(4-Cl)^{6}, Abu^{15}, Nle^{27}] hGHRH(1-28)Agm
MZ-5-156	[PhAc-Tyr^{1}, D-Arg^{2}, Phe(4-Cl)^{6}, Abu^{15}, Nle^{27}] hGHRH(1-28)Agm
MZ-4-243	[Nac0-Tyr^{1}, D-Arg^{2}, Phe(4-Cl)^{6}, Abu^{15}, Nle^{27}] hGHRH(1-28)Agm
JV-1-10	[PhAc-Tyr^{1}, D-Arg^{2}, Phe(4-Cl)^{6}, Arg^{9}, Abu^{15}, Nle^{27}, D-Arg^{29}] hGHRH(1-29)NH_2
JV-1-36	[PhAc-Tyr^{1}, D-Arg^{2}, Phe(4-Cl)^{6}, Arg^{9}, Abu^{15}, Nle^{27}, D-Arg^{28}, Har^{29}] hGHRH(1-29)NH_2
JV-1-38	[PhAc-Tyr^{1}, D-Arg^{2}, Phe(4-Cl)^{6}, Har^{9}, Tyr(Me)^{10}, Abu^{15}, Nle^{27}, D-Arg^{29}] hGHRH(1-29)NH_2
MZ-J-7-30	[HOOC-(CH2)12-CO-Tyr^{1}, D-Arg^{2}, Phe(4-Cl)^{6}, Arg^{9}, Abu^{15}, Nle^{27}, D-Arg^{28}, Har^{29}] hGHRH(1-29)NH_2
MZ-J-7-46	[CH3-(CH2)4-CO-Tyr^{1}, D-Arg^{2}, Phe(4-Cl)^{6}, Arg^{9}, Abu^{15}, Nle^{27}, D-Arg^{28}, Har^{29}] hGHRH(1-29)NH_2
MZ-J-7-110	[HOOC-(CH2)12-CO-Tyr^{1}, D-Arg^{2}, Phe(4-Cl)^{6}, Amp^{9}, Tyr(Me)^{10}, Abu^{15}, Nle^{27}, D-Arg^{28}, Har^{29}] hGHRH(1-29)NH_2
MZ-J-7-114	[CH3-(CH2)6-CO-Tyr^{1}, D-Arg^{2}, Phe(4-Cl)^{6}, Amp^{9}, Tyr(Me)^{10}, Abu^{15}, Nle^{27}, D-Arg^{28}, Har^{29}] hGHRH(1-29)NH_2
JMR-132	[PhAc-Tyr^{1}, D-Arg^{2}, Phe(4-Cl)^{6}, Ala^{8}, Har^{9}, Tyr(Me)^{10}, His^{11}, Abu^{15}, His^{20}, Nle^{27}, D-Arg^{28}, Har^{29}] hGHRH(1-29)NH_2
MIA-602	[(PhAc-Ada)0-Tyr^{1}, D-Arg^{2}, Fpa5^{6}, Ala^{8}, Har^{9}, Tyr(Me)^{10}, His^{11}, Orn^{12}, Abu^{15}, His^{20}, Orn^{21}, Nle^{27}, D-Arg^{28}, Har^{29}] hGHRH(1-29)NH_2
MIA-610	[PhAc0-Tyr^{1}, D-Arg^{2}, Cpa^{6}, Ala^{8}, Har^{9}, Fpa5^{10}, His^{11}, Orn^{12}, Abu^{15}, His^{20}, Orn^{21}, Nle^{27}, D-Arg^{28}, Har^{29}, Ada^{30}] hGHRH(1-30)NH_2

Ibu, Isobutyryl; Nac, 1-Naphthylacetyl; PhAc, Phenylacetyl; Cpa, 4-chloro-Phe; Agm, Agmatine, Decarboxy-Arg; Amp, *para*-Amidino-Phe; Har, Homo-Arg; Abu, 2-Aminobutyric acid; Tyr(Me), *O*-Methyl-Tyrosine; Orn, Ornithine; Fpa, Pentafluoro-Phenylalanine; Ada, 12-Aminododecanoyl.|

very high, about 100 fold higher than that of the standard antagonist. MZ-J series of antagonists with weaker endocrine effects (ex JMR-132) than JV-1-63, MZ-4-71 or MZ-

5-156 exhibited greater anticancer potency [28]. More recently, MIA-602 (MIA-series) was synthesized which exhibits one of the most potent antitumor effects reported so far. The structure of most commonly studied GHRH antagonists is shown in Table 1.

It is now widely accepted that GHRH antagonists can inhibit tumor growth by not only suppressing the production of the pituitary GH and subsequently IGF-I, but also by directly suppressing tumor cells growth at the tumor cellular level without any of its endocrine activities. The direct autocrine/paracrine mechanisms, based on the expression of GHRHR,may partly be based upon directly inhibiting the production of various endogenous growth factors such as IGF, epidermal growth factor (EGF) or vascular endothelial growth factor (VEGF), as well as upon affecting some of the signaling mechanisms involved in cell proliferation, survival, and the regulation of proapoptotic signaling mechanisms. For example, GHRH antagonists were found to inhibit the protein kinase C (PKC) - mitogen-activated protein kinases (MAPK) as well as the phosphatidylinositol-3-kinase (PI3K) – Akt kinase signaling. Furthermore, GHRH antagonists reduced the expression levels of c-Jun and c-fos proto-oncogenes as well as of the tumor-suppressor protein p53 in human SCLC, non-SCLC, and prostate cancer models, and decreased the telomerase activity in glioblastomas and other cancers [25, 26, 28-35]. In addition, GHRH antagonists were detected to reduce the levels of the apoptosis regulator B-cell lymphoma 2 (Bcl-2) and increase levels of the apoptosis regulator Bcl-2-like protein 4 (Bax) in non-SCLC and in the prostate cancer [36, 37]. Moreover, GHRH antagonists were shown to reduce the activity of the Janus kinase-signal transducer and activator of transcription (JAK-STAT) pathway in human prostate hyperplasia *in vitro* and *in vivo* [38]. Altogether, the possible antitumor mechanisms of GHRH antagonists are shown in Figure 1 [28].

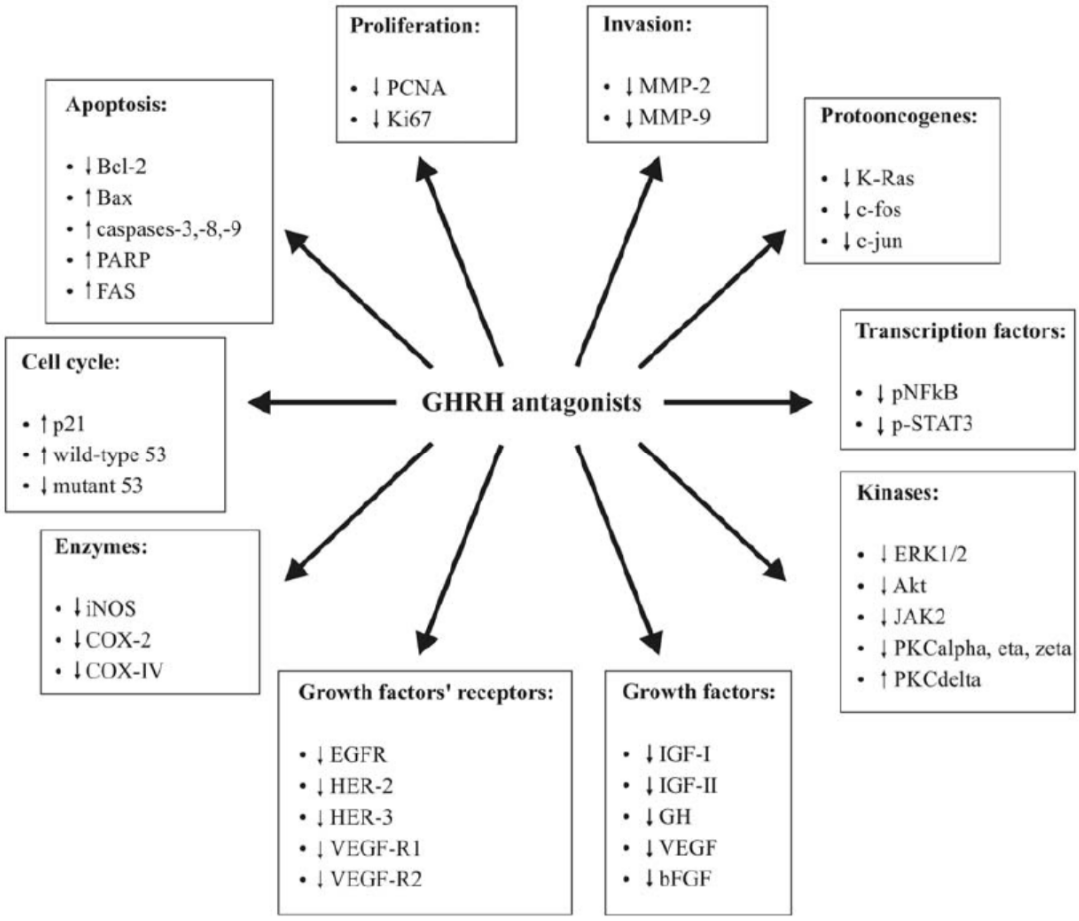

Figure 1: Schematic demonstration of selected mechanisms of action of GHRH antagonists. From Siejka *et al.* 2012 [28]**.**

Recently, GHRH and its receptors have also been found in endometriosis. Few studies were done to evaluate the proliferation inhibiting activity of GHRH antagonists in endometriosis and even no investigations about its underlying mechanisms. Endometriosis is generally assumed to be a benign disease, but it shows behaviors of increased cellular proliferation and invasion or decreased apoptosis which are similar to malignant tumors. Therefore, it might be proposed that the action mechanisms of tumors also operate on the cells involved in endometriosis.

However, the importance of these mechanisms seems to vary in different tumors and by different GHRH antagonists. Up to now, the molecular mechanisms involved in the antitumor or antiproliferative effects of GHRH antagonists on tumor cells or on cells involved in endometriosis have not been completely elucidated. In some tumors, more than one of these mechanisms might operate. The relative importance of these

mechanisms might be diverse in different cancers. Illuminated and identified the action mechanisms of GHRH antagonists might give new directions to their possible applications. Nevertheless, it is widely accepted that the effects of GHRH antagonists are receptor dependent and that SV1 with a high structural homology to pit-GHRHR is probably the main splice variant mediating the effects of GHRH and its antagonists in tumors.

1.4 Endometriosis

1.4.1 Epidemiology of endometriosis

Endometriosis is one of the most common benign gynecologic disorders, defined as estrogen-dependent lesions containing endometrial glands and stromal outside the uterus, strongly associated with intolerable dysmenorrhea, pelvic pain and subfertility. Endometriosis affects more than 176 million women of reproductive age worldwide and the annual cost per woman is estimated of 9,579 € per woman [39-41]. It is widely assumed that endometriosis lesions arise through retrograde endometrial tissue during menstruation, coelomic metaplasia and lymphatic spread in immunologically and genetically susceptible individuals. The mechanisms underlying the pathogenesis of endometriosis are still poorly understood; it is likely to be multifactorial including genetic, epigenetic and environmental influences [39-41]. As a consequence, the clinical cure rate (CCR) is usually quite low. Despite the use of surgery and endocrine therapy, systemic assisted reproduction, and novel pharmaceutical agents such as GnRH antagonists and aromatase inhibitor, the recurrence rate of endometriosis remains 30~50% after cessation of treatments within one year and increases over time [40]. Prospective observational studies indicate that hysterectomy with bilateral salpingo-oophorectomy is a successful strategy for women who are not pursuing to try getting pregnant but results in surgical menopause. Around one third of these women suffering from endometriosis, however, will require further surgery for symptoms at five years, compared with 10% of those who undergo hysterectomy with oophorectomy for endometriosis [42, 43]. In younger women (aged 30-39), however, removal of the ovaries does not significantly improve the surgery-free time and is likely

to lead to adverse symptomatic and health consequences associated with surgical menopause [39, 40]. Furthermore, medical therapy always can be prescribed only for a short time due to relatively short-term effects or unacceptable side effects. As for today, an optimal drug does not exist that allows both, pain management as well as continuing the attempt to conceive. Therefore, it is mandatory to develop more ideal treatment strategies for endometriosis which eliminate endometriotic lesions, prevent recurrence but not impede ovulation.

1.4.2 Expression of GHRH and GHRH receptor and response to GHRH antogonists in endometriosis

The actual findings of GHRH expression in normal endometrium, endometrial cancer and endometriotic tissues as well as of its influence on cell proliferation and invasion, suggest that GHRH may be involved in the pathogenesis endometriosis and furthermore in the development of endometrial cancers [44, 45]. Moreover, the presence of pit-GHRHR and its splice variant SV1 has also been detected in tissues including endometriosis and endometrial cancers. Wu *et al.* have shown that SV1 was expressed in human endometric cancer cell lines (Ishikawa, ECC-1 and HEC-1A) [46]. Chatzistamou *et al.* found SV1 expression in 63% (15/24) of the endometrioid tumors [47]. Fu *et al*. have detected SV1 mRNA in 63% (17/27) of patients with endometriosis in ectopic endometrial tissues but not in eutopic endometrial tissues and in 10% (2/20) of eutopic endometrial tissues in women without endometriosis. In endometriotic stromal cells (ESCs) derived from the endometrium of endometriosis patients the SV1 could be detected in 100% [48] . Annunziata *et al.* also found the SV1 mRNA expressed in normal endometrial tissues and ESCs. Moreover, the pit-GHRHR mRNA was expressed by ESCs and the endomeriotic cell line T-HESC. By using the GHRH antagonist JV-1-36 endometriotic cell proliferation and survival could be inhibited [49]. These findings imply that GHRH and its receptors (pit-GHRHR and SV1) appear to be involved in the proliferation and survivability of endometriotic cells and GHRH antagonists may be a potential new therapeutic agent in endometriosis.

GHRH receptors have been detected in various human cancers and cancer cell lines, however, reports about the expression levels of pit-GHRHR and its splice variant SV1

in normal endometrium as well as in ectopic and eutopic endometrial tissues from patients with endometriosis are still controversial. The expression level of functioning protein of GHRH receptors in ectopic and eutopic endometrial tissues in patients with endometriosis has not been identified so far. Furthermore, the effect of GHRH antagonists on endometriosis also remains to be elucidated.

In the actually conducted study of our department in Lübeck, the expression of SV1 in primary eutopic endometrial stromal cells (EESCs) isolated from patients with endometriosis has been demonstrated by reverse transcriptase-PCR and western blot analysis. Furthermore, an *in vivo* endometriosis xenotransplantation mouse model receiving a daily intra-peritoneal injection of 10µg MIA-602 over 28 days showed significantly reduced sizes of endometriosis lesions compared to the saline control. These results suggest the use of MIA-602 potentially as a new therapeutic agent for endometriosis. However, there are still questions about the involvement of GHRHR in pathogenesis of endometriosis and the way GHRH antagonists can modulate the biological behavior of endometriotic cells.

1.4.3 EGFR/ERK$_{1/2}$ and EGFR/Akt signaling pathways in endometriosis

A variety of genetic, endocrine, immunological, and environmental factors have been proposed to account for the development of endometriosis, but the exact pathogenesis still has not been fully elucidated [40]. The most accepted theory is that retrograded menstrual endometrial cells exhibit abnormal proliferative and apoptotic regulation in response to appropriate stimuli. Diverse signaling pathways have been studied in endometriosis that correlate with the abnormal proliferation or apoptosis of the endometrial cells [50, 51].

The most important of these signaling pathways are represented by the epidermal growth factor receptor (EGFR)/Akt kinase or the EGFR/extracellular-regulated kinase (ERK$_{1/2}$)-pathway. EGFR is a 170 kDa transmembrane glycoprotein, serving as the common receptor for its cognate ligand EGF and transforming growth factor. EGFR and its downstream effectors such as Akt, and ERK$_{1/2}$ play pivotal roles in normal and pathogenic cellular processes, including cell proliferation, apoptosis, angiogenesis, cell

migration, and invasion [52-56]. Overexpression of EGFR and its autophosphorylation followed by activation of $ERK_{1/2}$ and Akt signaling are linked to more aggressive behavior and correlate with poor prognosis of various human malignancies [52, 53, 55, 57]. Dysregulated EGFR expression is implicated in the pathogenesis of numerous diseases including endometriosis [58]. It has been reported that EGF and EGFR are present in ectopic endometrial tissues and EGF levels in peritoneal fluid are elevated in patients with severe endometriosis [58]. EGF and EGFR are also reported to be associated with the development of endometriosis, based on data showing that EGF increases DNA synthesis in cultured endometriotic cells and stimulates the *in vitro* proliferation of both eutopic and ectopic cells. Moreover, one EGFR gene polymorphism has been recently associated with susceptibility to endometriosis [51, 59-61]. In addition, activation of the $ERK_{1/2}$ and/or Akt pathway had been found in eutopic endometrium, ectopic endometriosis tissues and ESCs from patients with endometriosis [42, 43, 62, 63]. The hyperproliferative and aggressive phenotype of endometriotic cells were found to be associated with activation of the $ERK_{1/2}$ and/or Akt pathways. $ERK_{1/2}$ and/or Akt inhibition decreased ESCs proliferation, adhesion and invasion, both *in vitro* and *in vivo* [64, 65] . All of these evidences support that hyperactivated EGFR, $ERK_{1/2}$ and Akt signaling may be involved in pathogenesis of endometriosis. Thus blocking the EGFR, $ERK_{1/2}$ and Akt pathway offers new prospects for the treatment of endometriosis.

1.5 Triple negative breast cancer (TNBC)

1.5.1 Epidemiology of TNBC

In women, breast malignancies emerged as the most common cancer and accounts for the leading cause of cancer-related deaths among the world, according to the latest world cancer statistics from WHO's International Agency for Research on Cancer (IARC) [35, 36, 52, 55, 57, 66-71]. Since 2008 estimates, breast cancer incidence has increased by more than 20% and mortality increased by 14% [66-68]. Despite the use of radical operation, systemic chemotherapy, and molecular targeted treatments, the cancer-related mortality remains at an unacceptably high rate. Nowadays, it became obvious

that breast cancer is a disease composed of multiple subgroups characterized by their pathophysiological features, outcomes, and their ability to response to treatment [72]. The heterogeneity of the subgroups underscores the need for treatments being tailored for each individual patient, depending on the molecular characteristics of her malignancy.

Triple negative breast cancer (TNBC) is one of special subgroups of breast cancer that does not express the genes for estrogen receptor (ER), progesterone receptor (PR) and is not overexpressing the HER2-receptor. It accounts for approximately 10~17% of all breast cancer cases [72]. Compared to other subgroups of breast cancer, TNBC is frequently hereditary linked to early-onset, is more aggressive, growing faster and having an inferior prognosis also due to the lack of targeted treatment options [67, 73]. Although, most cases of TNBC appear to be responsive to chemotherapy, most patients cannot achieve a complete response (CR) and tend to relapse sooner than patients with other breast cancer subtypes. This suggests a crucial need to identify potential new agents and targeted therapies for TNBC.

1.5.2 Expression of GHRH and GHRH receptors and response to GHRH antagonist in TNBC

The presence of GHRH and its receptors has been demonstrated in various tumors, suggesting that this neuropeptide may be involved in the pathogenesis of neoplasms as an autocrine/paracrine growth factor [8, 74-76]. Various findings suggest that GHRH, GHRH receptors and its splice variants are present in breast cancer cell lines and surgical specimens of breast cancers [16, 69-71]. Moreover, the breast cancer cells, which possess the GHRHR, was proven to be stimulated by GHRH agonist and inhibited by GHRH antagonists [16, 69-71]. A previous study from our department had demonstrated that GHRH, pit-GHRHR and the splice variant SV1 mRNA were expressed in 80%, 25% and 75% of human triple-negative breast cancer specimens, respectively [77]. We also showed that GHRH antagonist MZ-J-7-118 efficiently inhibited the proliferation of triple-negative breast cancer cell HCC1806 which expressed the GHRHR [77]. Recently Roberto *et al.* reported that GHRH antagonist MIA-602 inhibited the growth of tumor in TNBC cells (HCC 1806 and MX-1) in a xenotransplantation

mouse model [78]. They also concluded that treatment with GHRH antagonists of TNBC xenografted mice reduced tumor growth through an action mediated by tumoral GHRH receptors. These results indicate that GHRH/GHRHR plays a role in modulates the growth in triple-negative breast cancer, moreover GHRH antagonist might be a new treatment regimen for TNBC.

1.5.3 EGFR/ ERK1/2 and EGFR/Akt pathway in TNBC

The human EGFR family comprises of four closely related receptors that are transmembrane glycoproteins containing an extracellular ligand binding domain and an intracellular receptor tyrosine kinase domain. The major signaling pathways activated by EGFR receptors are mediated by PI3 kinase/Akt and MAPK/$ERK_{1/2}$ pathways, resulting in a plethora of biological functions including cell proliferation, survival, angiogenesis, cell migration, and invasion [52-56]. EGFR overexpression in breast cancer is associated with large tumor size, poor differentiation, and poor clinical outcomes [52]. It has been reported that TNBC cells are often expressing high levels of EGFR and show abnormal activation of PI3 kinase/Akt and MAPK/$ERK_{1/2}$ signaling pathways [52]. TNBC overexpress EGFR in nearly half of the cases and the negative impact of EGFR overexpression is particularly pronounced in TNBC [57]. Moreover, Lee *et al.* recently showed that EGFR-targeted therapy may be used to enhance the initial sensitivity of TNBC cells to cytotoxic therapy [71]. Thus, EGFR and its signaling pathway components might be suitable supplementary sites for blocking tumorigenic signals in human TNBC.

1.6 Aims

MIA-602 is one of the newest series of highly potent GHRH antagonists with improved design and resistance to biodegradation. It has been proven to inhibit several human experimental cancers including prostate, lung and breast cancer, but the effects on endometriosis have not been reported so far. The knowledge about the underlying signaling mechanisms of MIA-602 in TNBC as well as in endometriosis is still under investigation.

Considering the importance of EGFR and its signaling pathway in both TNBC and

endometriosis as well as the antiproliferative function of the GHRH antagonist MIA-602 in breast cancer and endometriosis, we hypothesize that MIA-602 might inhibit the growth of human TNBC cells and the cells involved in endometriosis by interfering with the intracellular signaling pathways of EGFR/$ERK_{1/2}$ and/or EGFR/Akt (Figure 2).

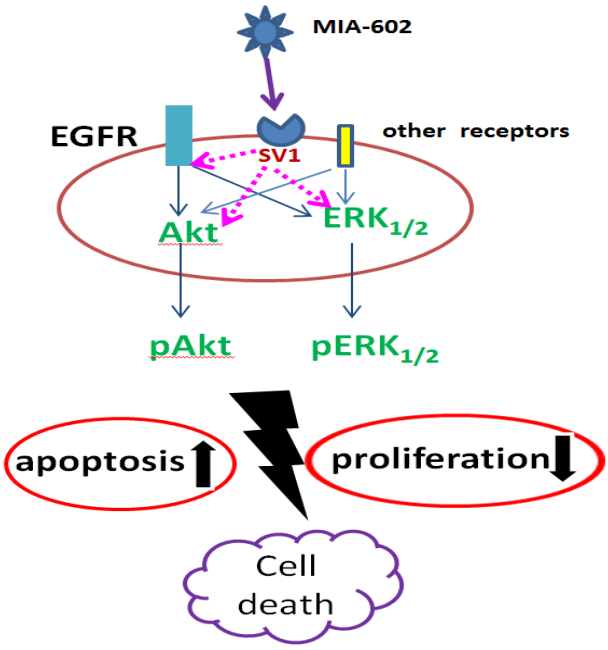

Figure 2: Presumed action pathway of MIA-602 in TNBC cells and the cells involved in endometriosis.

Special aims on endometriosis:

- To identify the expressions of GHRH, pit-GHRHR and SV1 in endometrium and endometriosis tissues from women with or without endometriosis.
- To evaluate the regulatory effects of MIA-602 on the immortalized human endometriotic epithelial cell lines.
- To demonstrate the inhibitory mechanisms of MIA-602 on the human endometriotic epithelial cell lines by interference with the EGFR pathway.

Special aims on TNBC:

- To explore the expression of pit-GHRHR and SV1 in TNBC cell lines.
- To evaluate the regulatory effects of MIA-602 on TNBC cell lines.
- To demonstrate the inhibitory effect of GHRH antagonist MIA-602 on TNBC cell lines by interference with the EGFR pathway.

2 Material and Methods

2.1 Materials

2.1.1 Chemicals

All chemicals and reagents were used in analytic degree of purity.

Acetic acid (glacial)	Merck, Darmstadt
Agarose	Invitrogen, Karlsruhe
Ammonium persulfate (APS)	Biorad, Munich
Bromophenol blue	Merck, Darmstadt
Dimethylsulfoxide	Merck, Germany
deionized H_2O	University of Lübeck
dNTP Mix(10Nm)	Invitrogen, Karlsruhe
Ethanol	Roth, Karlsruhe
Ethylenediaminetetraacetate (EDTA)	Sigma-Aldrich, Steinheim
Fetal calf serum (FCS)	Sigma, Steinheim
Filter paper	Whatman, GE Healthcare, Munich
Glycine	AppliChem GmbH, Darmstadt
Hydrochloric acid (HCl)	Merck, Darmstadt
Milk powder (blotting grade)	Roth, Karlsruhe
N,N-dimethylformamide	Merck Schuchardt OHG, Hohenbrunn
N,N,N',N'-tetramethylenediamine (TEMED)	Sigma-Aldrich, Steinheim
Penicillin/streptomycin (100x)	PAA Laboratories GmbH, Coelble
QIAZOL® reagent	Qiagen, Hilden
Re-Blot Plus	Chemicon International, Hofheim
RNAse-free water	Serva, Heidelberg
RPMI1640 Medium	Invitrogen, Karlsruhe
Serum replacement 1 (SR1, 50x)	Sigma, Taufkirchen
Sodium chloride	Roth, Karlsruhe
Sodium dodecyl sulfate (SDS)	Serva Electrophoresis, Heidelberg
Thiazolyl blue tetrazolium bromide (MTT)	Sigma-Aldrich, Taufkirchen
Tris-buffer	Roth, Karlsruhe
Trypsin-EDTA (1X)	PAA Laborateries GmbH, Coelble
Tween 20	Merck, Darmstadt
X-ray developer- / fixer-concentrate	Adefo-Chemie GmbH, Dietzenbach

2.1.2 Instruments and equipment

BioPhotometer plus	Eppendorf, Germany
Thermocycler T-personal 48	Biometra GmbH, Goettingen
TC20™ Automated Cell Counter	Biorad, Munich
CO_2 incubator	Binder GmbH, Tuttlingen
Cell culture pipettes (5, 10, 25ml)	Greiner, Frickenhausen
Cell culture flaskes (10, 25ml)	Greiner, Frickenhausen
Cell scrapers	Biochrom AG, Berlin
Cryovessels 1.5ml	Sarstedt, Nuembrecht
DNA Engine Opticon 2 Realtime-PCR-machine	MJ-Research, Biorad, Munich
Hyperfilm ECL	Millipore, Schwalbach
Electrophoresis Power supply device	Pharmacia Biotech, Hamburg
Gel Documentation System	Phase GmbH, Luebeck
Hoefer Semi-Dry Transfer Unit TE 70	GE Healthcare, Munich
Horizontal shaker Vibrax VXR S15	IKA Labortechnik, Staufen
Laboratory balance basic BA3105	Sartorius AG, Goettingen
Microcentrifuge MC6	Sarstedt, Nuembrecht
Microscope Axiovert 135M	Carl Zeiss, Goettingen
Microwave	Sharp Electronics, Hamburg
Mini-Sub® cell GT electrophoresis chamber	Biorad, Munich
Multiplate reader	Dynatech, Burlington, USA
Multipette Plus	Eppendorf, Hamburg
96-well cell culture plate	Sarstedt, Nuembrecht
6-well cell culture plate	Sarstedt, Nuembrecht
Neubauer-counting chamber	Brandt, Wertheim
Oven	Heraeus, Hanau
Orbital Shaker S4	ELMI Lab.Equipment, Riga, Latvia
pH Meter pH526 MultiCal®	WTW, Weilheim
Pipettor acccu-jet	Brand GmbH, Wertheim
PVDF transfer membrane	GE Healthcare, Munich
Refrigerated Centrifuge Biofuge®fresco	Heraeus, Hanau
Suction Gel Pump GP110	Savant, New York, USA
Thermomixer compact	Eppendorf, Wesseling-Berzdorf
Tubes (Safe Lock Tubes) (1.5ml, 2ml)	Eppendorf, Hamburg
Universal 32 centrifuge	Hettich,Tuttlingen
UV-transilluminator TI1	Biometra GmbH, Goettingen
Variable pipet Eppendorf Research®	Eppendorf ,Wesseling-Berzdorf
Vortex REAX2000	Heidolph, Schwabach

2.1.3 Solutions and Buffers

1 x TBST	8.18 g NaCl, 2.42 g Tris in 1 L dH_2O (pH=7.6), 0.1 % Tween 20
10 x TBS	81.8 g NaCl, 24.23 g Tris in 1 L dH_2O (pH=7.6)
MTT-solubilization solution	10% SDS, 50% N,N-dimethylformamide
MTT solution (50ml)	250 mg MTT, 50 ml PBS
10%MTT (10ml)	1 ml MTT solution, 9 ml DMEM medium
PBS	pH7.4; KH_2PO_4 1.4 mol/l, Na_2HPO_4 4.3 mol/l, NaCl 137 mol/l, KCl 2.7 mol/l and dh_2O
Paraformaldehyde/PBS	4.5% Paraformaldehyde in PBS
DNA electrophoresis buffer	0.5 TBE (0.5x45 Nm Tris-borate and 1Mm EDTA)
RIPA buffer	150 mM NaCl, 1.0% Igepal®, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris, pH 8.0
4xSDS-protein loading buffer	0.125 M Tris-HCL, pH 6.8, 20% glycine, 4% SDS, 0.01% bromphenolblue, 2% ß-mercaptoethanol
Protein electrophoresis buffer	25 mM Tris base, 192 mM glycine, 0.1% SDS, pH 8.3
Protein transfer buffer	25 mM Tris base, 192 mM glycine,10% methanol

2.1.4 Kits

BCA-Protein Assay Kit	Thermo Fisher Scientific, Schwerte
CellTiter 96®Aqueous One Solution Cell Proliferation Assay (MTS)	Millipore, Schwalbach
Precision Plus Protein Dual Color Standards	Biorad, Munich
Rneasy Mini Kit	Qiagen, Hilden
Rneasy Purified Kit	Qiagen, Hilden
SuperScript® II Reverse Transcriptase RT Kit	Life Technologies, Frankfurt
Immobilon western chemiluminescent HRP substrate	Millipore, Schwalbach

2.1.5 Antibodies and their characteristics

Primary GHRHR-antibody	Rabbit polyclonal antibody (ab28692; Abcam, Cambridge, UK). The antibody is produced with the synthetic peptide: 403-422 corresponding to amino acids RTRAKWTTPSRSAAKVLTSM of Human GHRHR. Predicted band size of pit-GHRHR and SV1 is 47 kDa and 39 kDa, respectively. It was used at 1:2000 dilutions in western blot for human endometrial tissues, and 1:5000 dilutions in western blot for TNBC cells.
Primary EGFR-antibody	Goat polyclonal antibody to the C-terminus of EGFR of human origin (sc-03-G, Santa Cruz Biotechnology, USA). Predicted band size is 170 kDa. It was used at 1:2000 dilutions in western blot.
Primary ERK-antibody	Rabbit polyclonal antibody, recommended for detection of ERK_1 p44 and, to a lesser extent, ERK_2 p42 of human origin (sc-94, Santa Cruz Biotechnology, USA). Predicted band size of ERK_1 and ERK_2 is 44 kDa and 42 kDa. It was used at 1:2000 dilutions in western blot.
Primary pERK-antibody	Mouse monoclonal antibody, recommended for detection of ERK_1 phosphorylated at Tyr 204 and correspondingly phosphorylated ERK_2 of human origin (sc-7383, Santa Cruz Biotechnology, USA). Predicted band size of $pERK_1$ and $pERK_2$ is 44 kDa and 42 kDa. It was used at 1:1000 dilutions in western blot.
Primary Akt-antibody	Mouse monoclonal antibody, raised against amino acids 345-480 of Akt of human origin (sc-5298, Santa Cruz Biotechnology, USA). Predicted band size of Akt is 62 kDa. It was used at 1:500 dilutions in western blot.
Primary pAkt-antibody	Rabbit polyclonal antibody, recommended for detection of Akt_1 phosphorylated Ser 473 and correspondingly Ser 474 phosporylated Akt_2 and Ser 472 phosporylated Akt_3 of human origin (sc-7985-R, Santa Cruz Biotechnology, USA). Predicted band size of $pAkt_1$, $pAkt_2$and $pAkt_3$ is 62 kDa, 56 kDa and 62 kDa. It was used at 1:1000 dilutions in western blot.
Primary Bax-antibody	Rabbit monoclonal antibody (ab10813; Abcam, Cambridge, UK). The antibody is produced with the synthetic peptide: 43-61 corresponding to amino acids CPELALDPVPQDASTKKLSE of Human Bax. Predicted

	band size of Bax is 21 kDa. It was used at 1:2000 dilutions in western blot.
Primary BCL-2-antibody	Goat polyclonal antibody to the C-terminus of BCL-2 of human origin (sc-03-G, Santa Cruz Biotechnology, USA). Predicted band size is 26 kDa. It was used at 1:1000 dilutions in western blot.
Primary ß-Actin-antibody	Mouse monoclonal antibody, recommended for detection of ß-Actin (Santa Cruz Biotechnology, USA). Predicted band size of ß-Actin is 42 kDa. It was used at 1:5000 dilutions in western blot.
Secondary antibodies	ECL™ anti-Rabbit IgG, anti-mouse IgG and anti-goat IgG from Amersham, GE Healthcare, and Munich. Those were used at 1:5000 dilutions.

2.1.6 Patients and samples

In the current study, the self-paired endometriotic tissues were collected in the Department of Gynecology and Obstetrics from 16 women with grade III-IV endometriosis (American Society for Reproductive Medicine classification of endometriosis, ASRM) diagnosed by the Pathology Department of the University of Lübeck. The ectopic endometrial tissues were obtained from the walls of ovarian endometriomas and/or peritoneal endometriotic lesions during therapeutic laparoscopy; their paired eutopic endometrial samples were collected by pipelle endometrium biopsies at the time of surgery. The normal endometrial tissues were collected from 16 non-endometriosis patients who underwent pipelle endometrium biopsies or hysterectomy (uterine myoma n=10, uterine cavity adhesion n=2, simple ovarian cysts n=4). All recruited patients were reproductive-aged women with regular menstrual cycles who had not received any endocrine or anti-inflammatory treatment at least 6 months before surgery or had undergone HRT. The collection of all samples was approved by the Ethical Committee for Clinical Research from the University of Lübeck and written informed consents were obtained. The mean age was 36.5± 3.2 years (range 26 to 44 years) in endometriosis patients and 37.4±2.7 years (range 26 to 47 years) in control group, the age range showed no significant difference between two groups. For expression analysis, eutopic and ectopic endometrial tissues were

snap-frozen in liquid nitrogen immediately after sampling and stored at -80 °C.

2.1.7 Cell Lines

Immortalized human endometriotic epithelial cell lines (12-Z and 49-Z) were a friendly gift by Prof. Starzinski-Powitz (University of Frankfurt, Germany). The triple negative breast cancer cell lines (HCC1806, MDA-MB-231, HCC1937 and MDA-MB-468) were obtained from the American Type Culture Collection and purchased at LGC-Standards, Wesel.

2.1.8 Peptides

The 44 amino acid form of human GHRH(1-44)NH_2 and 29 amino acid form of human GHRH(1-29)NH_2 were purchased from PeptaNova GmbH (Sandhausen, Germany) and stored at -20 °C.

The structure of GHRH(1-44)NH_2 is:

Tyr-Ala-Asp-Ala-Ile-Phe-Thr-Asn-Ser-Tyr-Arg-Lys-Val-Leu-Gly-Gln-Leu-Ser-Ala-Arg-Lys-Leu-Leu-Gln-Asp-Ile-Met-Ser-Arg-Gln-Gln-Gly-Glu-Ser-Asn-Gln-Gly-Arg-Gly-Ala-Arg-Ala-Arg-Leu-NH_2.

The structure of GHRH(1-29)NH_2 is:

Tyr-Ala-Asp-Ala-Ile-Phe-Thr-Asn-Ser-Tyr-Arg-Lys-Val-Leu-Gly-Gln-Leu-Ser-Ala-Arg-Lys-Leu-Leu-Gln-Asp-Ile-Met-Ser-Arg-NH_2.

The GHRH antagonist MIA-602 was synthesized and is a friendly gift by Andrew V. Schally and of the laboratory of the Endocrine, Polypeptide and Cancer Institute, Veterans Affairs Medical Center, Miami, FL, USA. MIA-602 is an antagonistic analog of human GHRH with the structure:

[(phAc-Ada)0- Tyr,1D-Arg,2 (phe[F]5),6 Ala,8 Har,9 Tyr(Me),10 His,11 Orn,12 Abu,15 His,20 Orn,21 Nle,27 D-Arg,28 Har29]hGHRH(1-29)NH_2

Three mg GHRH(1-44)NH_2,GHRH(1-29)NH_2 and MIA-602 were dissolved in 20 µl DMSO and further diluted with water ad 621 µl to a stock solution of 1 mM, respectively. For the control stocking solution, 20 µl DMSO was dissolved in 621 µl water. For incubation, the solutions were diluted to 1 µM and 0.1 µM with medium.

2.2 Methods

2.2.1 Cell Culture and Cell Concentrations

All cell culture work was carried out under a laminar airflow under sterile conditions. Cells were cultured as a monolayer in a humidified atmosphere with 5% CO_2 at 37 °C in RPMI1640 medium supplemented with 10% fetal bovine serum (FBS) and 1% antibiotics (100 U/ml penicillin G, 100 µg/ml streptomycin) and grown to confluence with regular changes of medium. For sub-cultivation and harvesting, adherent cells were washed twice with ice-cold 1x PBS and treated with 0.05% trypsin-EDTA for up to 10 min. Proteolysis was stopped with medium containing 10% FCS and cells were pelleted at 1500 rpm for 5 min at room temperature.

Cells were resuspended in culture media and counted in the TC20™ Automated Cell Counter (Biorad, Munich. One volume of Trypan Blue 0.1% (Merck, Darmstadt) was added to one volume of the cell suspension to mark dead cells. The polyanionic Trypan Blue crosses the membrane of dead cells and dyes them. Living cells remain undyed under the microscope. Cells in the four corner squares of the chamber were counted and averaged. Cell concentrations were determined according to the formula: average cell count x 2 (dilution factor) x 10^4 = cells/ml.

2.2.2 Cell Proliferation Assay

The cells were seeded onto 96-well plates at a density of 2,000~3,000 cells/well (3,000 cells/well for 49Z and MDA-MB-231 cells, 2,000 cells/well for12Z and HCC1806 cells) cultured in complete medium overnight, starved for 12-24 h in medium without FBS, and then treated for 96 h with GHRH antagonist MIA-602 (0.1-1 µM), GHRH agonists GHRH(1-44)NH_2 (1µM) and GHRH(1-29)NH_2 (1µM) in medium with 2% FBS respectively, as well as 0.01% DMSO as control.

For 12Z and 49Z cells, cell viability was evaluated by using the MTS-assay (CellTiter 96R AQueous One Solution Cell Proliferation Assay Kit, Promega) according to the manufacturer's instructions: 20 µl CellTiter 96R AQueous One Solution were added to hundred microliter samples in each well and the plates were incubated for a further 4 h at 37 °C. Absorbance was measured at 490 nm in a microplate reader. For TNBC cells,

cell viability was evaluated by using MTT assay. 100 µl of a 1:10 mixture of MTT (Sigma, Deisenhofen, Germany) in phenol red-free DMEM media (Invitrogen, Karlsruhe, Germany) was added to the cells and the plates were incubated for a further 4 h at 37 °C. Then 100 µl stop solution containing 10% N,N dimethyl formamide and 20% SDS were added. The solubilization of the formazan crystals was performed in the dark over-night. Absorbance was measured at 570 nm in a multiplate reader. All experiments were done in sextuplicate and were repeated 3 times. The inhibition of cell proliferation was expressed as the percentage of control.

2.2.3 Treatment of cell lines for GHRH antagonist responsiveness

For experiments involving GHRH antagonist MIA-602 and GHRH agonists [GHRH(1-44)NH_2 and GHRH(1-29)NH_2] stimulation, the cells were seeded onto 6-well plates at a density of 2×10^5 cells/w and cultured for 24 h prior to the experiment in serum-free RPMI supplemented with 1% penicillin and streptomycin. Inductions with 1 µM of MIA-602, 1 µM GHRH(1-44)NH_2 and 1 µM GHRH(1-29)NH_2 were performed in the same conditions for 5 min to 72 h, and 0.01% DMSO as control.

2.2.4 RNA Extraction and quantitative RT-PCR

RNA Extraction

After rinsing the cell monolayer with ice cold PBS twice, cells' total RNAs were extracted with the RNeazy-mini-kit (Qiagen, Hilden). The cells grown in 6-well were disrupted in 600 µl/well RLT-buffer and the lysate was transferred directly onto a QIAshredder spin column placed in a 2 ml collection tube, and centrifuged for 2 min at full speed. One Vol 70 % ethanol was added and mixed with the homogenized lysate. The mixture was added on an RNeasy spin column and the manufacturer´s procedure was followed until the elution of the RNA.

Tissues' total RNAs were extracted by using the Qiagen® reagent (Qiagen, Hilden). We homogenized the liquid nitrogen preserved samples in 1 ml of TRIZOL reagent per 50 mg of tissue using a homogenizer and incubated the homogenate for 5 min at room

temperature to permit the complete dissociation of nucleoprotein complexes. 0.2 ml of chloroform was added per 1 ml of Qiagen® reagent. After vigorous shaking for 15 s, the samples were incubated at room temperature for 10 min and centrifuged at 10,000 x g for 15 min at 40 °C. Then, the upper aqueous phase was carefully transferred into fresh tube without disturbing the interphase and incubated in 0.5 ml isopropyl alcohol at 15 to 30 °C for 10 min and centrifuged at no more than 12,000 x g for 10 min at room temperature. After removing of the supernatant, the RNA pellet was washed once with 1 ml 75% ethanol and centrifuged at 10,000 x g for 5 min at room temperature.

Next, the total RNAs were purified by RNA purification kit (Qiagen, Hilden). Firstly, we dissolved the above RNAs in 100 µl RNase-free water and added 350 µl buffer RLT and mixed thoroughly. Then the manufacturer´s protocol was followed until the elution of RNA with 30~50 µl RNase-free water.

The quality of isolated RNA was tested with a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific). The OD260/OD280 ratio was in the range of 1.8~2.1 for all samples. The RNA from the above purification is free from polysaccharides, but may still have some gDNA. For qRT-PCR assay, the RNA needed to be further treated with DNAaseI. Therefore, we used 1 unit of Amplification Grade DNase I (Life Technologies, Frankfurt) in a volume of 10 µl to treat each 1 µg of RNA. 1 µg RNA was mixed with 2 mM Mg2+, 1 unit DNase I, and 1 x PCR buffer and incubated for 37 °C for 30 min.

Quantitative reverse transcriptase PCR

cDNA synthesis

One microgram total RNA from each sample was reverse-transcribed into cDNA using the SuperScript® II Reverse Transcriptase (life technologies, Frankfurt). The RNA solution was mixed with 1 µl of oligo-dT Primers (0.5 µg/µl), 1 µl of dNTP and 12 µl H_2O. This mixture incubated in a PCR machine pre-heated at 70 °C. After 10 min, as temperature changed to 4 °C, 6 µl of master mix (2 µl of DTT, 4 µl of 5 x buffer; for one sample) was added to the tubes and allowed to incubate for 2 min at 25 °C. Finally, at 42 °C, 1 µl of Superscript II reverse transcriptase was added and allowed to react for 1

h. An RT-negative control was carried out using RNAse-free water instead of reverse transcriptase. The cDNA was diluted 1:5 and stored until further use at -20 °C.

Quantitative PCR

Quantitative PCR (qPCR) was used to evaluate the expression of mRNA for human GHRH-R, SV1, GHRH, EGFR, and HPRT. All primers were synthesized by Metabion GmbH, Martinsried and are described in Table 2. Quantitatve PCR reactions were performed using the Opticon-2 (Biorad, Munich). All the samples were run in triplicate, with each well containing a final volume of 25 µL, including 5 µL of cDNA, 5 nM gene-specific primers. Normal rat pituitary was used as a positive control, and HPRT served as housekeeping gene. RT-negative samples and no-cDNA controls were run in each PCR for each primer pair. The specificity of the PCR products was confirmed by melting curve analysis of the amplified product. Relative gene expression was analyzed by the 2-ΔΔCt method. The PCR conditions were:

step 1: 2 min polymerase activation at 50 °C
step 2: 2 min denaturation at 95 °C

step 3: 15 s denaturation at 95 °C
step 4: 30 s annealing and polymerization at 60 °C
step 5: Plate reading and return to step 3 for a total of 40 cycles

step 6: Melting curve from 60-94 °C, measured in increments of 0.1 °C → End
step 7: Cooling at 4.0 °C
The product was stored at -20 °C.

Table 2: The sense- and antisense-specific primers for GHRH-R, SV1, GHRH, EGFR, and HPRT

pit-GHRHR forward	5’- TTC TGC GTG TTG AGC CCG TTA C-3’
pit-GHRHR reverse	5’- TAA GGT GGA AAG GGC TCA GAC C-3’
SV1 forward	5’- TGG GGA GAG GGA AGG AGT TGT-3’
SV1 reverse	5’-TAA GGT GGA AAG GGC TCA GAC C-3’
HPRT1 forward	5’-TCA GGC AGT ATA ATC CAA AGA TGG-3’
HPRT1 reverse	5’-AGT CTG GCT TAT ATC CAA CAC TTC-3’
GHRH forward	5’-ATGCAGATGCCATCTTCACCAA-3’
GHRH reverse	5’-TGCTGTCTACCTGACGACCAA-3’
EGFR forward	5’-TCCTCTGGAGGCTGAGAAAA-3’
EGFR reverse	5’-GGGCTCTGGAGGAAAAGAAA-3’

2.2.5 Western Blot Analysis

Protein Isolation

We thawed frozen cells or tissues slowly on ice, and washed them with cold PBS twice. Cells or tumor samples were dissolved in lysis buffer (1 ml 1xRIPA buffer, 10 µl sodium orthovanadate, and 10 µl PMSF and 30 µl aprotinin) using 0.5 ml per 10^6 cells or 30 µl per 10 mg tissues and mixed gently with a pipette and incubated for 30 min on ice. Then cells were passage through a 21 gauge needle, and tissues were further disrupted and homogenized with an Eppendorf-tube homogenizer (Eppendorf, Hamburg), maintaining temperature at 4 °C. The lysate was transferred to a 1.5 ml microcentrifuge tube, and centrifuged at 10,000 x g for 10 min at 4 °C. The supernatant was transferred to a new tube for further analysis.

BCA Protein concentration evaluation

Protein concentration was evaluated by Bicinchoninic Acid (BCA) protein Assay Kit (Thermo Fisher Scientific, Bonn) following the manufacturer´s protocol. 200 µl of Reagent AB was added into every well of a 96 well plate each containing a sample. The plate was incubated at 37 °C for 30 min followed by measuring in a microplate reader at 560 nm. A standard curve was used to determine the concentrations of each unknown sample. For western blotting 7-25 µg of each sample were diluted up to 20 µl with water and 4 x SDS loading dye. The proteins were denaturated for 5 min at 95 °C followed by cooling on ice, immediately.

SDS-Polyacrylamide gel electrophoresis (SDS-PAGE) and wet-blotting

Proteins were separated by denaturing 8~15% SDS--polyacrylamide gel electrophoresis and transferred onto PVDF membrane. An electric current of 80-100 V was applied, causing the negatively charged proteins to migrate across the gel towards the anode. One membrane was blotted for 120 min at 20-25 mA. Membranes were blocked with 5% nonfat dry milk in TBS containing 0.1% Tween-20 (Sigma-Aldrich).

Antibodies were diluted in 5% nonfat dry milk in TBS containing 0.1% Tween-20. The primary antibodies used were anti- GHRH-R antibody (detecting both full-length GHRH-R and variant SV1)(1:2,000 or 1:5,000), anti-Bax (1:2,000), anti-cleaved PARP and anti-PARP(1:2,000) from Abcam Biotechnology, anti-$ERK_{1/2}$ (1:2,000), anti-$pERK_{1/2}^{Tyr204}$ (1:1,000), anti-AKT (1:1,000), anti-$pAKT_{1\text{-}3}^{Ser472\text{-}474}$ (1:1,000), anti-EGFR (1:2,000), anti-BCL-2(1:1,000), and anti-actin (1:5,000) as an endogenous control, all from Santa-Cruz Biotechnology, Heidelberg. Subsequently the membrane was incubated at 4 °C overnight on a shaker with the primary antibodies, and then washed with washing buffer for 3×10 min. The membrane was then incubated for another 1 h with the suitable secondary antibody. After repeated washing steps for 3×10 min, specific bands were visualized by the enhanced Chemiluminescence Western-Blot Detection Solution (Millipore, Schwalbach) and then by autoradiography (Hyperfilm ECL, GE-Healthcare, Schwalbach) and β-actin was measured as a loading control. The films were scanned and analyzed by densitometry with the program Quantity-One 4.2.1 (Bio-rad, Munich).

2.2.6 Statistical Analysis

The experiments were repeated at least three times. Analysis of variance with Bonferroni correction for multiple tests was used to determine significance. Results are expressed as mean ± S.E.M. An independent Student's t-test or an ANOVA was used to compare continuous variables. Pearson's correlation coefficient test was used to assess the correlation between two continuous variables. $p \leq 0.05$ was considered statistically significant.

3 Results

3.1 Part I: Endometriosis

3.1.1 Expressions of GHRH and GHRHR in endometrium and endometriotic tissues

To determine a possible involvement of GHRH and GHRHR splice variant 1 (SV1) in the development of endometriosis, we analyzed the expressions of GHRH and SV1 in self-paired eutopic and ectopic endometrial tissues from 16 patients with III-IV endometriosis, as well as endometrium tissues from 16 non-endometriosis patients as control group.

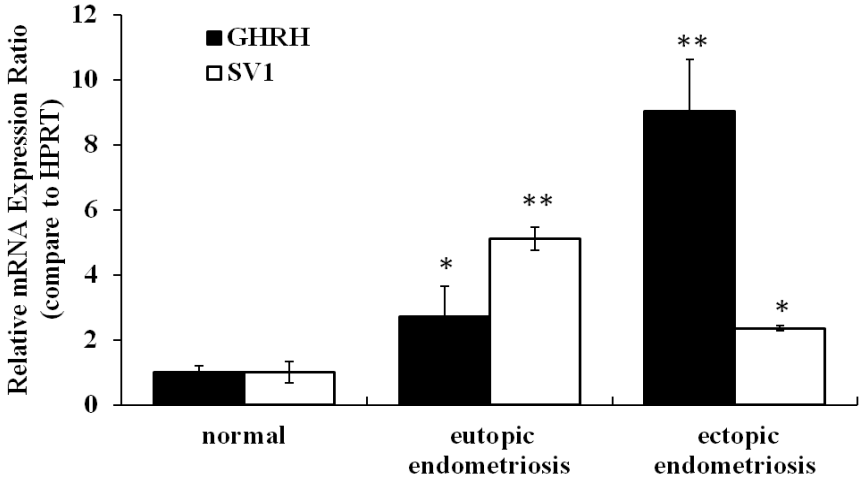

Figure 3: Quantitative RT-PCR analysis of the GHRH and SV1 in endometrium and endometriotic tissues. Relative mRNA expression of GHRH (black) and SV1 (white) in self-paired eutopic and ectopic endometrial tissues from 16 III-IV endometriosis patients compared to normal endometrium tissues from 16 non-endometriosis patients. All error bars indicate S.E.M.; statistical significance: ** p≤0.01, *p≤0.05.

By using qRT-PCR, we found that GHRH and SV1 mRNA were expressed in all ectopic endometrial tissues from women with endometriosis as well as in all endometrium from women with and without endometriosis (Figure 3). The mRNA expression of GHRH in ectopic and eutopic endometrial tissues were 9.02±1.06 (p≤0.01) and 2.72±0.32 (p≤0.05) higher compared to normal endometrium, respectively. Compared to normal endometrium, the expression of SV1 mRNA was 5.1±0.91 (p≤0.01) and

2.35±1.62 (p>0.05) higher in eutopic and ectopic endometrial tissues from patients with endometriosis.

We then analyzed the protein expression of pit-GHRHR and SV1 by western-blot. As shown in Figure 4A, the SV1 protein is expressed in 93.75%, 56.25% and 75% of the tissues of eutopic endometrial tissues, ectopic endometrial tissues and normal endometrium, respectively. Moreover, the mean relative SV1 protein expression in 16 eutopic endometrial tissues were significantly increased compared with that of 16 normal endometrium controls (p≤0.05) (Figure 4B). No significant difference was

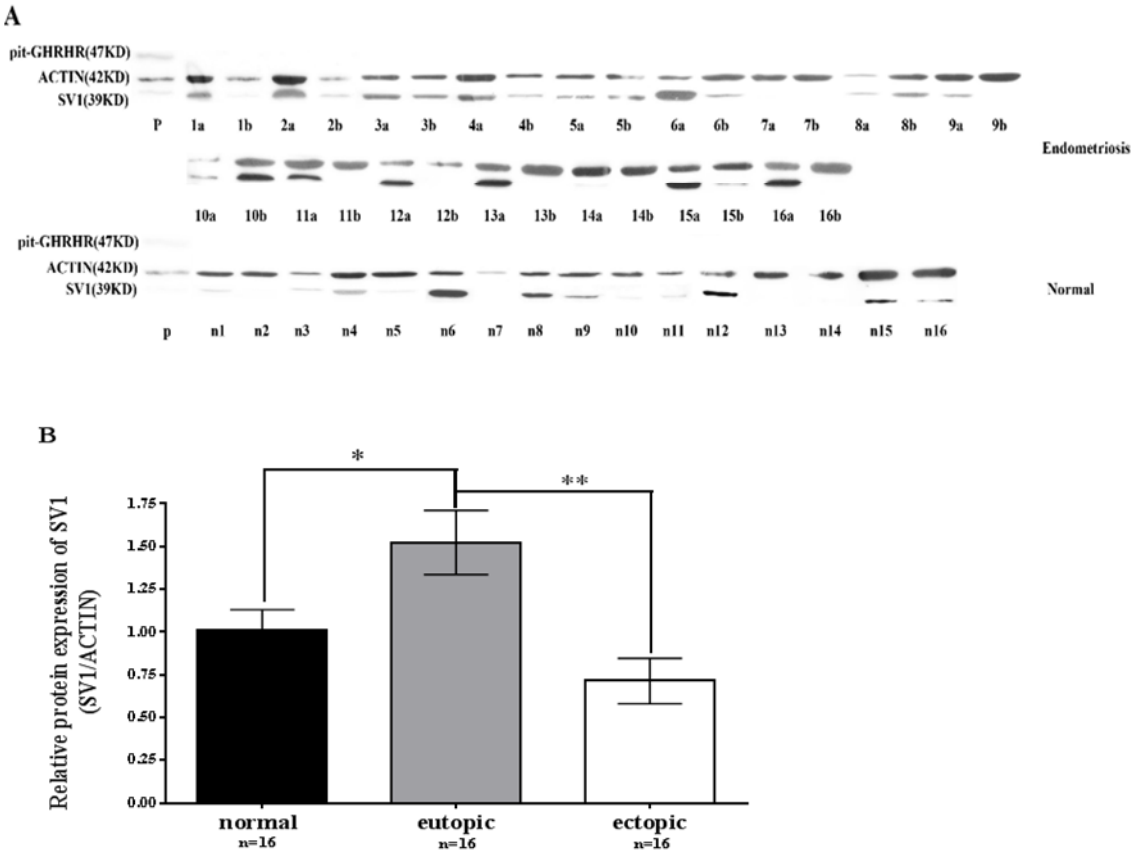

Figure 4: Western blot analysis of SV1 protein in endometrium and endometriotic tissues.
A. The representative specimens of self-paired eutopic and ectopic endometrial tissues from III-IV endometriosis patients (1a/b to 16a/b) as well as non-endometriosis normal endometrium controls (n1 to n16) are shown together with a positive control (p). Upper lane: p= positive control from pituitary tissue, upper and middle lane: 1-16a= eutopic endometrial tissue, 1-16b= ectopic endometrial tissues, lower lane: p= positive control from pituitary tissue, n1-16= non-endometriosis normal endometrium controls. **B.** The relative expression levels of SV1 protein detected by western blot were normalized to ß-actin. Data present the mean of triplicate western analysis. All error bars indicate S.E.M.; statistical significance: ** p≤0.01, *p≤0.05.

detected between ectopic endometrial tissues and normal endometrium control. In the self-paired endometrial tissues from 16 III-IV endometriosis patients, the relative expression levels of SV1 protein in eutopic endometrial tissues was significantly highter than in ectopic endometrial tissues (P≤0.01) (Figure 4B). The pit-GHRHR protein was rarely detectable in all the tissues and was too low to perform statistical analysis.

3.1.2 Expressions of GHRH and GHRHR in endometriotic epithelial cells

As far as we presume, the action of GHRH antagonist is initiated by binding to its receptor on target cells. To evaluate the suitability of the immortalized human endometriotic epithelial cells lines 12Z and 49Z as a research model, we firstly detected the expression of GHRH and SV1 type of the GHRHR in these cells by using qRT-PCR and western-blot. As shown in figure 5A, we found that the mRNAs of GHRH and SV1 were expressed in both of the two endometriotic epithelial cells. Moreover, the SV1 protein was detected in both, the 12Z and 49Z cell lines (Figure 5B). The expression levels of mRNA and protein for SV1 in 12Z cells were higher than in 49Z (p≤0.01). The existence of GHRH and the GHRHR splice variant SV1 approve the two endometriotic epithelial cell lines as a model for treatment experiments with GHRH antagonists.

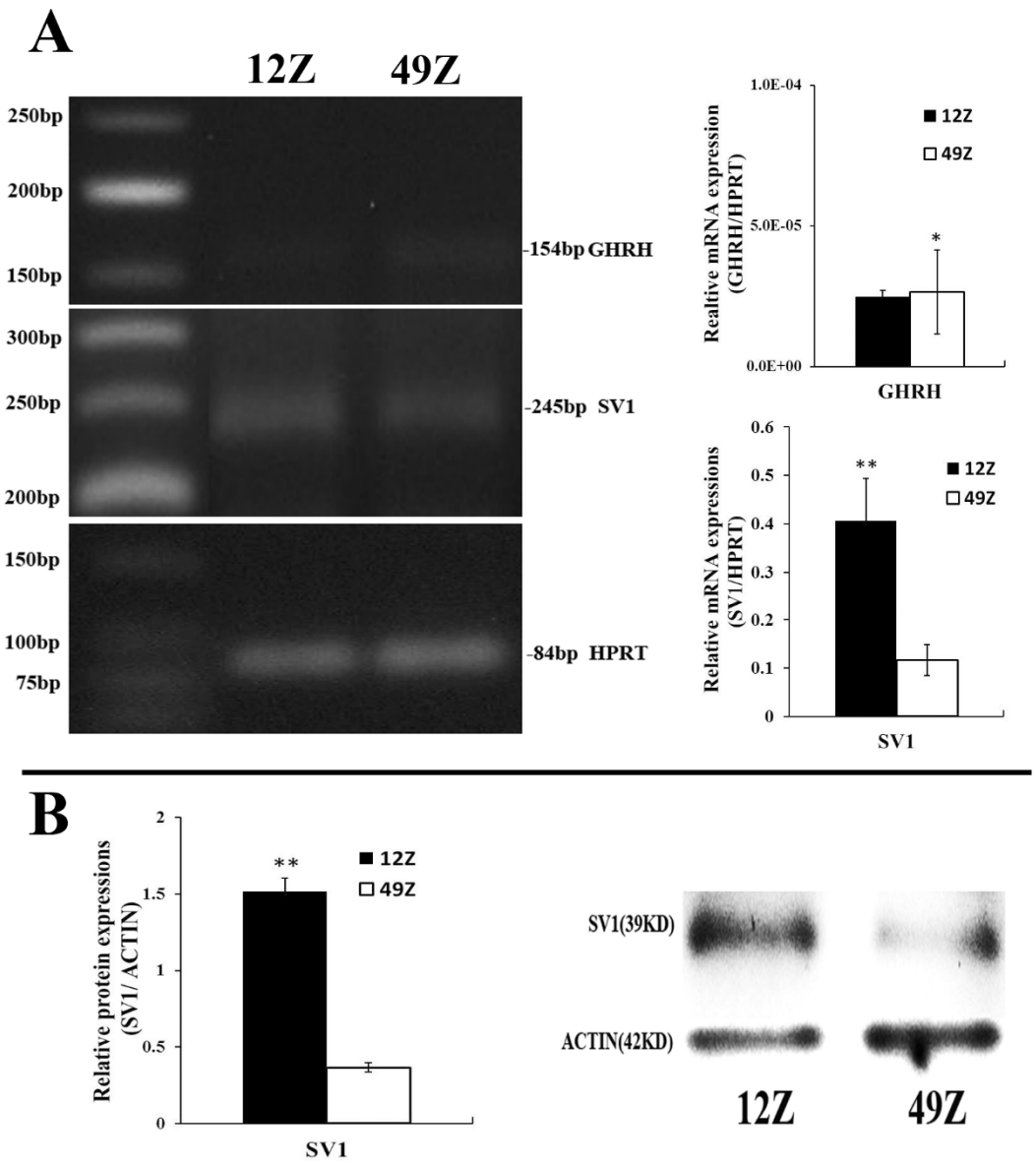

Figure 5: GHRH and SV1 expressed in endometriotic epithelial cells. A. The expression levels of GHRH and SV1 mRNA were detected in 12Z and 49Z cell lines by qRT-PCR.**B.** Determination of the SV1 protein levels by western-blot in 12Z and 49Z cells using specific anti-GHRHR and anti-ß-actin antibodies. A representative western-blot result was shown and the mean optical density ratio SV1 / ß-actin was calculated. Data present the mean of triplicate western blot analyses. All error bars indicate S.E.M.; statistical significance: ** p≤0.01, *p≤0.05.

3.1.3 Effects of GHRH antagonist and agonist on proliferation in endometriotic cells

To assess the effects of GHRH antagonists MIA-602 on proliferation in endometriotic cells, we choosed the human endometriotic epithelial cell lines 12Z and 49Z for this study. The GHRH agonists GHRH(1–29)NH_2 and GHRH(1–44)NH_2 were used together

with the GHRH antagonist MIA-602. The substances were examined by MTS assay. 12Z and 49Z cells were exposed to GHRH antagonists MIA-602 at 0.1, 1 µM concentrations, GHRH agonists GHRH(1–29)NH_2 and GHRH(1–44)NH_2 at 1µM for 96 h. The solvent DMSO and blank treated cells served as controls. As shown in figure 6, compared with the untreated control, a concentration of 1 µM MIA-602 exhibited a remarkably antiproliferative activity in both 12Z (13.2%, p≤0.01) and 49Z (8.34%, p≤0.01) cells while a concentration of 0.1 µM MIA-602 exhibited only an insignificant antiproliferative activity in both two cell lines. Moreover, GHRH(1–29)NH_2 exhibited a stimulating proliferative response in both cell lines (12Z: 18.9%, p<0.01; 49Z: 4.5%, p≤0.05), while GHRH(1–44)NH_2 exhibited a stimulating proliferative response only in 12Z (8.34%, p≤0.05).

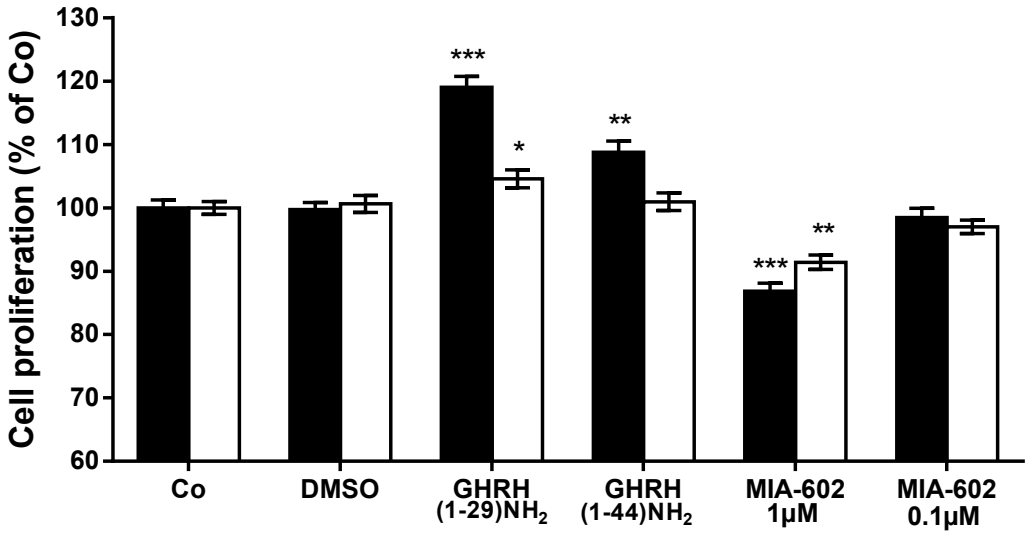

Figure 6: Effect of the GHRH antagonist MIA-602 and the GHRH agonists GHRH(1–29)NH_2 and GHRH(1–44)NH_2 on the proliferation in endometriotic cells. Data present the mean of three independent experiments. Each experiment was performed in sixtuplicate except blank controls, which were run three more times. White columns: 49Z; black columns: 12Z. All error bars indicate S.E.M.; statistical significance: *** p≤0.001, ** p≤0.01, *p≤0.05.

3.1.4 Effect of GHRH antagonist MIA-602 on expression of EGFR in endometriotic cells

To investigate the possible signal transduction mechanisms in the inhibitory effect of GHRH antagonist, we first focused on EGFR which is reported to be associated with the

development of endometriosis. After serum starving for 24 h, 12Z and 49Z cells were cultured with GHRH antagonist 1 µM MIA-602. Subsequently the expression of EGFR was revealed at 24 h, 48 h and 72 h using 0.01% DMSO solvent as control. As shown in figure 7, we found that 1 µM MIA-602 markedly suppressed the expression of EGFR at 48 h (12Z: 48.9±8.7%, p≤0.05; 49Z: 50.1±13.82%, p≤0.05) and 72 h (12Z: 67.3±5.23%, p≤0.01; 49Z: 65.2±11.31%, p≤0.01) both in 12Z and 49Z cells compared with the solvent DMSO control. Until 24 h, changes in the relative expression of EGFR did not reach statistical significance neither in 12Z nor in 49Z cells.

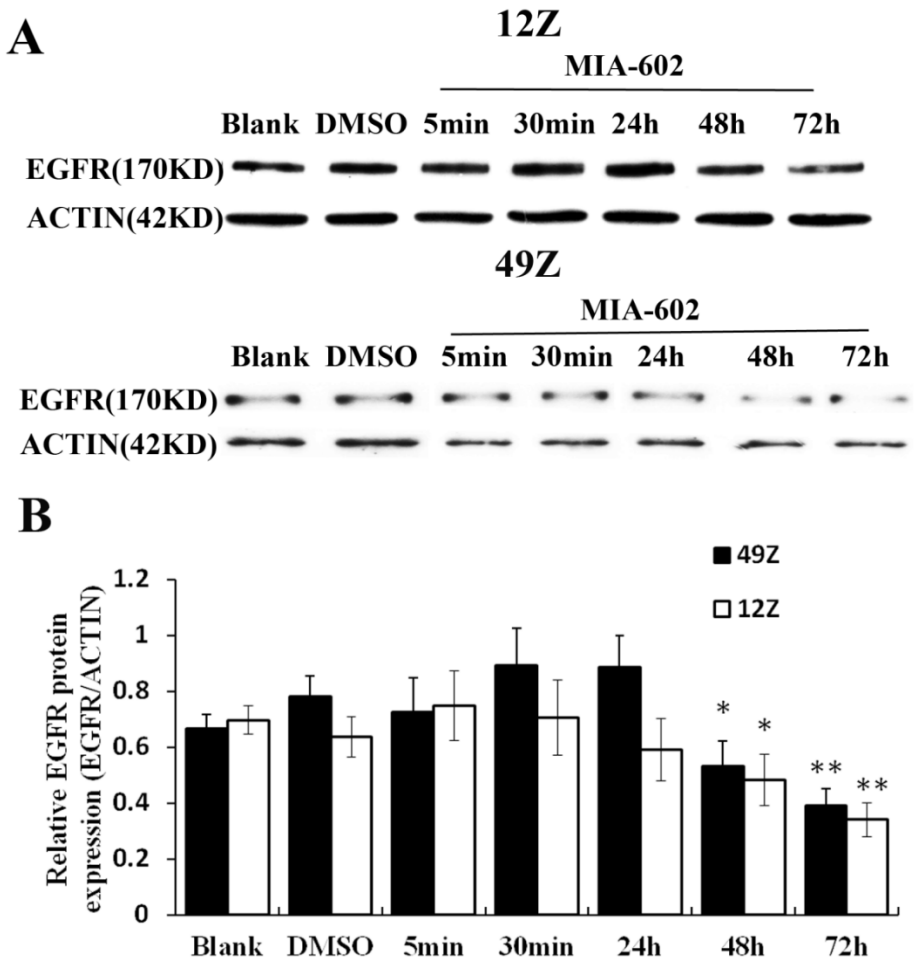

Figure 7: Effect of GHRH antagonist MIA-602 on the expression of EGFR in endometriotic cells. A. A representative western blot shows the protein of cells 12Z and 49Z treated with MIA-602 for 5 min to 72 h. **B.** The mean optical density ratio for EGFR/ ß-actin was calculated respectively in 12Z and 49Z cells for evaluating the expression of EGFR after treatment. Black columns: 49Z; white columns: 12Z. Data present the mean of from three independent experiments. All error bars indicate S.E.M., statistical significance: ** p≤0.01, *p≤0.05.

3.1.5 Effect of GHRH antagonist MIA-602 on $ERK_{1/2}$ pathway in endometriotic cells

We investigated the $ERK_{1/2}$ pathway in 12Z and 49Z cells after GHRH antagonist treatment. Changes of the ratio between $ERK_{1/2}$ protein and its phosphorylated form $pERK_{1/2}$ ^{Tyr204} in 12Z and 49Z cells after MIA-602 administration were revealed at 5, 10, 20, 40, 50 and 60 min by western blot (Figure 8). ERK activation, reflected by an elevated $pERK_{1/2}$ ^{Tyr204}/$ERK_{1/2}$ ratio, was decreased in 12Z and Z49 cells by 40~78%

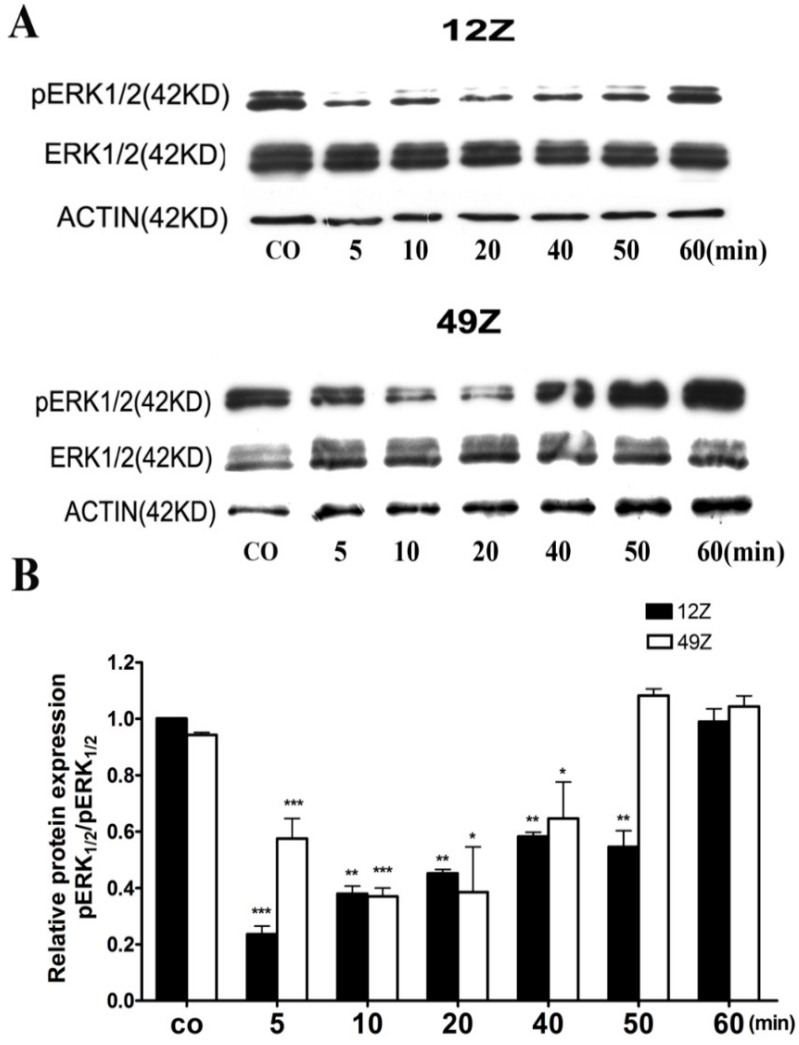

Figure 8: Effect of GHRH antagonist MIA-602 on $ERK_{1/2}$ pathway in endometriotic cells. A. Representative western blots show the protein of cells 12Z and 49Z treated with MIA-602 for 5 to 60 min. **B.** The mean optical density ratio for pERK1/2 ^{Tyr204}/ $ERK_{1/2}$ was calculated respectively in 12Z and 49Z cells for evaluating the activated $ERK_{1/2}$ after treatment. Black columns: 12Z, white columns: 49Z. Data present the mean of from three independent experiments. All error bars indicate S.E.M., statistical significance: ** p≤0.01, *p≤0.05.

between 5 and 50 min (p≤0.00~p≤0.05) and by 36.7~63.2% between 5 and 40 min (p≤0.00~p≤0.05), respectively, after treatment with 1 µM MIA-602 compared to the 0.01% DMSO solvent control. The ERK activation was inhibited at its deepest after 5 min treatment of 12Z cells with 1µM MIA-602 (78±4.21%, p≤0.00), and faded over time to reach the control level at 1 h. A similar effect was observed in 49Z cells, where the inactivation of $ERK_{1/2}$ by MIA602 reached the lowest ration at 10 min (63.2±3.89%, p≤0.00) and regained the control level after 50 min of treatment.

3.1.6 Effect of GHRH antagonist MIA-602 on Akt / apoptosis pathway in endometriotic cells

We examined the effect of the GHRH antagonist MIA-602 on the Akt / apoptosis pathway in immortalized endometriotic cells 12Z and 49Z. After treatment of the 12Z and 49Z cells for 5 min, 30 min, 24 h, 48h and 72h with 1 µM GHRH antagonist MIA-602, the Akt protein and its phosphorylated active form pAkt were analysed by western blot. Further, we analysed the downstream effectors proapoptotic protein Bax and antiapoptotic protein Bcl-2. All ratios were compared to the solvent DMSO control. The Akt, $pAkt_{1\text{-}3}$ $^{Ser472\text{-}474}$and Bax protein were highly present in both 12Z and 49Z cell lines, but Bcl-2 protein was expressed very low and could, therefore, not be investigated. No significant changes in the expression of the aforementioned protein levels and furthermore, no changes of the ratios at every time after treatment with 1 µM MIA-602 (Figure 9, 10).

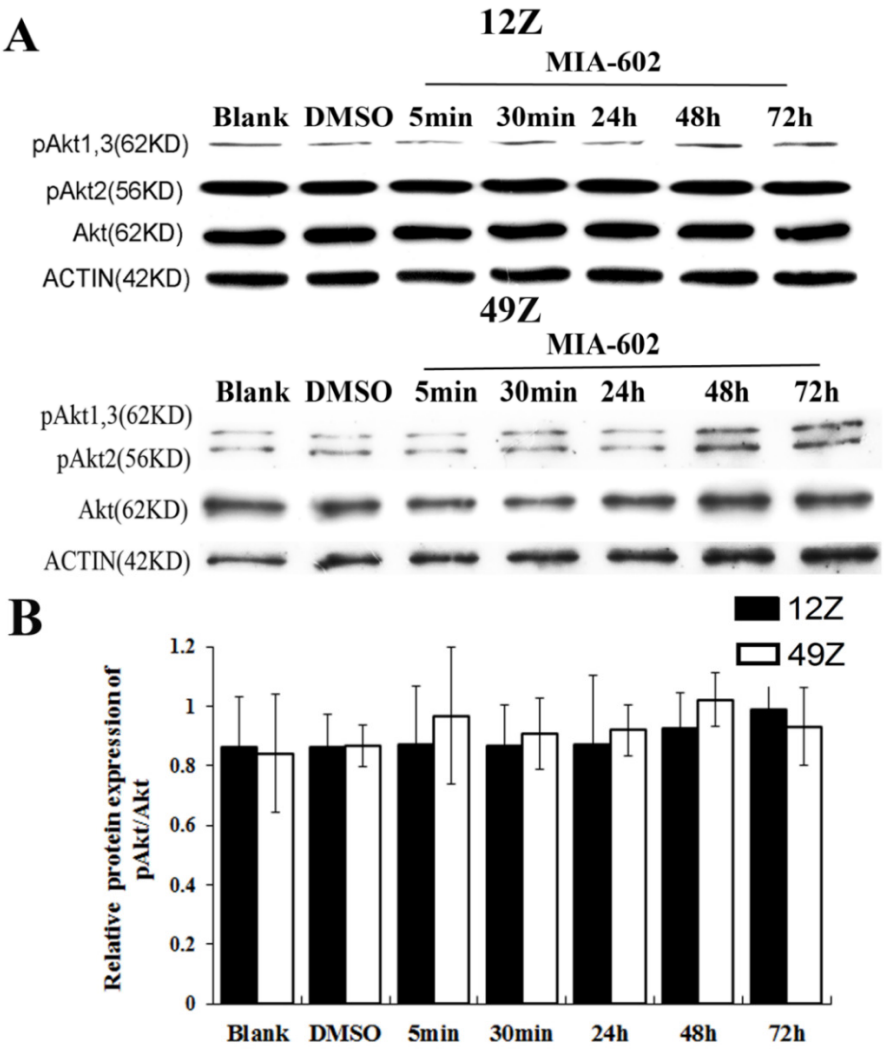

Figure 9: Effect of GHRH antagonist MIA-602 on Akt pathway in endometriotic cells.

A. Representative western blots show the protein of cells 12Z and 49Z treated with MIA-602 for 5min to 72h. **B.** The mean optical density ratio for $pAkt_{1\text{-}3}{}^{Ser472\text{-}474}$/ Akt was calculated both in 12Z and 49Z cells for evaluating the activated Akt after treatment. Black columns: 12Z, white columns: 49Z. Data present the mean of from three independent experiments. All error bars indicate S.E.M., statistical significance: ** p≤0.01, *p≤0.05.In all of the western blots the inactivated Akt was detected by using a specific monoclonal anti-Akt antibody showing one 62 kDa band: Akt(62KD); the anti- $pAkt_{1\text{-}3}$ polyclonal antibody shows two bands at 56 kDa for $pAkt_{1,3}$: $pAkt_{1,3}$(56KD) and at 62 kDa for $pAkt_2$: pAkt2(62KD). β-actin was used as loading control: ACTIN(42KD).

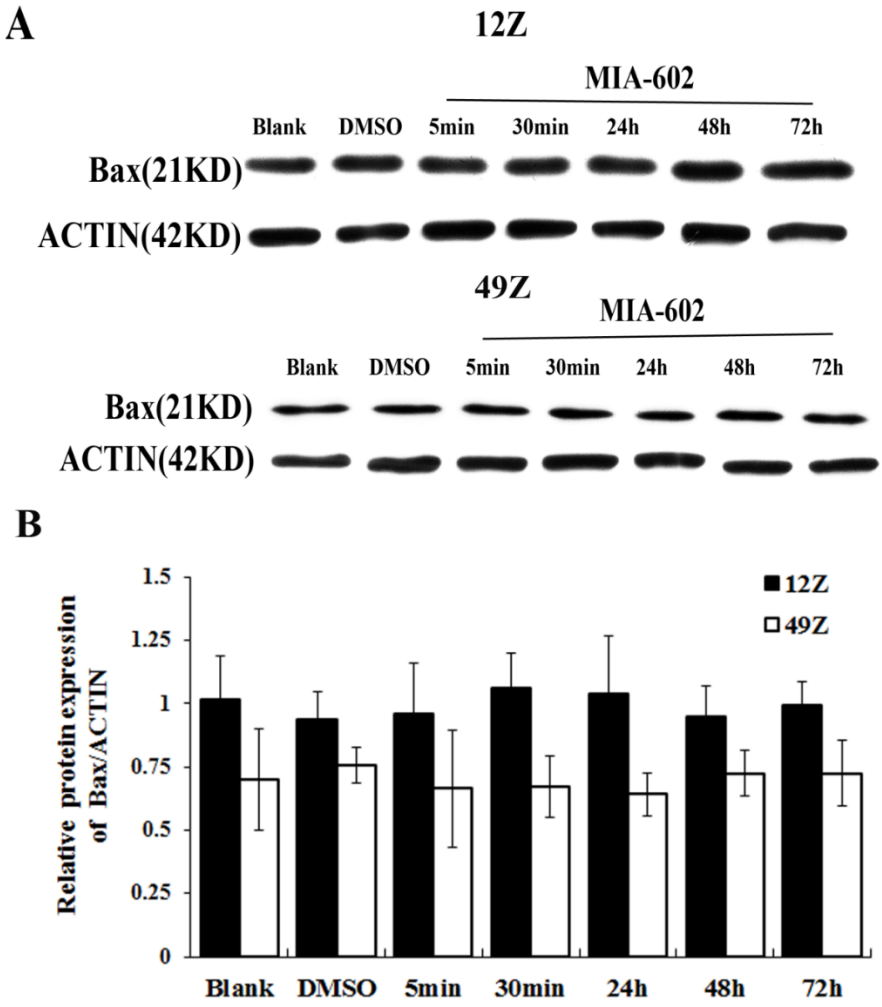

Figure 10: Effect of GHRH antagonist MIA-602 on Bax in endometriotic cells. A. A representative western blot shows the protein of cells 12Z and 49Z treated with MIA-602 for 5 min to 72 h. **B.** The mean optical density ratio for Bax / ß-actin was calculated respectively in 12Z and 49Z cells for evaluating the expression of Bax after treatment. Black columns: 12Z, white columns: 49Z. Data present the mean of from three independent experiments. All error bars indicate S.E.M., statistical significance: ** p≤0.01, *p≤0.05.

3.2 Part II: Triple-negative breast cancer

3.2.1 Expressions of GHRH-R and SV1 in TNBC cell lines

We used the breast cancer cell lines HCC1806, MDA-MB-231, HCC1937 and MDA-MB-468 representing triple negative breast cancer to evaluate the expression of pit-GHRHR, and its main splice variant, SV1. Proteins for pit-GHRHR and splice variant SV1 were detected in all four cell lines. The expression of SV1 is higher than pit-GHRHR in the HCC1806, MDA-MB-231, and MDA-MB-468 cells. HCC1806 and MDA-MB-468 cells express high levels of SV1. The protein expression level of SV1 was highest in MDA-MB-468 cell, followed by HCC1806, MDA-MB-231 and HCC1937 cells. The protein expression level of pit-GHRHR was highest in HCC1937 cell, followed by HCC1806, MDA-MB-231 and MDA-MB-468 cells (Figure11). These data suggested the existence of pit-GHRHR and its splice variant SV1 proteins in the TBNC cells.

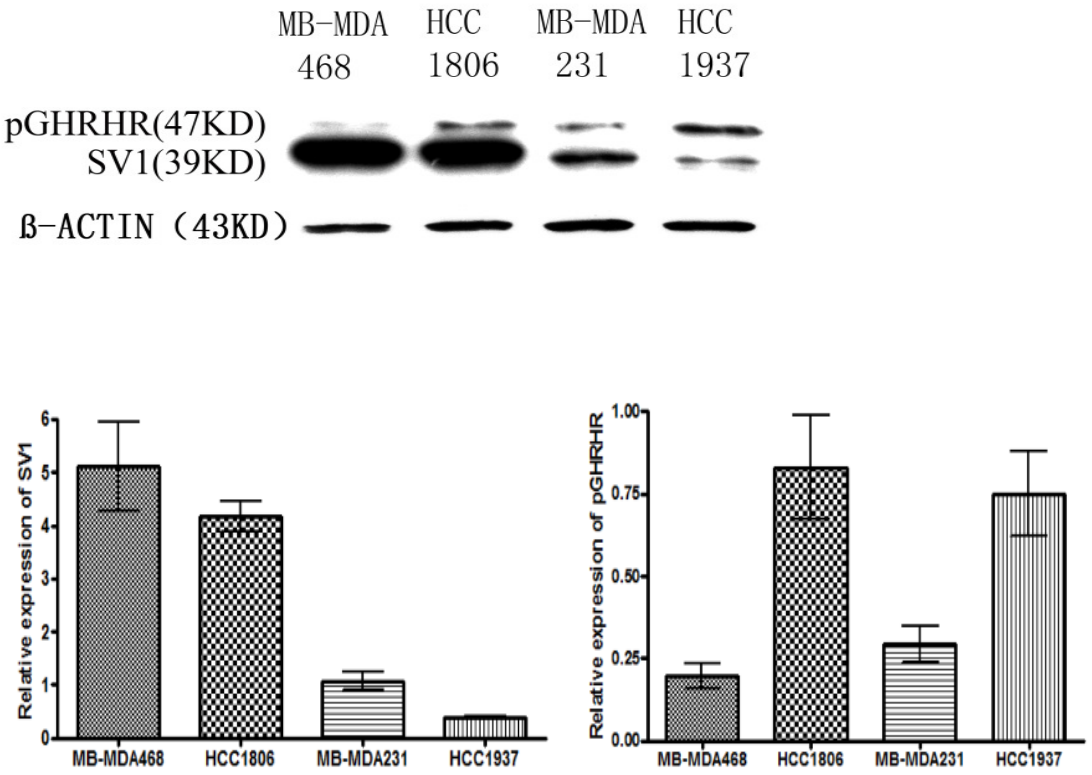

Figure 11: GHRHR expression in TNBC cell lines. A representative western-blot result is shown and the mean optical density ratio pit-GHRHR/β-actin and SV1/β-actin were calculated. Data present the mean of three western blot.

3.2.2 Effects of GHRH antagonist and agonist on proliferation in TNBC cells

To assess the effects of GHRH antagonists MIA-602 on tumor cells proliferation, we chose HCC1806 and MDA-MB-231 TNBC cells expressing high and medium levels of pit-GHRHR and SV1 for study. GHRH agonists GHRH(1–29)NH_2, GHRH(1–44)NH_2 and the antagonist GHRH MIA-602 were examined by MTT assay. HCC1806 and MDA-MB-231 cells were exposed to GHRH antagonist MIA-602 at 0.1 and 1 µM concentrations, GHRH agonists GHRH(1–29)NH_2 and GHRH(1–44)NH_2 at 1 µM all for 96 h. The untreated cells served as a control, and DMSO treated cells served as solvent control. As shown in figure 12, compared with the control, a concentration of 1 µM MIA-602 exhibited a remarkable anti-proliferative activity in both HCC1806 (17.3%, p≤0.01) and MDA-MB-231 (13.2%, p≤0.05) cells but a concentration of 0.1 µM MIA-602 exhibited a significant anti-proliferative activity only in HCC1806 (5.6%, p≤0.05).

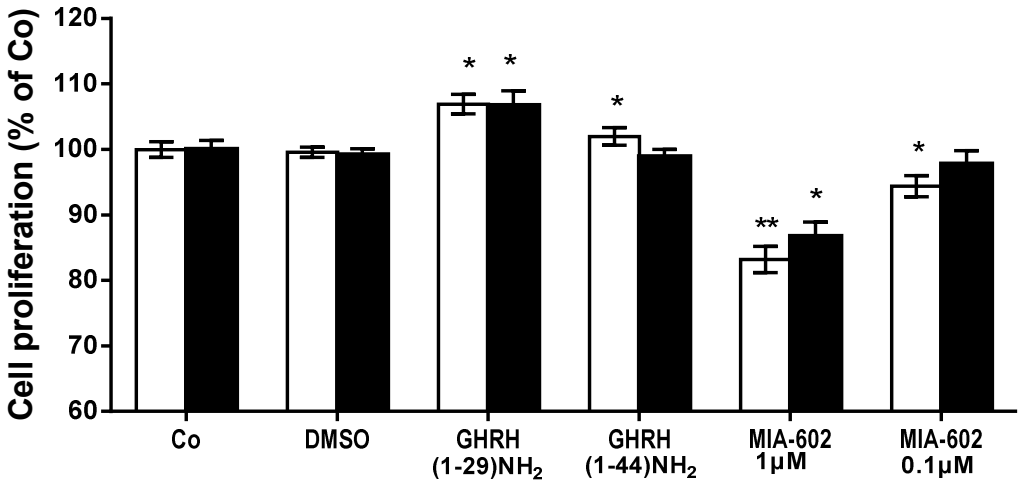

Figure 12: Effect of the GHRH antagonist and GHRH agonists on proliferation in TNBC cells. Data present the mean of three independent experiments. Each experiment was performed in sixtuplicate except blank controls, which were run three more times. White columns: HCC1806; black columns: MDA-MB-231. All error bars indicate S.E.M.; statistical significance: ** p≤0.01, *p≤0.05.

The agonist GHRH(1–29)NH_2 exhibited a stimulating proliferative response in both HCC1806 (6.9%, p≤0.05) and MDA-MB-231 (6%, p≤0.05) cells, while GHRH(1–44)NH_2 exhibited a significant stimulating proliferative response in HCC1806 (2.9%, p≤0.05),

only. GHRH receptor expression levels and the anti-proliferative activity of MIA-602 were positively correlated. These data show that triple negative cells HCC1806 and MDA-MB-231 could directly be inhibited by the GHRH antagonist MIA-602 to varying degrees according to the expression level of pit-GHRHR and SV1 receptors.

3.2.3 Effect of GHRH antagonist and agonist on the expression of EGFR in TNBC cells

To investigate the possible signal transduction mechanisms in the inhibitory effect of the GHRH antagonist, we first focused on EGFR which is overexpressed in triple negative breast cancer and linked with poor prognosis. After serum starvation for 24 h, HCC1806 and MDA-MB-231 cells were treated with 1 µM GHRH antagonist MIA-602 and 1 µM of the GHRH agonists GHRH(1–44)NH_2 or GHRH(1–29)NH_2. Subsequently, the expression of EGFR was revealed at 24, 48 and 72 h. As shown in figure 13, we found that 1 µM MIA-602 markedly suppressed the expression of EGFR after 48 h by 14% in MDA-MB-231 ($p \leq 0.05$) and 54% in HCC1806 ($p \leq 0.01$) and after 72 h by 48% in MDA-MB-231 ($p \leq 0.01$) and 55 % in HCC1806 cells compared with the solvent DMSO control. After 24 h this inhibitory effect only reached a statistic significant decrease of 39% in HCC1806 cells ($p \leq 0.01$), but no such effect in MDA-MB-231 cells. Furthermore, GHRH(1–29)NH_2 significantly upregulated the expression of EGFR at 48h by 11% and 9% and at 72 h by 47% and 40% in HCC1806 ($p \leq 0.01$) and MDA-MB-231 ($p \leq 0.05$), respectively. Changes in the relative expression of EGFR did not reach statistical significance at 24 h after GHRH(1–29)NH_2 treatment, and at all the 3 time points after GHRH(1–44)NH_2 treatments. These data strongly indicated that anti-proliferative effects of MIA-602 were associated with a down-regulation of EGFR receptors in TNBC cells.

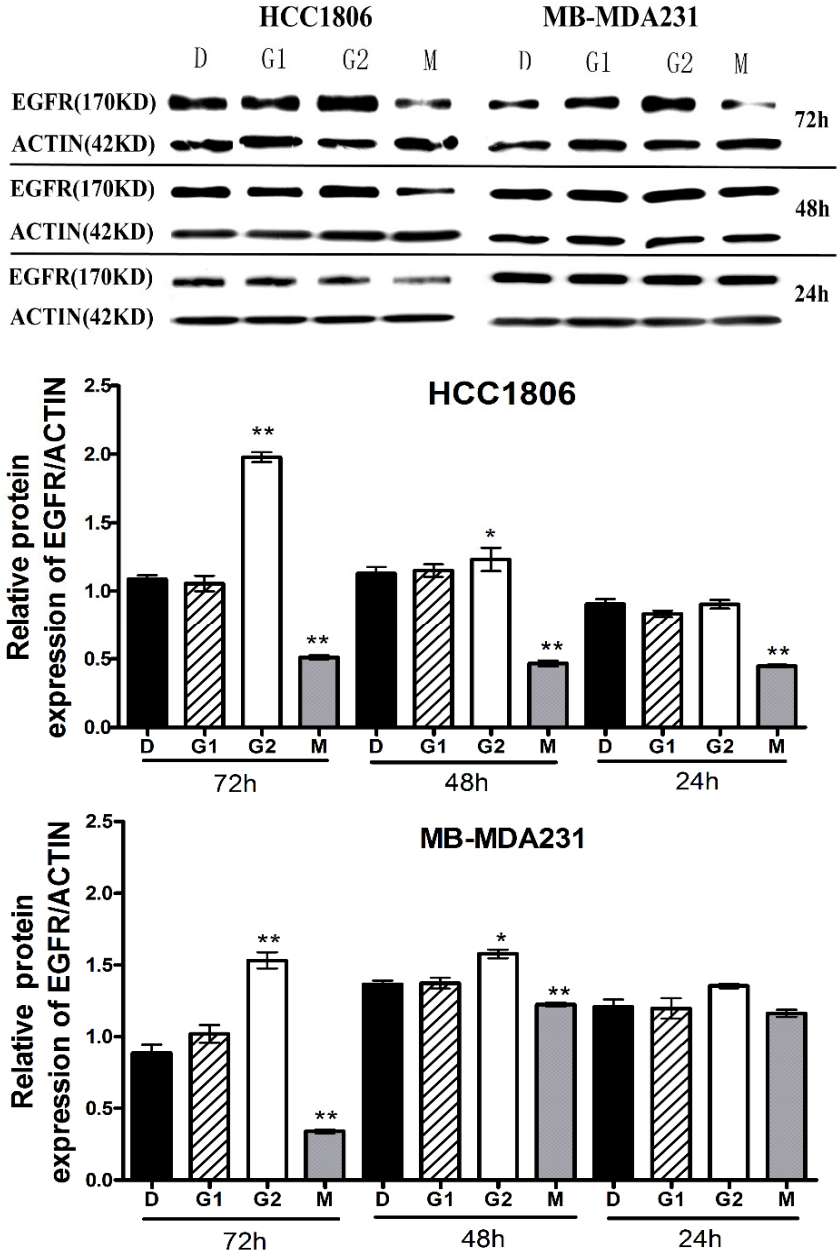

Figure 13: Effect of GHRH antagonist and agonists on the expression of EGFR in TNBC cells. A representative western blot shows the treated in cells at three different time points. The mean optical density ratio for EGFR / ß-actin was calculated in HCC1806 and MDA-MB-231 cells from three independent experiments. D: DMSO, G1: GHRH(1–44)NH_2,G2: GHRH(1–29)NH_2, M: MIA-602. Data express the relative protein content of EGFR / ß-actin compared to DMSO control. All error bars indicate S.E.M.; statistical significance: ** p≤0.01, *p≤0.05.

3.2.4 Effect of GHRH antagonist and agonists on the phosphorylation of $ERK_{1/2}$ in TNBC cells

Subsequently, we examined the activation of $ERK_{1/2}$, one of the main downstream effectors of EGFR, in HCC1806 and MDA-MB-231 cells after GHRH antagonist and agonist treatment as well as a combination of both. After serum starvation for 24 h, the cells were incubated with 1 µM MIA-602, 1 µM GHRH(1–29)NH_2 for 5 to 30 min or with 1 µM MIA-602 for 5 to 30 min after a pretreatment with 1 µM GHRH(1–29)NH_2

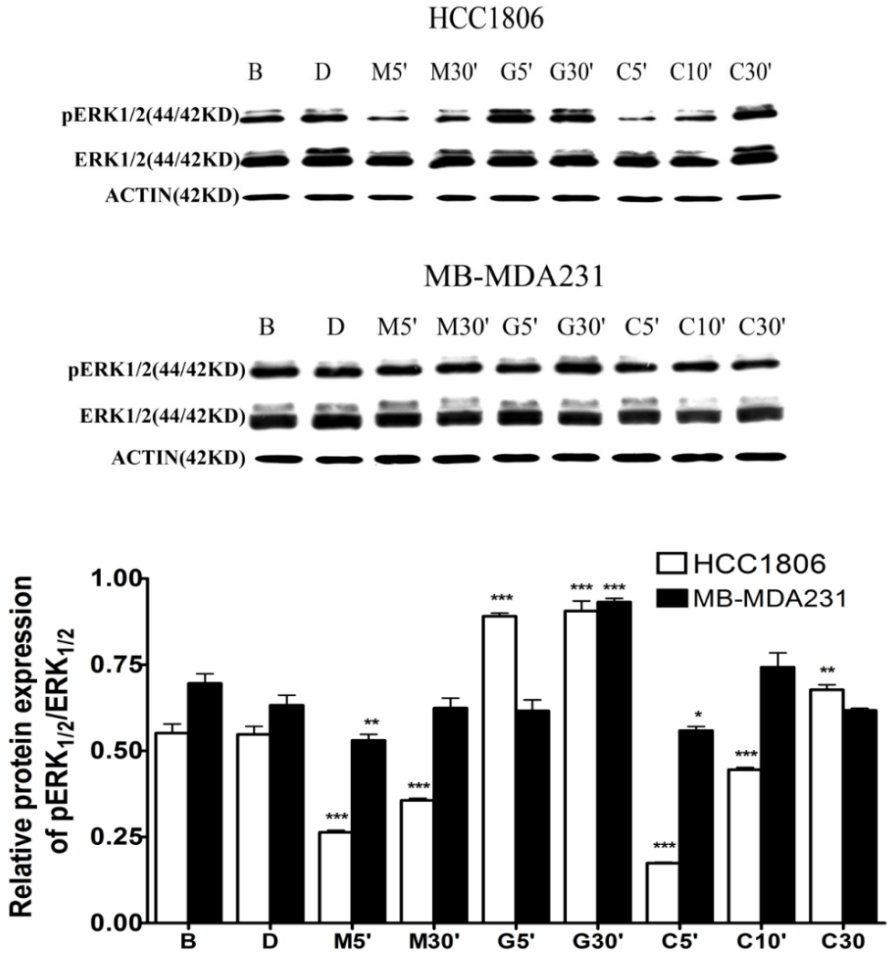

Figure 14: Effect of GHRH antagonist and agonist on phosphorylation of $ERK_{1/2}$ in TNBC cells. A representative western blot shows the cells after treatment treated in one experiment. The mean optical density ratio for $pERK_{1/2}^{Tyr204}$/ $ERK_{1/2}$ was calculated in HCC1806 and MDA-MB-231 as the mean of three western blot analyses. B: blank, D: DMSO, M5': MIA-602, 5 min, M30': MIA-602, 30 min, G5': GHRH(1–29)NH_2, 5 min, G30': GHRH(1–29)NH_2, 30 min, C5', C10',C30': pretreatment with 1 µM GHRH(1–29)NH_2 for 30 min and then added 1 µM MIA-602 for 5 min (C5'); 10 min (C10'); and 30 min (C30'). White columns: HCC1806; black columns: MDA-MB-231. All error bars indicate S.E.M.; statistical significance: *** p≤0.001, ** p≤0.01, *p≤0.05.

for 30 min. DMSO at 0.01% served as solvent control and only medium as blank control. Stimulation of HCC1806 cells with GHRH agonist 1 µM GHRH(1–29)NH_2 caused a significant increase of the ratio between phosphorylated $ERK_{1/2}$ ($pERK_{1/2}^{Tyr204}$) and $ERK_{1/2}$ compared to the ratio found in the solvent control. Treatment with the GHRH antagonist 1 µM MIA-602 caused a significant decrease in ratio of $pERK_{1/2}^{Tyr204}$ and $ERK_{1/2}$ compared to solvent control. The dephosphorylation of $ERK_{1/2}$ by treatment with MIA-602 was highest after 5 min and faded until 30 min in HCC1806. After pretreatment with 1 µM GHRH(1–29)NH_2 for 30 min the administration of MIA-602 still dephosphorylated the $ERK_{1/2}$ after 5 min in HCC1806. Unlike the single treatment, the ration of $pERK_{1/2}^{Tyr204}$ and $ERK_{1/2}$ response faded earlier and reached an elevated level already at 30 min compared to the control (Figure 14). These data suggest that MAP-kinases $ERK_{1/2}$ can be activated by GHRH agonist GHRH(1–29)NH_2 in HCC1806 and MDA-MB-231 triple-negative breast cancer cells as well as inactivated by using the GHRH antagonist MIA-602. The GHRH agonist is not able to block the activity of the antagonist, but it seemingly shortens its effect.

3.2.5 Effect of GHRH antagonist and agonist on the Akt/apoptosis pathway in TNBC cells

The Akt pathway is another downstream effector of EGFR. Akt, officially named protein kinase B, is a central regulating kinase of cell survival. Activated Akt is prohibiting apoptosis by direct interference with members of the proapoptotic protein family Bcl-2. Therefore, Akt and its phosphorylated activated form $pAkt_{1-3}^{Ser472-474}$ were examined after GHRH antagonist and agonist application. Furthermore, the associated apoptosis molecules Bcl-2 and Bax as well as PARP and cleaved PARP were investigated. According to the changes in phosphorylated $ERK_{1/2}$, HCC1806 and MDA-MB-231 cells were incubated with 1 µM MIA-602, 1 µM GHRH(1–29)NH_2 from 5 to 30 min, or pretreatment with 1 µM GHRH(1–29)NH_2 for 30 min followed by 1 µM MIA-602 application for 5 and 30 min. All incubations were compared to 0.01% DMSO as a solvent control. As shown in figure 15A, the Akt protein and $pAkt_{1-3}^{Ser472-474}$ were not significantly changed after any of the treatments with 1 µM MIA-602, 1 µM GHRH(1–29)NH_2 or 1 µM MIA-602 pretreatment followed by with 1 µM GHRH(1–29)NH_2 from

5 to 30 min compared with the control. In a longer time course, the Akt and $pAkt_{1\text{-}3}$ $^{Ser472\text{-}474}$ proteins were investigated after 24 h to 72 h of treatment. No significant changes could be detected in the protein levels of Akt and $pAkt_{1\text{-}3}$ $^{Ser472\text{-}474}$ after any of the treatments.

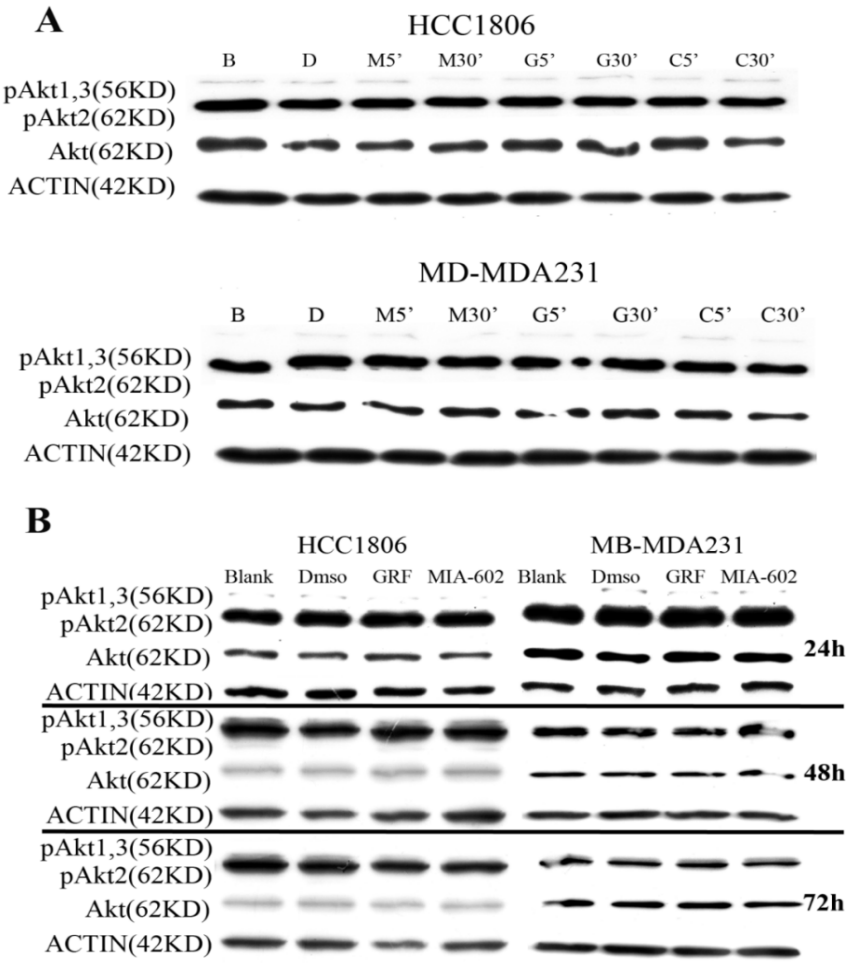

Figure 15: Effect of GHRH antagonist and agonist on AKT pathway in TNBC cells.
A. A representative western blot shows the proteins of cells after treatment for 5 to 30 min. B: blank, D: DMSO, M5': MIA-602, 5 min, M30': MIA-602, 30 min, G5': GHRH(1–29)NH_2, 5 min, G30': GHRH(1–29)NH_2, 30 min, C5', C30': pretreatment with 1 µM GHRH(1–29)NH_2 for 30 min and then added 1 µM MIA-602 for 5 min (C5') and 30 min (C30'). **B.** A representative western blot shows the proteins of cells treated with 1 µM MIA-602 or GHRH(1–29)NH_2 and blank as well as DMSO solvent control for 24 to 72 h. All experiments were repeated three times. All of the western blots were performed on lysates of the cells using specific anti- Akt, anti- $pAkt_{1\text{-}3}$ and anti-β-actin antibodies. Inactivated Akt was detected by using a specific monoclonal anti-Akt antibody showing one 62 kDa band: Akt(62KD); the anti- $pAkt_{1\text{-}3}$ polyclonal antibody shows two bands at 56 kDa for $pAkt_{1,3}$: pAkt1,3(56KD) and at 62 kDa for $pAkt_2$: pAkt2(62KD). β-actin was used as loading control: ACTIN(42KD).

After treatment with 1µM MIA-602 for 24 h to 72 h the 116 kDa PARP protein was activated resulting in a higher proportion of the cleaved PARP fragment at 89 kDa (Figure 16A). The change of the relation after MIA-602 treatment was statistically significant (p≤0.01) at 24, 48 and 72 h in both tested cell lines (Figure16B).

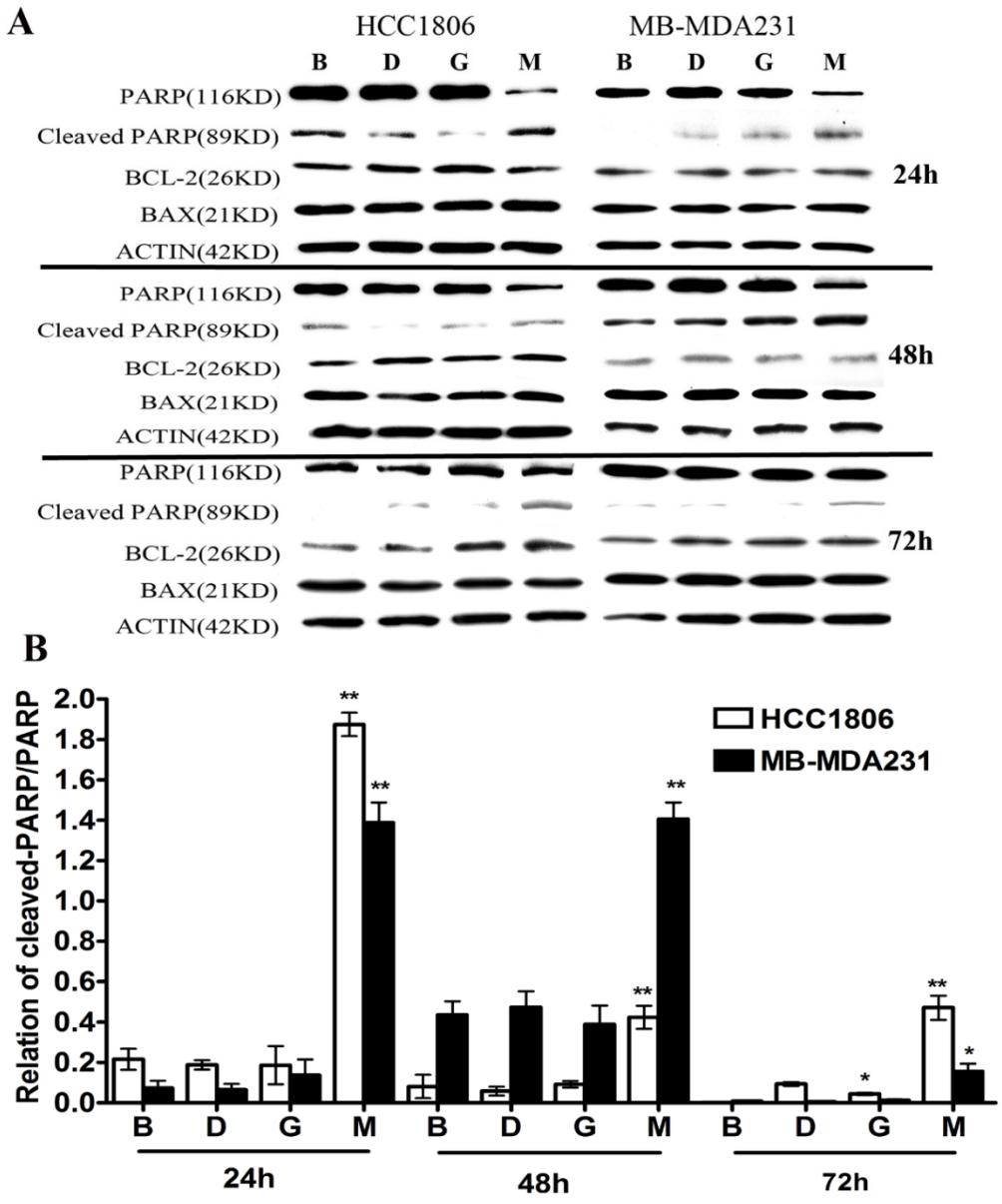

Figure 16: Effect of GHRH antagonist and agonist on apoptosis in TNBC cells.

A. A representative western blot shows the proteins of HCC1806 and MDA-MB-231 cells treated with 1 µM MIA-602 or GHRH(1–29)NH_2 for 24 to 72 h. **B.** The mean optical density ratios for cleaved PARP/ PARP were calculated as the mean of three experiments. White columns: HCC1806; black columns: MDA-MB-231. All error bars indicate S.E.M.; statistical significance: ** p≤0.01, *p≤0.05. B: blank, D: DMSO, G: GHRH(1–29)NH_2, M: MIA-602.

The proapoptotic enzymes Bcl-2 and Bax have not been changed at any time after any of the treatments in neither cell line. These data might indicate that GHRH antagonist MIA-602 promotes apoptosis in triple negative breast cancer cell lines independent of the $pAkt_{1\text{-}3}{}^{Ser472\text{-}474}$ pathway.

4 Discussion

4.1 Overview of experimental results

Here, for the first time, we demonstrated both, in mRNA expression and protein levels that SV1, a variant of the pit-GHRHR, is present in eutopic and ectopic endometrium as well as endometriotic tissues. Further, we found that the GHRH and SV1 expression in eutopic endometrium from patients with endometriosis were significantly higher than that in normal endometrium tissues.

Using the four breast cancer cell lines HCC1806, MDA-MB-231, HCC1937 and MDA-MB-468 that are representing triple negative breast cancer we demonstrated the existence of pit-GHRHR and its splice variant SV1 proteins in the TBNC cells.

Moreover, the effects of the new GHRH antagonists MIA-602 and its mechanisms of action were demonstrated. While the GHRH agonist GHRH(1-29)NH_2 promoted proliferation, the GHRH antagonist MIA-602 inhibited the proliferation both in immortalized endometriotic epithelial cells and triple-negative breast cancer cells. Further, we could show that the efficiency of GHRH antagonist MIA-602 and GHRH agonist GHRH(1-29)NH_2 seems to be correlated with the expression level of SV1 receptors. In addition, the activation of the EGFR/$ERK_{1/2}$ pathway, which is pivotal in the pathogenesis and progression of TNBC and endometriosis, was investigated and it was proven to be markedly involved in the antiproliferative activity of MIA-602. Our data also indicate that MIA-602 promotes apoptosis in triple negative breast cancer cell lines independent of the $pAkt_{1-3}$ $^{Ser472-474}$ pathway.

4.2 Expressions of GHRH and GHRHR in endometrium and endometriosis tissues

Endometriosis is defined as the presence of ectopic endometrial glands and stroma, which are often accompanied by fibrosis. The pathogenesis of endometriosis is remained mystifyingly elusive, but it is widely accepted that the ectopic endometrial-type tissue are characterized by enhanced proliferation and decreased apoptosis compared to normal endometrium [42, 51, 60]. Accumulating evidence suggests that

hereditary properties or acquired substances such as cytokine, chemokine, hormonal and growth factors are associated with the aberrant growth of this ectopic endometrial-type tissue [44, 49, 58]. Growth factors may play a fundamental role in stimulating ectopic endometrial growth and differentiation, such as epidermal growth factor (EGF) , insulin-like growth factors (IGF), platelet derived growth factor, and basic fibroblast growth factor are potent mitogens for endometrial stromal cells in vitro [79-81]. IGF-1 is an anti-apoptotic growth factor and may enhance cell survival [80, 82, 83]. GHRH, a traditional neuroendocrine factor for regulating the secretion of GH from the pituitary followed by the production and secretion of IGF-1 in the liver, is now recognized as a locally acting growth factor for various cancers. GHRH receptors including its splice variant SV1 are expressed in various primary human cancers and established cancer cell lines and appear to mediate the proliferative effects of GHRH. Recently, GHRH also has been found in normal endometrium, endometrial cancer, and endometriotic tissues, where it promotes cell proliferation and may function as an autocrine/paracrine growth factor [84, 85]. Moreover, Annunziata M *et al.* [86] detected pit-GHRHR and SV1 mRNA in ectopic endometric stromal cells ESCs. Fu L *et al.* [48] detected a low rate of SV1 mRNA in eutopic (26%) and ectopic (24%) endometrial tissues from patients with endometriosis, they also found that GHRH stimulates cyclic adenosine monophosphate (cAMP) production and proliferation in SV1-expressing endometriotic stromal cells (ESCs), suggesting that GHRH and SV1 could promote the development of endometriosis. These results suggested a role of GHRH in the development of endometriosis and the potential use of antagonistic analogues of GHRH as therapeutic agents. In the actual running study performed by our group we also had demonstrated that SV1 and pit-GHRHR are expressed in all tested eutopic endometial stromal cells EESCs which isolated from endometriosis patients. However, there are still many obscure questions to become explored. For example, is there any different expression of GHRH and its receptor between normal endometrium versus eutopic and ectopic endometrial-type tissue with endometriosis? Do GHRH and its receptor have any pathogenic effect on the development of endometriosis?

Our study is the first one that demonstrates the expression of GHRH and the GHRHR

splice variant SV1 together in human eutopic and ectopic endometrial tissues by qRT-PCR and western blot. Relative expression of GHRH and SV1 in self-paired eutopic and ectopic endometrial tissues from 16 III-IV endometriosis patients were compared to endometrium tissues from 16 non- endometriosis patients. The expressions of GHRH mRNA in ectopic and eutopic endometriosis tissues were significantly higher than in normal tissues. The expression level of SV1 mRNA was highest in eutopic endometrial tissues in patients with endometriosis, followed by ectopic endometriosis tissues and normal control. Moreover, the protein expression of SV1 was consistent with the results in mRNA level. Although previous studies have shown that the GHRHR-antibody used can identify both pit-GHRHR and SV1 on western blot assays bands at 47 kDa and 39 kDa, but in this study the expressions of pit-GHRHR protein in all the tissues were too low to have statistical analysis. However this result was consistent with Fu L *et al*. [48] reported that only SV1, but not pit-GHRHR mRNA was detected in eutopic and ectopic endometrial tissues from patients with endometriosis. This difference between the activity of SV1 and the pit-GHRHR may be explained by the fact that SV1 is the predominant type of GHRH-R on both normal endometrium and endometrial tissues in patients with endometriosis.

We further analyzed the GHRH mRNA, the mRNA and protein expression of SV1 in the immortalized endometriotic epithelial cells 12Z and 49Z using RT-PCR and western blot methods in order to prove their suitability for used as the research model for endometriosis. The results show that GHRH and SV1 were expressed in both of the two cells; however, the expression of GHRH mRNA was very low. Moreover, SV1 was expressed at a significantly higher level in both 12Z and 49Z cell lines. The expression levels of SV1 in 12Z cell were higher than in 49Z. But pit-GHRHR was expressed very low and could, therefore, not be investigated.

Our findings strongly suggest that GHRH and SV1 could be involved in the pathogenesis of endometriosis and SV1 type GHRH receptors might be function as the main therapeutic target for the antiproliferation effect of GHRH antagonists.

4.3 Activity of MIA-602 in endometriosis

MIA-602 is a novel GHRH antagonist which has exhibited more potent antitumor effect than previous analogues in various cancers, but its inhibitory effect in endometriosis is still unknown. In regard to endometriosis, there have been no appropriate *in vivo* and *in vitro* models available for studying the characteristics of the active phase of endometriosis in humans. The immortalized endometriotic cells 12Z and 49Z were established from active endometriotic lesions from women with endometriosis, and these lines retained the phenotypic characteristics and several in vivo properties of the active phase of endometriosis [82, 86, 87]. In the present study, after approved the existence of GHRH and SV1 in both of the two endometriotic epithelial cell lines, 12Z and 49Z were used as the experimental model to evaluate the effect and action mechanisms of MIA-602 in endometriosis. Little previous studies had evaluated the antiproliferative effect of GHRH antagonist dealing with endometriosis. Annunziata M *et al.* [88] had demonstrated that the GHRH antagonist JV-1-36 inhibited ESCs proliferation and survival. Yet, no study exists evaluating the inhibitory effect of the new GHRH antagonist MIA-602 in endometriosis. Here, by using MTS-assay we demonstrated that 1 μM MIA-602 exhibited a remarkably antiproliferative activity in both 12Z and 49Z cells while 1μM GHRH agonist GHRH(1–29)NH_2 resulted in a stimulating proliferative response in both cell lines . Moreover, the effects in 12Z were more stronger than 49Z whatever exposure to GHRH antagonist MIA-602 or GHRH agonist GHRH(1–29)NH_2 and GHRH(1–44)NH_2. In accordance with studies in cancer cell lines, which reported improved cell survival following treatment with GHRH as well as inhibited cell growth following treatment with GHRH antagonist MIA-602, we observed the enhancement of endometriotic cell viability after exposure to GHRH agonists and a significant decrease in proliferation after treatment with the GHRH antagonist MIA-602 for 96 h. Our data support the view that GHRH is a growth factor in endometriosis as previously found in endometriosis tissues and the proliferation of endometrial cells depends at least in part on GHRHR signaling. GHRH antagonist MIA-602 might be a new therapeutic option in the treatment of endometriosis; however its function is depended on the expression of GHRHR, especial the expression of SV1.

4.4 Expression of GHRHR in TNBC cells

In a previous study, GHRH antagonists blocked the acitvity of tumoral autocrine or paracrine GHRH to pit-GHRHR or GHRHR splice variants on tumors; thus, both types of GHRHR should be considered potential targets for anticancer therapy with GHRH antagonists. Here, we demonstrated the presence of both pit-GHRHR and SV1 proteins on the TNBC cell lines. MDA-MB-468 cells expressed the highest level of SV1 followed by HCC1806, MDA-MB-231 and HCC1937 cells. While the highest expression levels of pit-GHRHR was in HCC1937 cell followed by HCC1806, MDA-MB-231 and MDA-MB-468 cells. Our results were consistent with the previous findings that most of TNBC cells possess the GHRHR, but at different levels [77, 89-92] .

As the direct effects of GHRH antagonists on cancer cells should be mediated by GHRH receptor (pit-GHRHR and SV1), we chose HCC1806 and MDA-MB-231 TNBC cell lines with medium strong expression of SV1 and pit-GHRHR among the four cell lines for following studies.

4.5 Activity of MIA-602 in TNBC cells

MIA-602 is a novel GHRH antagonist which has exhibited more potent antitumor effects than previous analogues in various cancers. Here we showed that 1 μM MIA-602 exhibited a remarkably antiproliferative activity in both HCC1806 and MDA-MB-231 cells compared with controls, moreover 0.1 μM MIA-602 exhibited a significant antiproliferative activity only in HCC1806. Treatment with 1 μM GHRH agonist GHRH(1–29)NH_2 exhibited a stimulating proliferative response in both HCC1806 and MDA-MB-231 cells. The correlation between the higher SV1 and pit-GHRHR level in HCC1806 compared to MDA-MB-231 and the sensitivity to MIA-602 and GHRH agonist seemed to be apparent. This proliferation inhibiting effect of MIA-602 is consistent with previous studies in lung and ovarian cancers [93, 94]. Altogether, these data show that the proliferation of TBNC cells depends at least in part on GHRHR binding. GHRH antagonist MIA-602 can directly inhibited the proliferation of TBNC cells, and its effects are receptor depended; SV1 with a high structural homology to pit-GHRHR is probably the main SV that mediates the effects of GHRH and its antagonists in tumors.

4.6 Action mechanisms of MIA-602 in endometriotic and TNBC cells

Over the past decade, GHRH antagonists have been proved to inhibit the growth of a wide range of human experimental malignancies [25, 95, 96]. Their mechanisms of action include suppression of the pituitary GH/ hepatic IGF-I axis and the inhibition of autocrine and/or paracrine production of IGF-I and IGF-II in tumors as well as the blockage of the direct stimulatory action of local GHRH on tumors [25]. Importantly, GHRH antagonists directly affect some of the signaling mechanisms involved in cell proliferation, survival, metastasis, and the activation of proapoptotic signaling mechanisms. However, the molecular mechanisms are varied in different GHRH antagonists and different diseases [18, 27, 32, 97]. Moreover, the intracellular signal transduction mechanisms involved in the antiproliferative effects of GHRH antagonists are still obscure. Yet, there exists no study evaluating the molecular mechanisms of the inhibitory effect by GHRH antagonist MIA-602 in endometriosis or in TNBC.

Considering the pivotal role of EGFR and its signaling pathway components in the pathogenesis and progression of TNBC and endometriosis, as well as the antiproliferative function of the GHRH antagonist MIA-602 in both of them, we here focused on the interaction between MIA-602, EGFR and EGFR pathway components (Akt, $pAkt_{1\text{-}3}^{Ser472\text{-}474}$, $ERK_{1/2}$, $pERK_{1/2}^{Tyr204}$) in TNBC and the immortalized endometriotic cell lines. Firstly, by using western-blot, we showed that treated with 1 μM MIA-602 markedly suppressed the expression of EGFR at 48 h and 72 h both in TNBC and the immortalized endometriotic cells. By contrast, GHRH(1–29)NH_2 significantly upregulated the expression of EGFR at 48 and 72 h in TNBC HCC1806 and MDA-MB-231 cells. This result gives the suggestion that the anti-proliferative effect of MIA-602 might be associated with the down-regulated expression of EGFR. Our observation was in accordance with Guo *et al.* [35] and Kanashiro *et al.* [96] who had demonstrated that anti-proliferative effect of JMR-132 in ovarian cancer and JV-1-65 in H-69 small cell lung carcinoma were also associated with EGFR downregulation. They found that treatment with GHRH antagonist JV-1-65 can inhibit the mRNA expression for EGFR by 27-75.4% and decrease the protein levels for EGFR by 21-34%.

As previous studies showed, a time-dependent elevation of $ERK_{1/2}$ phosphorylation can

be observed after treatment with GHRH(1–29)NH_2 and an opposite effect can be detected upon treatment with GHRH antagonists in cancer cells [77]. Accordingly, we detected a significantly decrease of $ERK_{1/2}$ phosphorylation in a time-dependent manner followed by treatment with GHRH antagonists MIA-602 after 5-10 min both in TNBC and the immortalized endometriotic cells. These data suggest that MAP-kinases $ERK_{1/2}$ can be inactivated by GHRH antagonist MIA-602 in both TNBC and the immortalized endometriotic cells. Moreover, we also found that stimulation of HCC1806 cells with 1 μM GHRH agonist significantly activated $ERK_{1/2}$ by phosphorylation. Furthermore, MIA-602 was still able to inactivate $ERK_{1/2}$ after pretreatment with 1 μM GHRH(1–29)NH_2 for 30 min, but this inactivating effect faded more earlier. Altogether, our findings not only confirmed that the binding of GHRH to its receptor results to the activation of the MAPKs $ERK_{1/2}$, but also implied that MIA-602 could counteract the effects of GHRH probably by competitive binding to the GHRHR. Our data also indicate that MIA-602 inhibits the proliferation of TNBC and the immortalized endometriotic cell lines might involve the inactivation of the EGFR/$ERK_{1/2}$ pathway.

It is well known that the activation of the EGFR pathway further activates its downstream effectors, which play various important roles in regulating cell apoptosis [56, 98]. The Akt as another downstream effector of EGFR is a central regulating kinase of cell survival. Activated Akt is prohibiting apoptosis by direct interference with members of the proapoptotic protein family Bcl-2. Therefore, we next examined Akt and its phosphorylated activated form $pAkt_{1\text{-}3}^{Ser472\text{-}474}$ as well as the associated apoptosis molecules (PARP, cleaved PARP, Bcl-2 and Bax) by western blot. Although Eva Pozsgai *et al.* [99, 100] had reported that a major decrease in the levels of $pAkt_{1\text{-}3}$ $^{Ser472\text{-}474}$ was detected at 5 and 10 min following treatment with the GHRH antagonists JMR-132 or MIA-602 in human glioblastoma cell lines, we had not observed any significant change in Akt, $pAkt_{1\text{-}3}$ $^{Ser472\text{-}474}$, as well as Bcl-2 and Bax proteins after treatment with 1 μM MIA-602. This difference in constitutive activation of Akt pathway might be explained by variable molecular mechanisms of the inhibitory effect by GHRH antagonist MIA-602 in different kinds of cells. However, we detected a markedly increase in cleaved PARP product after treatment with 1 μM MIA-602 in TNBC cell lines.

These data might indicate that GHRH antagonist MIA-602 promotes apoptosis in triple negative breast cancer cell lines independent of the $pAkt_{1\text{-}3}$ $^{Ser472\text{-}474}$ pathway.

4.7 Conclusion and prospects

The current study clearly shows the effectiveness of the GHRH antagonists MIA-602 for inhibiting the proliferation and survival of TNBC and immortalized endometriotic cells, suggesting that MIA-602 might not only be a useful option for the patients suffering from TNBC but also likely to have diverse no-oncological applications such as endometriosis. Moreover, our findings also provide compelling evidence that GHRH antagonists affect cell proliferation through the following key pathways: the reduction of phosphorylated $ERK_{1/2}$ and the cleavage of PARP.

In addition, the taxanes in combination with GHRH antagonists recently entered phase I trials for the treatment of patients with TNBC expressing the SV1 and/or pit-GHRHR, encouraging results in terms of efficacy and safety have been reported [90]. These data further suggest to combine the GHRH antagonists with standard chemotherapeutic agents for an enhanced antitumor effect because of its different pharmacological principles.

GHRH antagonists create a new potential therapeutic option for various neoplasms and diseases. Based on these preliminary results, additional clinical studies are urgently needed to define the effectiveness and lack of toxicity of this novel GHRH antagonist based pharmacological formulations to improve the therapeutic options for neoplasms bearing GHRHR for which the available treatments are still limited. Furthermore, a deeply understanding of their mechanisms of action might give new directions to their possible applications. With ongoing research efforts, we will one day be able to translate these molecular targets into effective personalized therapies for TNBC and endometriosis.

5 Summary

MIA-602 is a highly potent growth hormone releasing-hormone (GHRH) antagonist. Besides its hypothalamic endocrine actions, GHRH is known to regulate the growth of several human experimental cancers on the cellular level by binding to their intrinsic GHRH receptors (GHRHR). Especially, a splice variant (SV1) of the pituitary type GHRHR was found to be expressed on cells of tumors and endometriosis. The knowledge about the underlying signaling mechanisms of the GHRH antagonist MIA-602 in endometriosis as well as in triple negative breast cancer (TNBC) is still under investigation.

Here, by using qRT-PCR and western blot we detected the expression of GHRH and the tumor standing GHRHR splice variant SV1 in human eutopic and ectopic endometrial tissues. We found the mRNA expression of GHRH and SV1 as well as the protein of SV1 being significantly higher in eutopic and ectopic endometrium from patients with endometriosis than in healthy endometrium tissues. In TBNC cells we demonstrated the existence of the pituitary type GHRHR and its splice variant SV1.

Further, by using proliferation assays, we demonstrated that the GHRH agonist GHRH(1-29)NH2 promoted the growth while the GHRH antagonist MIA-602 inhibited the proliferation in the immortalized endometriotic epithelial cell lines 12Z and 49Z as well as in the TNBC cells HCC1806 and MDA-MB-231. Moreover, the intracellular signaling effects of MIA-602 were investigated in endometriosis and TNBC performing western blots with densitometric analysis. We found the inactivation of the EGFR/$ERK_{1/2}$ pathway being involved in the antiproliferative action mechanism of MIA-602, but the EGFR/Akt pathway was not. The application of MIA-602 lead to a significant downregulation of the EGFR after 48 and 72 h and a decrease in the proportion of phosphorylated $ERK_{1/2}$ after 5 min. Further, we detected apoptosis by showing enhanced cleavage of PARP after treatment with MIA-602.

As a conclusion, GHRH and SV1 are most probably involved in the pathogenesis of endometriosis and TNBC; SV1 might function as the main target for GHRH and its antagonist. MIA-602 might provide effective treatment of patients suffering from endometriosis or TNBC, partly depend on the inactivation of the EGFR/$ERK_{1/2}$ pathway and by leading the cells in to apoptosis.

Zusammenfassung

MIA-602 ist ein höchst wirksamer Antagonist des Growth-Hormon-Releasing-Hormons (GHRH). Neben seiner Wirkweise über die hypothalamisch-hypophysär-endokrine Achse reguliert GHRH das Wachstum von mehreren humanen Tumormodellen auch auf zellulärer Ebene durch Bindung an tumorintrinsische GHRH-Rezeptoren (GHRHR). Insbesondere eine Splicevariante (SV1) des GHRHR wurde auf endometrialen Zellen von Endometriosepatientinnen sowie in triple-negativen Brustkrebszellen (TNBC) nachgewiesen. Über die zu Grunde liegenden intrazellulären Wirkmechanismen von MIA-602 ist noch wenig bekannt.

In dieser Studie konnte mit qRT-PCR die Expression der mRNA von GHRH und SV1 und zusätzlich in Westernblots das SV1-Protein in humanem eutopischem und ektopischem Endometriosematerial nachgewiesen werden. Die mRNA von GHRH und SV1 sowie das SV1 Protein kam stärker in eutopischem und ectopischem Gewebe von Patientinnen mit Endometriose vor, als in Endometrien von gesunden Frauen. In TNBC Zellen wurde der hypophysäre GHRHR und SV1 nachgewiesen.

In Wachstumsversuchen zeigte der GHRH Agonist GHRH(1-29)NH_2 eine wachsstumsteigernde Wirkung, während der Antagonist MIA-602 das Wachstum sowohl in den immortalisierten endometrioiden epithelialen Zelllinien 12Z und 49Z als auch in den TNBC Zelllinien HCC1806 und MDA-MB-231 inhibierte.

Weiterhin wurden Effekte auf die intrazelluläre Signalweiterleitung in Westernblots mit densitometrischer Auswertung untersucht. Mit Antikörpern gegen den epithelialen Wachstumshormonrezeptor (EGFR) und gegen phosphorylierte MAP-Kinase (p$ERK_{1/2}$) und Akt-Kinase (pAkt) konnte festgestellt werden, dass eine Inaktivierung des EGFR/$ERK_{1/2}$ Signalwegs in die wachstumsinhibierende Wirkung von MIA-602 in volviert ist, jedoch der Akt Signalweg nicht involviert ist. MIA-602 führte zu einer signifikanten Herunterregulation von EGFR nach 48 und 72 h und verminderte den Anteil phosphorylierter $ERK_{1/2}$ nach 5 min. Weiterhin führte die Gabe von MIA-602 zur Apoptose, wie durch einen verstärkten Anteil von gespaltener Poly(ADP-ribose)-Polymerase 1 zu erkennen war.

GHRH und SV1 spielen höchstwahrscheinlich bei der Entstehung von Endometriose und TNBC eine Rolle. Der GHRH-Rezeptor SV1 könnte als Rezeptor für GHRH und

dessen Antagonisten ein geeignetes Ziel für eine Therapie gegen Endometriose und TNBC darstellen. MIA-602 wäre dabei ein geeignetes Mittel, das betroffene Zellen durch Inaktivierung des EGFR/$ERK_{1/2}$ Signalwegs und Aktivierung der Apoptose bei der Ausbreitung hindern könnte.

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7 Attachment

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im Hause

nachrichtlich:
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Aktenzeichen: 10-179
Datum: 08. Oktober 2010

Sitzung der Ethik-Kommission am 28. September 2010
Antragsteller: Herr Dr. Finas / Herr Prof. Diedrich
Titel: Rezeptorvermittelte Therapie der Endometriose mit Peptidantagonisten des Growth-Hormon-Releasing-Hormon (GHRH)

Sehr geehrter Herr Dr. Finas,

der Antrag wurde unter berufsethischen, medizinisch-wissenschaftlichen und berufsrechtlichen Gesichtspunkten geprüft.

Die Kommission hat nach der Berücksichtigung folgender **Hinweise** keine Bedenken: Die vorgelegte Patienteninformation ist unvollständig und bricht im Absatz zur datenschutzrechtlichen Information unvermittelt ab. Es ist darüber aufzuklären, welche Daten erfasst werden, der Zeitpunkt der Vernichtung der Proben ist zu nennen. Die Information ist weiterhin um einen Absatz zu Nutzenchancen und Schadenrisiken zu ergänzen.

Bei Änderung des Studiendesigns sollte der Antrag erneut vorgelegt werden. Über alle schwerwiegenden oder unerwarteten und unerwünschten Ereignisse, die während der Studie auftreten, muß die Kommission umgehend benachrichtigt werden.

Nach Abschluß des Projektes bitte ich um Übersendung eines knappen Schlussberichtes (unter Angabe unseres Aktenzeichens), aus dem der Erfolg/Misserfolg der Studie sowie Angaben darüber, ob die Studie abgebrochen oder geändert bzw. ob Regressansprüche geltend gemacht wurden, ersichtlich sind.

Die ärztliche und juristische Verantwortung des Leiters der klinischen Studie und der an der Studie teilnehmenden Ärzte bleibt entsprechend der Beratungsfunktion der Ethikkommission durch unsere Stellungnahme unberührt.

Mit freundlichem Gruß bin ich
Ihre

M. Schrader

Prof. Dr. med. Marianne Schrader
Stellv. Vorsitzende

anwesende Kommissionsmitglieder: ☒

☒ Prof. Dr. Dr. H.-H. Raspe
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9 Curriculum Vitae

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2. **Shen Y**, Wang P, Li Y, Ye F, Wang F, Wan X, Cheng X, Lu W, Xie X: **miR-375 is upregulated in acquired paclitaxel resistance in cervical cancer.** *Br J Cancer* 2013, **109**(1):92-99.
3. **Shen Y**, Li Y, Ye F, Wang F, Wan X, Lu W, Xie X: **Identification of miR-23a as a novel microRNA normalizer for relative quantification in human uterine cervical tissues.** *Exp Mol Med* 2011, **43**(6):358-366.
4. **Shen Y**, Li Y, Ye F, Wang F, Lu W, Xie X: **Identification of suitable reference genes for measurement of gene expression in human cervical tissues.** *Anal Biochem* 2010, **405**(2):224-229.