Aus der Klinik für Frauenheilkunde und Geburtshilfe

der Universität zu Lübeck

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A Growth Hormone-Releasing Hormone Antagonist Inhibits the Growth of Endometrial Tissue from Patients of Endometriosis in *In vitro* and *In vivo* Models

Inauguraldissertation

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Abbreviations

APS	ammonium persulfate
cAMP	cyclic adenosine monophosphate
cDNA	complementary DNA
CREB	cAMP response element binding protein
CBP	CREB binding protein
DAG	diacylglycerol
DMSO	dimethyl sulfoxide
DMEM	Dulbecco minimal essential medium
DTT	dithiothreitol
EDTA	ethylenediamine tetraacetate
EESCs	eutopic endometial stromal cells
ESCs	endometrial endometrial stromal cells
FCS	fetal calf serum
Fig	Figure
GH	growth hormone
GHRH	growth hormone-releasing hormone
GHRHR	growth hormone-releasing hormone receptor
GnRH	gonadotropin-releasing hormone
GPCR	G-protein-coupled receptor
HCl	hydrogen chloride
HESCs	human endometrial stromal cells
h	hour
IP_3	inositol triphosphate
KCl	potassium chloride
IGF-I	Insulin-like growth factor I
MAPK	mitogen-activated protein kinase
min	minute

ml	milliliter
mRNA	messenger RNA
MTT	thiazolyl blue tetrazolium bromide
NaCl	sodium chloride
NaOH	sodium hydroxide
OC	oral contraceptives
PACAP	pituitary adenylate cyclaseactivating peptide
PBS	phosphate-buffered saline
PLC	phospholipase C
PKA	cAMP-dependent kinase
PKC	protein kinase C
PFA	paraformaldehyde
Pit-GHRHR	pituitary type GHRH receptor,
rpm	revolutions per minute
RT-qPCR	reverse transcriptase quantitative polymerase chain reaction
RT-PCR	reverse transcriptase polymerase chain reaction
SR I	serum replacement I
SDS	sodium dodecyl sulfate
s	second
SV1	splice variant 1
TBS	Tris buffered saline
TEMED	N, N, N ', N'-tetramethylenediamine
µl	microliter
VIP	vasoactive intestinal peptide

1 Introduction

1.1 Endometriosis

1.1.1 Definition

Endometriosis, a benign chronic gynecological disease, is prevalent in approximately 10% of women of childbearing age. It is defined as the presence of endometrial-like tissue outside the uterine cavity, typically leading to painful symptoms such as dysmenorrhea, dyspareunia, pelvic pain, as well as infertility and malignant transformation (Rogers PA *et al.*, 2009). The surgeon Thomas Cullen described the full morphological and clinical picture of endometriosis and adenomyosis for the first time. He laid out a scheme with the classic sites of adenomyotic lesions in the pelvis in 1920 (Cullen TS, 1921). In 1927, John A. Sampson introduced the "endometriosis" after his fundamental paper on ovarian endometriomas (Sampson JA, 1927). Endometriotic tissues growing outside the uterine cavity are called endometriosis implants. These implants are most commonly attached to peritoneal surfaces, the fallopian tubes, uterus or intestines, and to the ovaries. They can also infiltrate, although less frequently, the bladder, cervix and vagina. Very seldom, endometriosis implants can be found outside the pelvis, for example in old surgery scars, on the liver, and even in or around the lung or brain.

1.1.2 Etiology and pathogenesis

The most widely accepted theory on the etiology of this disease indicates that retrograde menstrual flux is responsible for the implantation and growth of endometrial-like tissue in the peritoneal cavity. Other mechanisms have been proposed such as transportation through veins or lymphatic vessels, peritoneal metaplasia, the presence of embryonic vestiges, or the transformation of endometrial stem cells and bone marrow. But the menstrual regurgitation with the subsequent implantation of endometrial cells can explain most ectopic localizations. The specific characteristics of the ectopic endometrium and the local environment are thought to play important roles in the development of endometriosis.

It is tempting to speculate that the endometriosis is a disease of exaggerated endometrial preconditioning primarily, which not only confers protection against hyper-inflammation and oxidative stress associated with pregnancy, but also endows endometrial cells with the mechanism to survive in the ectopic locations. In 1997, Ryan and Taylor found evidence of a local peritoneal inflammatory process, including elevated cytokine and growth factor concentrations in the peritoneal fluid of affected patients (Ryan IP *et al.*, 1997). Some studies have, by means of Colour-Doppler sonography, shown that endometriosis is associated with an increased intraendometrial and subendometrial blood flow during the late secretory phase of the menstrual cycle (Xavier P *et al.*, 2005). Perhaps the most interesting evidence for cyclic endometrial preconditioning is derived from the observation that stromal cells which are purified from eutopic endometrial biopsy specimens from patients with and without endometriosis show different responses to a decidual stimulus, even after prolonged culture (Klemmt PA *et al.*, 2006), and the cells retained phenotypic (e.g. IGF binding protein-1, vimentin) and functional (e.g. estrogen, progesterone, and epidermal growth factor receptors) markers of their endometrial and endometriotic origin in vivo (Ryan IP *et al.*, 1994).

1.1.3 Treatments

In 1994 Adamson and Pasta conducted a meta-analysis and found that in the moderate and severe stages of this disease, both operative laparoscopy and laparotomy seemed to yield comparable results (Adamson GD *et al.*, 1994). Another study published at the same time proved that laser laparoscopy was a safe, effective and simple treatment in alleviating pain in patients with stage I, II and III endometriosis (Sutton CJ *et al.*, 1994). In 1997, Marcoux *et al.* published the results of a randomized, controlled trial and postulated that laparoscopic surgery enhanced fecundity in infertile women with minimal and mild endometriosis. In conclusion, surgical therapy is considered an appropriate treatment, especially for the advanced stages of the disease, and laparoscopy is an effective surgical approach with the objective of excising visible endometriosis.

As endometriosis is a chronic condition with very high recurrence rates, and as hysterectomy or oophorectomy are not a practical option for many patients, it is possible that a combination of surgical and medical management might provide better outcomes. The primary objective of the

medical treatment of endometriosis is to prevent the growth and activity of endometriotic lesions. It is noteworthy that the first attempts at the hormonal management of the symptoms associated with endometriosis started almost 70 years ago. As soon as synthetic steroids became available, some clinicians began to utilize these in an attempt to find a medical cure for endometriosis. In 1941, the first suggestion came from Geist and Salmon who advocated the use of androgens in gynaecological disorders (Geist SH *et al.*, 1941). In 1958, commenting on the use of androgens, Kistner noted that although a direct effect of the androgenic substances upon the endometriotic area had been suggested, the substances probably exerted their effect through the inhibition of gonadotropic substances (Kistner RW, 1958). This great discovery consequently prompted endocrinologists and gynaecologists to test other gonadotropin – inhibiting substances.

In 1958, Kistner proposed a more practical alternative: induce a state of "pseudopregnancy" to reproduce the improvement of endometriotic symptoms during and after pregnancy (Kistner RW, 1958). He postulated that the positive effect of pregnancy was due to decidualization which resulted in necrosis and elimination of superficial endometriotic implants. This concept bears striking similarity to the approach taken at the same time by Garcia in his quest for a hormonal contraceptive (Garcia CR *et al.*, 1956). This estrogen-progestin regimen has been used extensively for the treatment of endometriosis ever since. Symptomatic relief of the disease was reported in a majority of cases and pregnancy rates in women suffering from infertility as well as endometriosis ranged from 10 to 53 per cent (Moghissi KS, 1988).

Since present studies show that decreased estrogen levels result in cessation of endometriotic implant growth and pain symptoms associated with the disease, conventional therapies focus on reducing systemic levels of estrogen. The most widely utilized medication for endometriosis involves gonadotropin-releasing hormone (GnRH) agonists and oral contraceptives (OC). Due to the chronic nature of endometriosis, long-term or repeated courses of medication may be required to control its related symptoms.

In conclusion, GnRHa, oral contraceptives, androgenic agents, estrogens, progestins, antigonadotropic agents and antiprogestins have all been used successfully. While endometriosis still remains an enigmatic disease, increasing knowledge about the pathogenesis of endometriosis

at cellular and molecular levels will enable us to use new, specific agents for treatment, including GnRH antagonists, progesterone antagonists, aromatase inhibitors, GHRH antagonists and new pharmaceutical agents affecting inflammation, angiogenesis, and matrix metalloproteinase activity (Huang HY，2008; Annunziata M *et al.*, 2010). The purpose of these promising new agents is to prevent or inhibit the development of endometriosis. Further clinical trials will prove whether these new therapies are superior to current medical treatment (Huang HY，2008).

1.2 Growth hormone releasing hormone

Growth hormone releasing hormone (GHRH) was first described in the 1960s (Schally AV *et al.*, 1965). It is a peptide hormone secreted by the hypothalamus in a pulsatile manner. It stimulates the release of growth hormone (GH) by binding onto GHRH receptors on somatotrophs in the anterior pituitary. The amino acid sequence (44 long) of human GHRH is: HO - 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 - Glu - Arg - Gly - Ala - Arg - Ala - Arg - Leu - NH2. In addition to its endocrine role, this peptide has been shown to act as a growth factor in various malignancies. Recent studies indicate that GHRH is also acting as a regulator of several key physiological processes. These processes including the regulation of the metabolism of the reactive oxygen and nitrogen radicals are similarly enhanced by GHRH agonists. In contrast, GHRH antagonists can decrease the growth factor effects of GHRH. The full intrinsic biological activity of GHRH is retained by the NH2-terminal 29-amino acid sequence (Guillemin R *et al.*, 1982; Rivier J *et al.*, 1982).

GHRH is expressed in a variety of normal peripheral tissues, including lymphocytes, ovary, uterus, testis, placenta, cerebral cortex, pituitary, kidney, prostate, liver and lung (Moretti C, 1990). GHRH belongs to a family of related peptides that include vasoactive intestinal peptide (VIP), pituitary adenylate cyclase activating peptide (PACAP), secretin, gastric inhibitory peptide, glucagon, and glucagon-like peptides-1 and -2 (Guillemin R *et al.*, 1982). These peptides in this family act to stimulate the intracellular accumulation of cAMP with the resultant activation of protein kinase A (Gaylinn BD, 1999; Ramirez JL *et al.*, 1999).

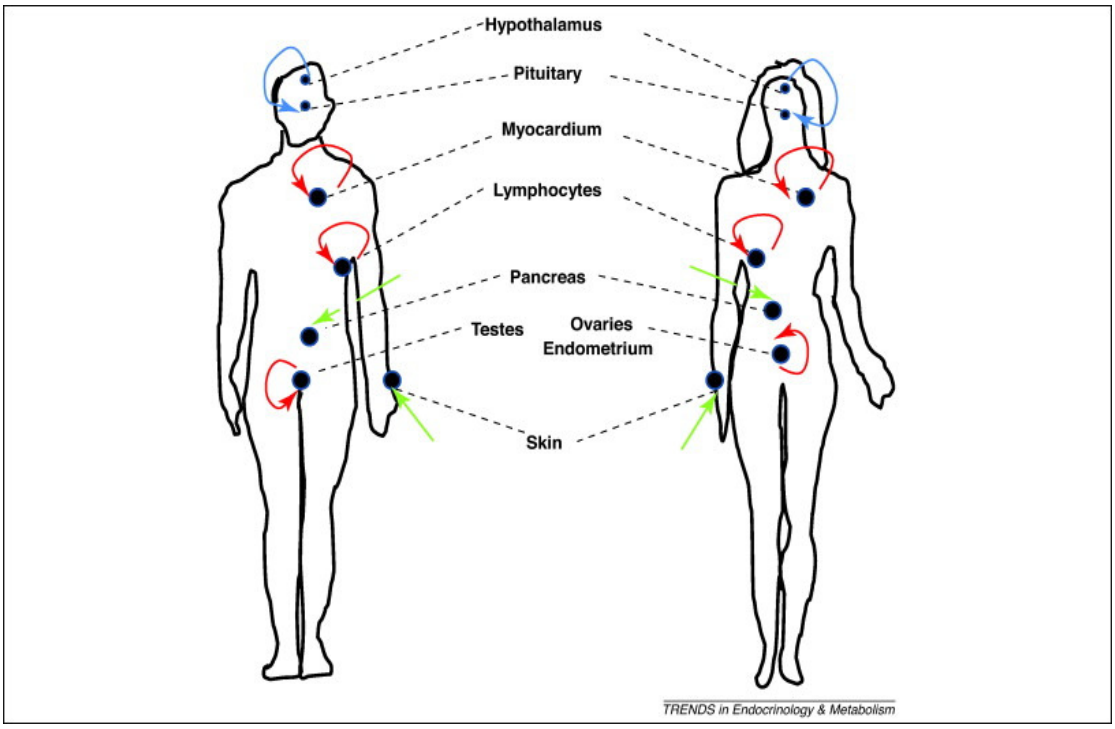

Figure 1. The response to GHRH in tissues and organs. The blue arrow between the hypothalamus and the pituitary indicates the main, neuroendocrine action in regulating GH production. Red arrows show that both production and activity of GHRH in the corresponding tissues has been demonstrated, suggesting autocrine activity. Green arrows correspond to tissues in which activity of GHRH has been demonstrated, but no clear evidence regarding local production has been obtained. This probably implies the existence of paracrine and/or endocrine loops. Drawing by Kiaris H *et al.*, Trends in Endocrinology & Metabolism 2011.

1.3 GHRH receptor

Pituitary type of GHRHR, pit-GHRHR, is a class II G-protein-coupled receptor (GPCR) with seven transmembrane domains and is homologous with the receptors for VIP, PACAP and calcitonin (Gaylinn BD, 1999; DeAlmeida VI *et al.*, 2001). It is predominantly expressed in the anterior pituitary, but it is also found in the hypothalamus, kidney and placenta as well as a variety of established cancer cell lines and tumors (Schally AV *et al.*, 2008). GHRHR is essential for normal somatotroph cell proliferation and the synthesis and secretion of growth hormone (Martari and Salvatori, 2009). Activation of the G-protein complex stimulates the adenyl cyclase, which results in the conversion of ATP to cAMP. CAMP, acting as the second messenger in the GHRH signal transduction, induces phosphorylation of intracellular and membrane-associated proteins (Mayo KE *et al.*, 1996).

At present, several mutations have been identified in the GHRHR gene including nonsense mutations, splice site mutations, microdeletions as well as several different missense mutations and mutations in the promoter region of GHRHR (Castro-Feijóo L *et al.*, 2005; Dattani MT, 2005; Martari M, 2009). At least, four functionally active splice variants of pit-GHRHR have been found through isolation and sequencing of complementary DNA molecules (cDNAs) corresponding to the tumoral GHRHR mRNAs (Rekasi Z *et al.*, 2000). The most prevalent splice variant of GHRHR, the SV1 is a functional receptor. It differs from the pit-GHRHR only in the N-terminal extracellular domain. In SV1, the first 89 amino acids in the aminoterminal extracellular domain of the pit-GHRHR are replaced by a different 25-amino acid sequence which is coded in the 5' untranslated region of the GHRHR gene. Besides its ligand-dependent activity, a ligand-independent activity of SV1 has also been proposed (Kiaris H *et al.*, 2003). The mRNA sequence of SV1 and the molecular mass of the receptor protein from western-blot assays are consistent with a seven-transmembrane receptor which has a third intracellular loop critical for the interaction with G proteins (Rekasi Z *et al.*, 2000; Toller GL *et al.*, 2004). This receptor has also been shown to possess a strong ligand independent activity (Kiaris H *et al.*, 2005; Barabutis N *et al.*, 2007; Barabutis N *et al.*, 2008; Kiaris H *et al.*, 2003). The expression of SVs has been shown in several cancers, including prostatic (Barabutis N *et al.*, 2007; Plonowski A *et al.*, 2002; Halmos G *et al.*, 2002; Barabutis N *et al.*, 2008; Heinrich E *et al.*, 2008), breast carcinomas (Barabutis N *et al.*, 2007; Köster F *et al.*, 2009; Chatzistamou I *et al.*, 2001; Bellyei S *et al.*, 2010), ovarian and endometrial carcinomas (Fu L *et al.*, 2009; Wu HM *et al.*, 2010; Bellyei S *et al.*, 2010; Engel JB *et al.*, 2005), colorectal, gastric, pancreatic (Busto R *et al.*, 2002; Theophanous E *et al.*, 2009; Hohla F *et al.*, 2008) and renal cancers (Halmos G *et al.*, 2000), lung cancers (Barabutis N *et al.*, 2007; Kanashiro CA *et al.*, 2007), bone sarcomas (Busto R *et al.*, 2002), glioblastomas (Havt A *et al.*, 2005; Kiaris H *et al.*, 2000; Bellyei S *et al.*, 2010; Kovacs M *et al.*, 2010; Pozsgai E *et al.*, 2010), non Hodgkin's lymphomas (Keller G *et al.*, 2005), primary human melanomas (Chatzistamou I *et al.*, 2008), and transformed mucosa of the oesophagus (Hohla F *et al.*, 2008). In addition, SVs expressed in normal tissue is also abundantly expressed in tumors and shows high intrinsic, ligand independent activity (Canzian F *et al.*, 2005; Mulhall C *et al.*, 2005; Wagner K *et al.*, 2006). Positive immunostaining of the GHRH receptor in human endometrium has been reported recently (Gallego R *et al.*, 2005).

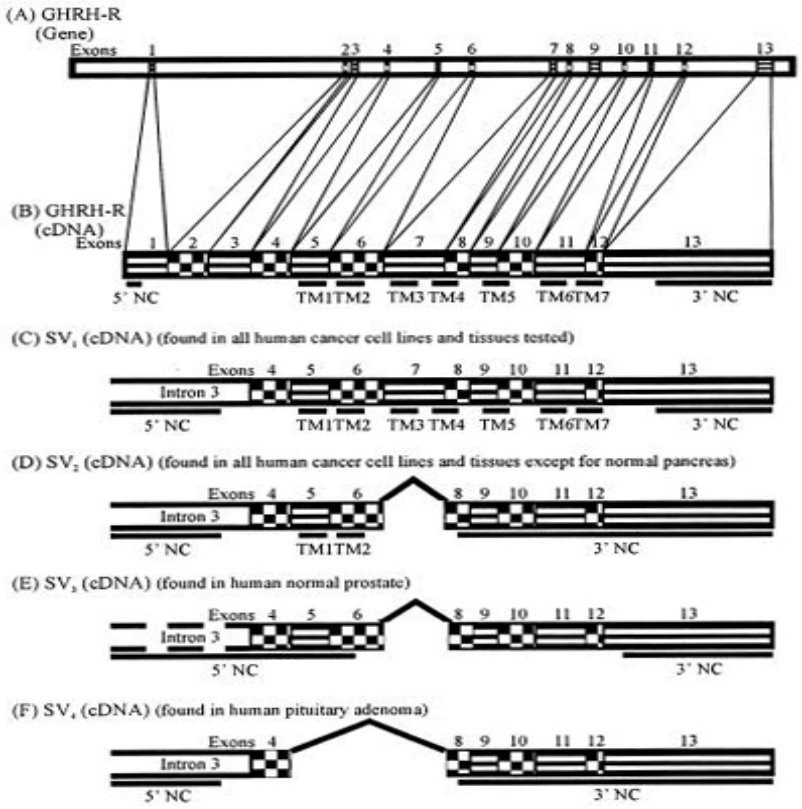

Figure 2: Schematic of the gene structure of various types of GHRHR. The exon–intron structure of the GHRHR gene (A); cDNA structures of the full-length pituitary GHRH-R (B), GHRH-R SV1 (C), SV2 (D), SV3 (E), and SV4 (F). The thick underlines indicate the 5´ and 3´ noncoding regions (NC) of the full length GHRH-R as well as the transmembrane domains (TM). The blank areas connecting the underlines represent the other receptor protein domains. Image by Rekasi Z *et al.*: Isolation and sequencing of cDNAs for splice variants of growth hormone-releasing hormone receptors from human cancers. PNAS 2000, p.97.

1.4 GHRH & GHRHR

GHRH binding to GHRHR results in GH production mainly by the cAMP dependent pathway, but also by the phospholipase C pathway (IP3/DAG pathway) (Frohman LA *et al.*, 1999; Silverman BL *et al.*, 1988; Vasilatos-Younken R *et al.*, 1992; Radecki SV *et al.*, 1994). The cAMP-dependent pathway is initiated by GHRH binding to its receptor. This binding alters receptor conformation, which activates the G_s alpha subunit of the closely associated G-Protein complex on the intracellular side. This G-Protein complex stimulates the membrane-bound adenylyl cyclase and increases intracellular cyclic adenosine monophosphate (cAMP). CAMP binds to and activates the regulatory subunits of protein kinase A (PKA) thus allowing the free catalytic subunits to translocate to the

nucleus and to phosphorylate the transcription factor cAMP response element binding protein (CREB). Phosphorylated CREB, together with its coactivators, p300 and CREB binding protein (CBP), binds to cAMP-response elements (CREs) in the promoter region of the GH gene and enhances the transcription of GH. It also increases transcription of the GHRHR gene, providing positive feedback.

In the phospholipase C pathway, GHRH stimulates phospholipase C (PLC) through the βγ-complex of heterotrimeric G-proteins. PLC activation produces both diacylglycerol (DAG) and inositol triphosphate (IP3) leading to the release of intracellular Ca^{2+} from the endoplasmic reticulum, thus increasing cytosolic Ca^{2+} concentration which results in vesicle fusion and release of growth hormone from the secretory vesicles (Smith RG *et al.*, 1996; Shaner R. *et al.*, 2002). Some Ca^{2+} influx is also a direct action of cAMP, which is distinct from the usual cAMP dependent pathway of activating protein kinase A (Chen C *et al.*, 1995).

1.5 GHRH antagonists

GHRH antagonists were first advocated in the early 1990s due to the fact that somatostatin analogues do not adequately suppress GH and IGF-I levels in patients with neoplasms potentially dependent on IGF-I (Pollak MN *et al.*, 1990; Pollak M *et al.*, 1992). Inhibitory effects of GHRH antagonists have been evaluated *in vitro* and in a variety of human experimental cancer models in nude mice (Schally AV and Varga JL, 1999; Schally AV and Varga JL, 2006; Varga JL and Schally AV, 2006). In recent years, various new GHRH antagonists were synthesized by replacing certain positions with different substituends. Among them, JV-1-63 and JV-1-36 exhibited potent endocrine activity (Varga JL *et al.*, 2004). GHRH antagonists with histidine and ornithine replacements exhibit some of the most potent antitumor effects reported so far (Schally AV and Varga JL, 2006; Varga JL and Schally AV, 2006; Buchholz S *et al.*, 2007; Stangelberger A *et al.*, 2007; Stangelberger A *et al.*, 2005). GHRH antagonists affect some of the signaling mechanisms involved in cell proliferation, survival, and metastasis, and activate proapoptotic signaling mechanisms. The inhibitory effect of antagonistic analogues of GHRH is exerted in part by endocrine mechanisms through the suppression of GHRH-evoked GH release from the pituitary, which, in turn results in the reduction of hepatic production of IGF-I and a decrease in the serum IGF-I levels. The antitumor effects of GHRH antagonists can also be mediated through direct mechanisms. One of these mechanisms is based upon the inhibition of the secretion of autocrine/paracrine IGF-I or IGF-II from the tumors, while probably the most influential pathway involves the blockade of action of autocrine GHRH in tumors. The antitumor activity of GHRH antagonists is especially noteworthy oncologically because of the wide expression of the intrinsic GHRH, pit-GHRHR and SVs of GHRHR in various cancers.

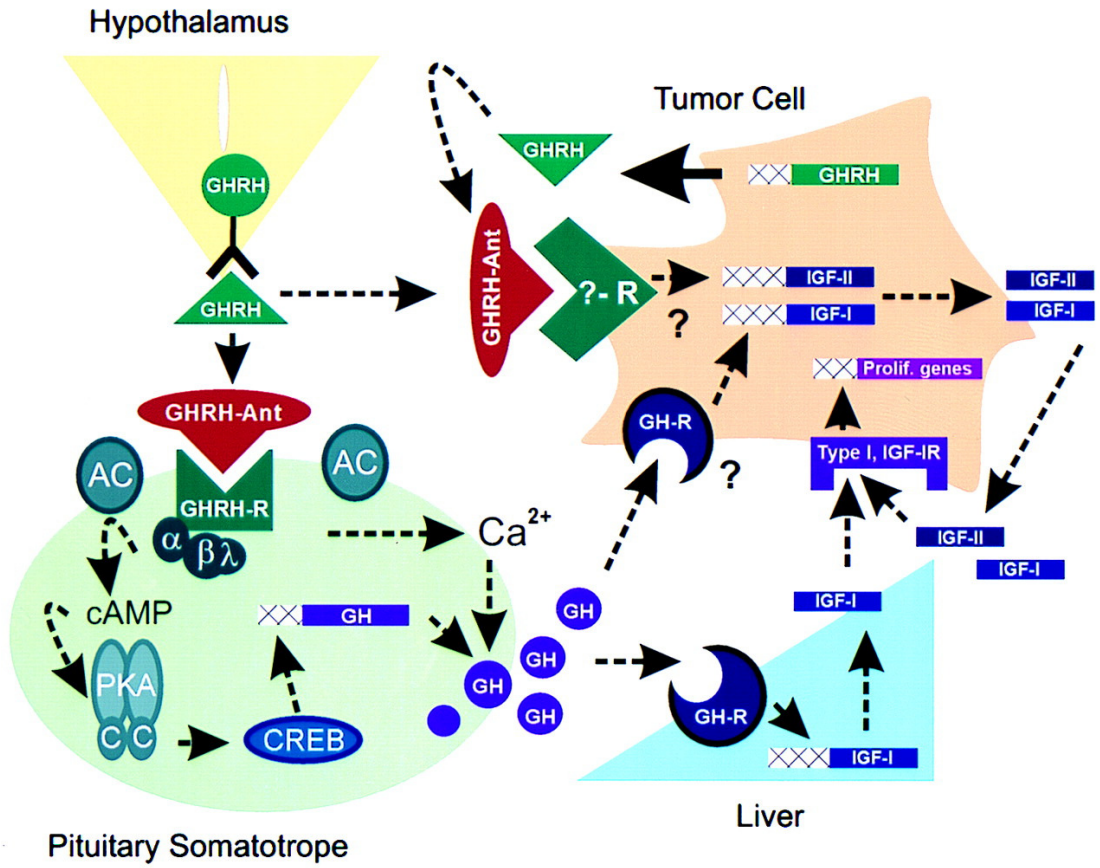

Figure 4: Potential mechanisms mediating the antitumorigenic actions of GHRH antagonists (GHRH-Ant). Image by Kineman RD: Antitumorigenic actions of growth hormone-releasing hormone antagonists. Proc Natl Acad Sci USA 2000, 97.

1.6 Aim of the current study in endometriosis

To this day, the etiology of endometriosis remains uncertain, and the most widely accepted theory on the pathogenesis is the attachment of endometrial tissue from retrograde menstruation through the fallopian tubes in the peritoneal cavity. The proliferation of such endometrial tissue depends on hormonal factors, inducing inflammation and angiogenesis (Giudice LC and Kao LC, 2004). Compared to normal endometrium the cells of ectopic endometrium are characterized by increased proliferation and survival (Giudice LC and Kao LC, 2004; Gebel HM *et al.*, 1998). Recently, GHRH expression 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 (Chatzistamou I *et al.*, 2002; Fu L *et al.*, 2008; Kahán Z *et al.*, 1999; Khorram O *et al.*, 2001). GHRH was found to be synthesized in human endometrial, ovarian, and breast cancers (Kahan Z *et*

al., 1999) suggesting that GHRH may play a role in the development of endometrial cancers and endometriosis. GHRHR has also been detected in peripheral tissues besides the central nervous system (Havt A *et al.*, 2005). Among the different SVs, SV1 has the greatest structural similarity to the pit-GHRHR and may mediate the main effects of GHRH and its analogues in peripheral tissues (Rekasi Z *et al.*, 2000; Barabutis N and Schally AV, 2008; Schally AV *et al.*, 2008). Splice variant 1 has been detected in endometrial carcinoma (Kiaris H *et al.*, 2003) and, recently, in ectopic and eutopic endometrial tissues from endometriosis patients (Fu L *et al.*, 2008). Furthermore, Fu L *et al.* have 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 (Fu L *et al.*, 2008). Based on this finding, it is conceivable that pit-GHRHR or SVs may play a role in endometriotic cell proliferation and survival. GHRH antagonists inhibit proliferation and survival of endometrial cancer cells and counteract the growth of endometrial cancers (Engel JB *et al.*, 2005). In a recent study, Marta Annunziata *et al.* evaluated pit-GHRHR and SV1 mRNA expression in eutopic and ectopic endometrial tissues from endometriosis patients, primary ectopic ESCs from patients with endometriosis, and the T-HESC human ESC line. They found that the GHRH antagonist JV-1-36 inhibited the proliferation and survival of ESCs and T-HESCs (Annunziata M *et al.*, 2009). This exciting finding suggests the potential use of antagonistic analogues of GHRH as therapeutic agents.

In our study, we will evaluate the effect of a new GHRH antagonist MIA-602 in some cancer cell line and primary eutopic ESCs from patients with endometriosis. We will determine the inhibitory effect of MIA-602 on the development of endometriosis *in vivo* as well.

2 Materials 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
Acrylamide / bisacrylamide 37.5:1	Roth, Karlsruhe
Agarose	Invitrogen, Karlsruhe
Ammonium persulfate (APS)	Biorad, Munich
Bromophenol blue	Merck, Darmstadt
Collagenase	Sigma, Taufkirchen
Dimethylsulfoxide (DMSO)	Merck, Germany
dH_2O	University of Lübeck
dNTP Mix (10 mM)	Invitrogen, Karlsruhe
Ethanol	Roth, Karlstruhe
Ethylenediaminetetraacetate (EDTA)	Sigma-Aldrich, Steinheim
Faramount aqueous mounting medium	Dako-Cytomation , Denmark
Fetal calf serum (FCS)	Sigma, Steinheim
Filter paper	Whatman, GE Healthcare, Munich
Gentamicin (10mg/ml)	PAA Laborateries GmbH, Cölble
Glycerol	Merck, Darmstadt
Glycine	AppliChem GmbH, Darmstadt
Hematoxylin	Merck, Darmstadt
Hydrogen peroxide (H_20_2)	Thermo Fischer Scientific, Schwerte
Goat serum	Vector, Biozol, Hamburg
Hydrochloric acid (HCl)	Merck, Darmstadt
Hyperfilm ECL	Amersham, GE Healthcare, Munich
Immobilon Western Chemiluminescent HRP Substrate	Millipore, Schwalbach
Liquid permanent red	DakoCytomation , Hamburg
Low molecular weight DNA ladder	New England Biolabs, Frankfurt
Midori Green Direct	Biozym Scientific GmbH, Oldendorf
Milk powder (blotting grade)	Roth, Karlsruhe
Nitrocellulose transfer membrane	Whatman, GE Healthcare, Munich
N, N-dimethylformamide	Merck Schuchardt OHG, Hohenbrunn
N, N, N ', N'-tetramethylenediamine (TEMED)	Sigma-Aldrich, Steinheim

Penicillin / streptomycin (100×)	PAA Laborateries GmbH, Cölble
Precision Plus Protein Dual Color Standards	Biorad, Munich
Random Primer	Invitrogen, Karlsruhe
Re-Blot Plus	Chemicon International, Hofheim
RNAse-free water	Serva, Heidelberg
Rompun 2%	Bayer, Monheim
RPMI1640 Medium	Invitrogen, Karlsruhe
17β-estradiol tablet	Innovative Research of America, Sarasota, FL, USA
Serum replacement 1 (SR1, 50×)	Sigma, Taufkirchen
Sodium chloride	Roth, Karlsruhe
Sodium dodecyl sulfate (SDS)	Serva Electrophoresis, Heidelberg
Sulfuric acid (H_2SO_4)	Baker Chemicals, Gross-Gerau
SuperScript® II Reverse Transcriptase (RT)	Invitrogen, Karlsruhe
Taq DNA Polymerase (5 000 U/ml)	New England Biolabs, Frankfurt
Thiazolyl blue tetrazolium bromide (MTT)	Sigma-Aldrich, Taufkirchen
Tris	Roth, Karlsruhe
Trypsin-EDTA (1×)	PAA Laborateries GmbH, Cölble
Tween 20	Merck, Darmstadt
X-ray developer/ fixerconcentrate	Adefo-Chemie GmbH, Dietzenbach

2.1.2 Kits

BCA-Protein Assay Kit	Thermo Fisher Scientific, Schwerte
Red Alkaline Phosphatase Substrate Kit I	Vector, Biozol, Hamburg
RNeasy Mini Kit	Qiagen, Hilden

2.1.3 Solutions and Buffers

1×TBST:	8.18g NaCl, 2.42g Tris in 1L dH_2O(PH=7.6), 0.1% Tween 20
10×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 (50 ml):	250 mg MTT, 50 ml PBS
10% MTT (10 ml):	1 ml MTT solution, 9 ml DMEM medium
PBS:	pH 7.4; 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 1 mM EDTA)
RIPA buffer:	150 mM NaCl, 1.0% Igepal®, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris, pH 8.0.

4×SDS-protein loading buffer:	0.125 M Tris-HCL, pH 6.8, 20% glycine, 4% SDS, 0.01% bromphenolblue, 2% β-mercaptoethanol
Protein electrophoresis buffer:	25 mM Tris base, 192 mM glycine, 0.1% SDS, pH 8.3
Transfer buffer:	25 mM Tris base, 192 mM glycine, 10% methanol

2.1.4 Antibodies and their characteristics:

Primary GHRHR-antibody:	Rabbit polyclonal antibody to GHRHR (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 1:20,000 dilution in western blot, and 1:200 dilution in immunohistochemistry.
Secondary antibody for western blot:	ECL™ Anti-Rabbit IgG from Amersham, GE Healthcare, Munich. It was used at 1:5,000 dilution.
Secondary antibody for immunohistochemistry:	Biotinylated Anti-Rabbit IgG (H+L), made in goat, from Vector Laboratories, Biozol, Hamburg. It was used at 1:250 dilution.

2.1.5 Instruments and equipment

BioPhotometer plus	Eppendorf, Germany
Biometra Thermocycler T-personal 48	Biometra GmbH, Göttingen
CO_2 incubator	Binder GmbH, Tuttlingen
Cell culture pipettes (5, 10, 25 ml)	Cell Star, Greiner Bio-One, Frickenhausen
Cell culture flaskes (10, 25ml)	Cell Star, Greiner Bio-One, Frickenhausen
Cell scrapers	Biochrom AG, Berlin
Centrifuge tubes (plastic, 50 ml, 15 ml)	Greiner Bio-One, Frickenhausen
Cryovessels 1.5 ml	Sarstedt, Nümbrecht
Current source Pharmacia Biotech EPS 200	GE Healthcare, Munich
DNA Engine Opticon 2 Realtime-PCR-machine	MJ-Research, Biorad, Munich
Electrophoresis System	Owl Scientific Inc, Hamburg
Electrophoresis Power supply device	Pharmacia Biotech, Hamburg
Gel Documentation System	Phase GmbH, Lübeck
Gel-sealing tape	Macor, Hamburg

Hoefer Semi-Dry Transfer Unit TE 70	GE Healthcare, Munich
Horizontal shaker Vibrax VXR S15	IKA Labortechnik, Staufen
Magnetic stirrer MR82	Heidolph Instruments, Schwabach
Microcentrifuge MC6	Sarstedt, Nümbrecht
Microscope (Axiovert 135M) (cell count)	Carl Zeiss, Göttingen
Microtome	Leica, Germany
Microwave	Sharp Electronics (Europe) GmbH, Hamburg
Mini-Sub ® Cell GT electrophoresis chamber	Biorad, Munich
MRX multiplate reader	Dynatech Laboratories, Burlington, USA
Multipette Plus	Eppendorf, Hamburg
96-well tissue culture plate	Sarstedt, Nümbrecht
Neubauer-counting chamber	Brandt, Wertheim
Oven	Heraeus, Hanau
Orbital Shaker Shaker S4	ELMI Lab. Equipment, Riga, Latvia
pH Meter pH526 MultiCal ®	WTW, Weilheim
pipettor accu-jet	Brand GmbH, Wertheim
Refrigerated Centrifuge Biofuge ® fresco	Heraeus, Hanau
Sartorius laboratory balance basic BA310S	Sartorius AG, Göttingen
Cellstrainer (40,100um)	Becton Dickinson Falcon, BD Biosciences, Heidelberg
Suction Gel Pump GP110	Savant, New York, USA
Superfrost plus microscopeslide	Thermo Fischer Scientific, Schwerte
Tissue culture dishes (10ml)	Becton Dickinson Falcon, BD Biosciences, Heidelberg
Thermomixer compact	Eppendorf, Wesseling-Berzdorf
Tubes (Safe Lock Tubes) (1.5 ml, 2 ml)	Eppendorf, Hamburg
Universal 32 centrifuge	Hettich, Tuttlingen
UV transilluminator TI 1	Biometra GmbH, Göttingen
Variable single-channel Eppendorf Research ®	Eppendorf, Wesseling-Berzdorf
Vortex REAX 2000	Heidolph Instruments, Schwabach

2.1.6 Cell lines

Breast cancer cell lines HCC1806, MDA-MB-231, ovarian cancer cell line OVCAR-3, and choriocarcinoma cell line JEG3 were obtained from American Type Culture Collection (ATCC). These cell lines were used for cell MTT-proliferation assay, RT-PCR and western-blot analyses to establish the methods for the detection of GHRHR and the use of the substances.

2.1.7 Nude mice

In this study, we used five to seven weeks old female CD1-Nude mice from Charles River, Sulzfeld. The animals were housed (two/cage) with a 12:12-hour light-dark cycle. Housing material, food and water were sterilized before use. *In vivo* experiments were done with the permission of the "Ministerium für Landwirtschaft, Umwelt und Ländliche Räume des Landes Schleswig-Holstein".

2.1.8 GHRH antagonist MIA-602

MIA-602 was synthesized in the laboratory of Andrew V. Schally, at the University of Miami, FL, USA. MIA-602 is an antagonistic analog of human GHRH. Its structure is [(PhAc-Ada)^{0}- Tyr,^{1} D-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} Har^{29}]hGHRH(1-29)NH_2. For the *in vivo* study, 2 mg MIA-602 were dissolved in 20 µl DMSO, and diluted with 2 ml propylene glycol and filled with 17.98 ml water *ad* 20 ml. The daily injection dose contained 10 µg MIA-602 in 100 µl volume. The control solvent (100 µl vehicle/day) was composed of 20 µl DMSO, 2 ml propylene glycol and 17.98 ml water *ad* 20ml.

For the in vitro study, 3 mg MIA-602 were dissolved in 20 µl DMSO and further diluted with water ad 621 µl to a stock solution of 1 mmol/L. For the control stocking solution, 20 µl DMSO were dissolved in 621 µl water. For incubation the solutions were diluted to 1 µmol/L, 0.1µmol/L, and 0.01 µmol/L with medium.

2.1.9 Endometrial biopsies

Endometrial biopsies were collected using a "Pipelle" device under sterile conditions from 17 women with endometriosis (aged 33.8±7.96) undergoing surgical explorative laparoscopy for endometriosis and infertility problems. Final diagnosis was confirmed by histopathological examination. Prior to surgery, each patient signed an informed consent which was approved by the Ethics-Committee of the University of Lübeck. All recruited patients were reproductive-aged women with regular menstrual cycles who had not received any endocrine or anti-inflammatory therapy for at least 6 months before surgery. We used 8 of 17 endometrium tissues for primary

cell culture and 14 for xenotransplantation into nude mice. Tissue samples were placed in cold, sterile RPMI-1640 medium containing 100 IU/ml penicillin, 100 µg/ml streptomycin and 100 µg/ml gentamicin, and immediately transported to the laboratory for primary cell culture and *in vivo* studies.

2.2 Methods

2.2.1 Cell Culture

All culture work was carried out under a laminar airflow workbench under sterile conditions. The bench was prepared with UV light 20 min prior to cultivation and thoroughly cleaned with an appropriate disinfectant. All consumables such as pipette tips were sterilized. Cells were cultured in a humidified atmosphere at 37 °C containing 5% CO2 in RPMI medium (Life Technologies, Frankfurt) supplemented with 10% FCS and 1% penicillin G streptomycin (50 mg/ml) and grown to confluence with regular changes of medium. For sub cultivation and harvesting, adherent cells were washed twice with ice-cold 1 x 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 1.500 rpm for 7 min at room temperature.

2.2.2 Cell Concentrations

Cells were resuspended in culture media and counted in a Neubauercounting chamber (Brandt, Wertheim). 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.

2.2.3 Primary eutopic endometrial stromal cells (EESCs) culture

Eutopic endometrial stromal cells (EESCs) from patients with endometriosis were cultured under established conditions (Ryan IP *et al.*, 1994). Endometrial biopsies were collected under sterile

conditions and transported to the laboratory on ice. There, the endometrial tissue was briefly minced into small pieces briefly, digested with collagenase (2 mg/ml) for 1 h at 37 °C, and separated using serial filtration. Debris was removed by 100 µm aperture strainer and epithelial glands were retained in the 40 µm aperture cell strainer (Becton Dickinson, Heidelberg). The endometrial stromal cells in the filtrate were plated onto cell culture dishes (Becton Dickinson, Heidelberg) and allowed to adhere for 60 min, after which blood cells and epithelial cells were removed with rinses of phosphate-buffered saline. The cells were grown to confluence and subcultured twice to eliminate contamination by epithelial cells, macrophages and other leukocytes. These cells, along with the cancer cell lines mentioned above, were also used in cell proliferation assays, RT-qPCR, and western blot.

2.2.4 MTT-proliferation Assay

The MTT-test is a colorimetric assay to determine cell proliferation and viability. In this work, it was used to demonstrate the influence of the GHRH antagonist MIA-602 in various concentrations on cell growth.

Cells were seeded into 96-well plates at a density of 3000 cells per well in complete medium. After 24 h, the medium was removed and cells were serum starved for 24 h, followed by treatment for 72 h with decreasing concentrations of MIA-602 (from 1 µmol/L to 0.01 µmol/L) as well as DMSO at an appropriate concentration in fresh medium containing 1% serum replacement I (Sigma, Germany). Hereafter, the cells were incubated with 10% MTT in medium for 4 h or until crystalline precipitates became visible after which the reaction was stopped by adding MTT-solubilization solution (1:1 ratio) and incubation in the dark overnight. At last, readings were taken at an absorption wavelength of 560 nm and a reference wavelength of 650 nm on an MRX plate reader (Dynatech Laboratories). All data represent cumulative results of several readings (n= 6).

In EESCs, the assays were carried out in the same way but without the serum-starving step, instead of which cells were always incubated in the presence of 3% serum after the first 24-h-incubation.

2.2.5 RNA Extraction, Reverse Transcription Polymerase Chain Reaction

2.2.5.1 RNA Extraction

After harvesting and counting the cells, total RNA was extracted with RNeazy-mini-kit (Qiagen, Hilden). Briefly, the cells were disrupted in 600 µl 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. 1 vol of 70% ethanol was added to the homogenized lysate and mixed well by pipetting up and down. The sample was transferred to an RNeasy spin column placed in a 2 ml collection tube and centrifuged for 15 sec at >10,000 rpm. The flow-through was discarded, 500 µl Buffer RPE were added to the column and was centrifuged again for 15 s at >10,000 rpm to wash the spin column membrane. The column was dried by centrifugation after the flow-through was discarded. The RNeasy spin column was placed into a new 1.5 ml collection tube and 40 µl RNase-free water were added directly to the spin column membrane. The column was centrifuged for 1 min at >10,000 rpm to elute the RNA.

For the photometric determination of concentration and purity, the RNA was diluted 1:20 in TE-buffer, pH 7.5. The optical density (OD) was measured in the BioPhotometer plus at a wavelength of 260 and 280 nm and blanked with TE-buffer, pH 7.5. The ratio of OD_{260} /OD_{280} indicates the purity of the isolated RNA. Samples with insufficient purity (<1.5) were discarded. Finally, the extracted RNA was stored at -80 °C.

2.2.5.2 cDNA synthesis

Two micrograms of the RNA were reverse transcribed (RT-reaction) to synthesize cDNA using the enzyme, SuperScript® II Reverse Transcriptase (RT, life technologies, Frankfurt). The cDNA thus obtained served as template in polymerase chain reaction. Briefly, 2 µg RNA were brought to the total volume of 10 µl with water and pipetted into a PCR tube. After the addition of 1 µl random primer and 1 µl of 10 mM dNTP mix per PCR tube, the samples were mixed and centrifuged briefly. Subsequently, the mixture was incubated at 65 °C for 5 min in a thermocycler to melt the secondary structure of the RNA and quick chilled on ice. During this time, one per batch of 6 µl RT mix was created and pipetted as follows: 5x Buffer 4 µl , 0.1 M DTT 2µl. After careful mixing, 1 µl

superscript II reverse transcriptase added. A negative control was carried out using RNAse-free water instead of reverse transcriptase. The mixtures were then incubated for 50 min at 42 °C in a thermocycler followed by heated for 15 min at 70 °C. The cDNA was stored until further use at -20 °C.

2.2.5.3 Polymerase chain reaction

A housekeeping gene was amplified along with the target genes as an internal standard in order to compensate for variations of the starting amount of cDNA,. In this study, the hypoxanthine phosphoribosyl transferase (HPRT) gene was chosen. The negative control contained HPLC water instead of cDNA. The specificity of the PCR products was confirmed by a melting curve analysis of the amplified product. The specific primer pairs for the pituitary GHRHR (pit-GHRHR), GHRHR SV1 and SV1-I2-I3 (stretching from Intron 2 to Intron 3) as well as the housekeeping gene HPRT were synthesized by Metabion GmbH, Martinsried:

pit-GHRHR	forward 5'- TTC TGC GTG TTG AGC CCG TTA C-3'
	reverse 5'- TAA GGT GGA AAG GGC TCA GAC C-3'
SV1	forward 5'- TGG GGA GAG GGA AGG AGT TGT-3'
	reverse 5'-TAA GGT GGA AAG GGC TCA GAC C-3'
HPRT	forward 5'-TCA GGC AGT ATA ATC CAA AGA TGG-3'
	reverse 5'-AGT CTG GCT TAT ATC CAA CAC TTC-3'

For each batch, the following master mix was prepared: 12.5 µl Platinum SYBR Green qPCR SuperMix-UDG, 2 µl primer pair (5 µM forward / reverse) and 9.5 µl HPLC water to a total volume of 24 µl. One µl cDNA was pipetted into each well of a 96-well microtiterplate with one well containing water instead of cDNA for the negative control. After adding 24 µl master mix to each well the microtiterplate was sealed andbriefly centrifuged at 1000 rpm to remove air bubbles and finally placed in a thermo cycler operating with the OpticonMonitor ™ Analysis Software, version 3.1.32 (Biorad-laboratories, Inc., 2005).

For amplification of the 25-hydroxylase, 1α-hydroxylase and 24-hydroxylase following real-time PCR programs were used:

1. 2 min polymerase activation at 50 °C
2. 2 min denaturation at 95 °C
3. 15 s denaturation at 95 °C
4. 30 s annealing and polymerization at 60 °C
5. Plate reading and return to step 3 for a total of 40 cycles
6. Melting curve from 60-94 °C, measured in increments of 0.1 °C → End
7. Cooling at 4.0 °C

The product was stored at -20 °C.

Agarose gel electrophoresis

Agarose gel electrophoresis is a method for DNA molecules separate into an electric field according to their size. In this study, we used Midori Green Direct (Biozym Scientific GmbH, Oldendorf), a new and safe class of nucleic acid stains to replace toxic Ethidium Bromide. MIDORI GREEN DIRECT can be used with regular UV transilluminators and Blue Light LED illuminators as well. MIDORI GREEN DIRECT stains are provided in a form of 6X sample loading dyes added to samples only. We prepared a 2% agarose gel in TBE buffer. PCR product (12 µl) and the LMW DNA ladder (6 µl) were mixed with Midori Green Direct at 1:10 (dye:sample) dilution rate. The samples were electrophoretically separated for 50 min at 90 volts. Using the DNA ladder, the size of the PCR products were assessed. The individual bands were photographed under UV light with a digital camera system (Phase, Lübeck).

2.2.6 Western Blotting

2.2.6.1 Protein Isolation and concentration evaluation

Protein of cells and tissues was extracted by RIPA buffer. RIPA buffer enables the extraction of cytoplasmic, membrane and nuclear proteins and is compatible with many applications, including reporter assays, protein assays, immunoassays and protein purification. We thawed frozen cells or tissues slowly on ice, and washed them with cold PBS. Cells or tissues were collected again by

centrifugation at 1,500 rpm for 7 min. We added 10 µl PMSF solution, 10 µl sodium orthovanadate solution and 30 µl aprotinin (Sigma) per ml of 1X RIPA buffer to prepare complete RIPA immediately before applying. 0.5 ml per 10^6 cells of RIPA buffer was added to cells or tissues (30 µl per 10 mg tissues) and mixed gently with a pipette and incubated for 30 min on ice. Then cells were passaged through the 21 gauge needle, and tissues were further disrupted and homogenized with a Eppendorf-tube homogenizer (Eppendorf, Hamburg), maintaining temperature at 4°C. 10 mg/ml PMSF stock was applied to mixture (10 µl per 10^6 cells or 0.5 µl per 10 mg tissue) followed by incubating 30 min on ice. The lysate was transfered to a 1.5 ml microcentrifuge tube, and centrifuged at 10,000 x g for 10 min at 4 °C. This separates the total protein (supernatant) from the cellular debris (pellet). The supernatant was transferred to a new tube for further analysis.

Protein concentrations were evaluated by using the Bicinchoninic Acid (BCA) Protein Assay Kit (Thermo Fisher Scientific, Bonn). First, we prepared a set of BCA standards to make a standard curve under the protocol offered by company. Every unknown sample was diluted from 3 µl into 30µl with water. Mix Reagent A with Reagent B in a 50:1 ratio immediately before use. 10 µl of each BCA standard and unknown sample were added into microplate wells in duplicate. Then 200 µl of Reagent AB was added into every well containing a sample. The plate was incubated at 37 °C for 30 min followed by measuring in a microplate reader at 560 nm. The standard curve was used to determine the concentrations of each unknown sample. Every 20 µg sample was 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 the ice immediately.

2.2.6.2 SDS-Polyacrylamide gel electrophoresis (SDS-PAGE) and semi-dry blotting

The running gel was 10% PAGE and the the stacking gel was 4%. 20 µg of sample protein and a molecular weight marker (Biorad) were subsequently loaded onto the gel submerged in 1x electrophoresis buffer. An electric current of 110 V was applied, causing the negatively charged proteins to migrate across the gel towards the anode. Depending on their size, each protein moves differently through the matrix. In order to make the proteins accessible to antibody detection they were moved from gel to nitrocellulose membranes (Whatman). This was facilitated using the

Semi-Dry Transfer Unit TE 70 Semi-Dry Blotter (Hoefer). The gel was placed on the membrane without any air bubbles between the membrane and the gel. They were then placed between two stacks of filter papers that had been presoaked in transfer buffer. One membrane was blotted for 90 min at 35 mA.

The membrane was blocked with fresh blocking buffer for 1 h. The blocking buffer was removed and the membrane was washed with washing buffer for 3×5 min on a shaker. Subsequently the membrane was incubated at 4 °C overnight on a shaker with the Anti-GHRHR antibody (10 ml, 1: 20,000), then washed with washing buffer for 3×5 min. The membrane was then incubated for another 1 h with secondary antibody (HRP- (horseradish peroxidase) conjugated anti-rabbit IgG, 10ml, 1:5,000). After repeated washing steps for 3×10 min, specific bands were visualized by the enhanced chemiluminescence autoradiography (Hyperfilm ECL; Amersham, GE healthcare). As a protein loading control, the expression of β-actin as housekeeping gene was always checked.

2.2.7 *In vivo* model of endometriosis

Endometrial tissue was washed twice in phosphate buffered saline (PBS), dissected into five small cubes (~2×1×1 mm^3) per mouse. Animals were anesthetized with a mixture of Rompun® and Ketavet® (1:3, Bayer) by intramuscular injection. The corneas were protected by using an ointment (Bepanthen®). The peritoneal cavity was opened carefully, and the small endometrial tissue fragments were sutured onto the peritoneum (3 in the right, 2 in the left) with 5-0 PDS*II sutures (Ethicon Johnson & Johnson) as shown in **Figure 5**. Before suturing the incision with 5-0 PDS*II sutures, we would check the mice again to make sure that the biopsies fragments were fixed firmly, and there was no bleeding. We did xenotransplantation in 26 mice totally. We subcutaneous implanted the estrogen tablets (Innovative Research of America) in the neck area of the last 8 mice in order to increase the estrogen level, so that the endometrial lesion could grow better as shown in **Figure 6**.

Implanted mice were monitored daily for survival and comfort until day 14 postimplantation. Starting on day 14 animals received a daily intraperitoneal injection of MIA-602 (10 µg in 100 µl) (treated group, n=13) or only solvent alone used as vehicle (control group, n=13) (**Fig. 5 d**). Injections were performed for 28 days of treatment, and animals were sacrificed by decollation.

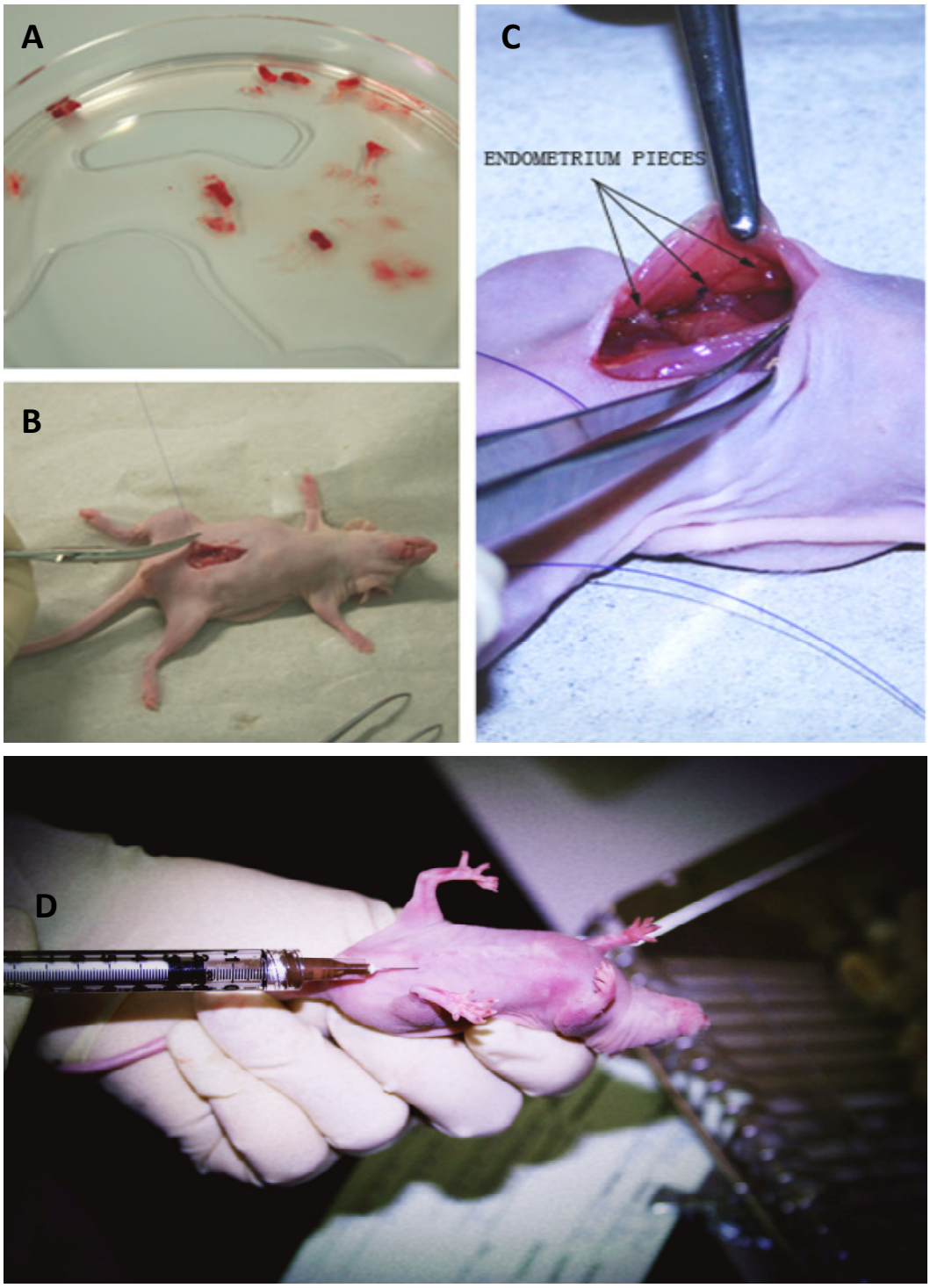

Figure 5: The xenotransplantation and intraperitoneally injection to the nude mouse. The surgery was administered on the day we received the human endometrium tissue from the surgery. The tissue was sutured to the peritoneal cavity of mice and left for 14 days before starting treatment. MIA-602 was then intraperitoneally administered for 28 consecucutive days before euthanizing the animals. A): The human endometrium fragments (~2×1×1 mm^3 for each size) were washed with PBS before xenotransplantation. B): Suturing the fragments to the peritoneum in the abdominal cavity. C): The fragments were sutured on the peritoneum (3 on the right, 2 on the left). D): A daily intraperitoneal injection was exerted.

Then, the abdominal cavity of each mouse was explored for localization and measure of endometriotic lesions using a caliper precision instrument (**Figure 5**). We also checked whether there was any macroscopic change of the heart, liver, spleen, intestines, or kidneys of tested animals. Lesions were photographed *in situ* using a Canon HD camera. Then, we separated the lesions carefully followed by washing the lesions in PBS. Finally, some lesions were stored at −80 °C for the latter analysis and others were fixed in cold formaldehyde (4.5% in PBS) and were paraffin-embedded for immunohistochemistry analyses.

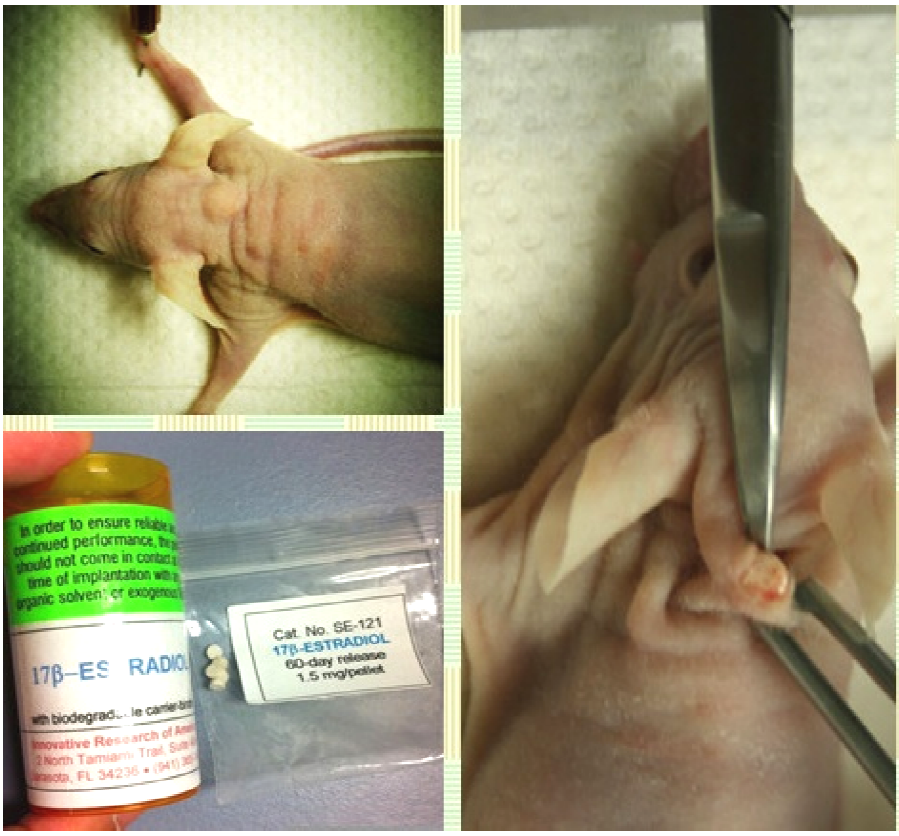

Figure 6: The subcutaneous implant of an estrogen tablet. We implanted the estrogen tablet which supplies a 1.5 mg/pellet and 60-day release under the neck skin.

2.2.8 Protocol for immunohistochemistry

The lesions isolated from the mice were washed in PBS and fixed more than 24 h in 4% PFA/PBS at 4 °C, then dehydrated and embedded in paraffin. We cut the paraffin block into slides of 4 µm. Sections were collected on Superfrost-plus slides (Thermo, Germany) and dried in 60 °C for 1 h. The slides were kept at room temperature for later staining. Paraffin sections were deparaffinized

and rehydrated by immersion in xylene (3X5 min), 100% isopropanol (3X5min), 100% ethanol (1X5 min), 96% ethanol (1X5 min), 70% ethanol (1X5 min), dH_2O (1X5 min). Antigen retrieval was done by heating the citrate buffer (pH=6) with 0.1% tween 20 in a slide container in the microwave for at least 20 min. The slide was cool ed down at room temperature for 20 min and then incubated for 2X5 min with TBS+0.1% tween 20 (TBST). After blocking with 10% goat serum in TBST for 30 min at room temperature, slides were drained for a few second. Sections were incubated overnight with the primary antibody (rabbit polyclonal antibody against human GHRHR, 1:200 diluted in blocking serum) at 4 °C in humidified container, washed for 2X5 min with TBST, followed by the application of the secondary antibody (goat anti rabbit, 1:200 diluted in blocking serum) for 1 h at room temperature. Slides were washed for 2x5 min with TBST and the streptavidin-alkaline phosphatase reagent (1:250 diluted in TBST) was applied to the slides and incubated for 20min, washed for 2x5 min with TBST. Substrate chromogen “Liquid Permanent Red” (DAKO) was applied to the slides as directed by the manufacturers´ manual. The reaction was stopped by washing slides in TBST as soon as the desired color intensity had developed. The slides were counterstained for 3 min in hematoxylin, then rinsed in softly running tap water to develop counterstain colour. The slides were incubated in increasing concentrations of ethanol: 3 min each in 70%, 96%, 100% ethanol, and 3 min in xylene. When the tissue sections were dried, 2 drops of mounting medium were applied to tissue section followed by placing a cover slip. Slides were set at room temperature for 2 h on a flat surface and microscoped. Omission of the primary antibody on parallel sections was used as a negative control.

2.2.9 Statistical Analysis

Results are expressed as mean ± SE. Statistical analysis were performed using Student t-test or one-way analysis of variance. IBM-SPSS Version 19 (IBM-Germany) was used for statistics and significance was established when $P<0.05$.

3 Results

3.1 *In vitro* study

3.1.1 Expression of GHRHR in cell lines

In order to establish a test for the mRNA expression of the genes encoding GHRHR, we performed RT-PCR of full-length GHRHR (pituitary GHRHR, pit-GHRHR) and SV1 of the GHRHR in the cell lines from triple-negative breast carcinoma HCC1806 and MDA-MB-231, from chorion carcinoma JEG3 as well as OVCAR-3 from ovarian carcinoma. The length of the amplified products is 293 bp for pit-GHRHR, 103 bp for SV1, and 84 bp for HPRT which was used as a housekeeping gene. We could detect pit-GHRHR and SV1 and mRNA in all cell lines named above (**Fig. 7**).

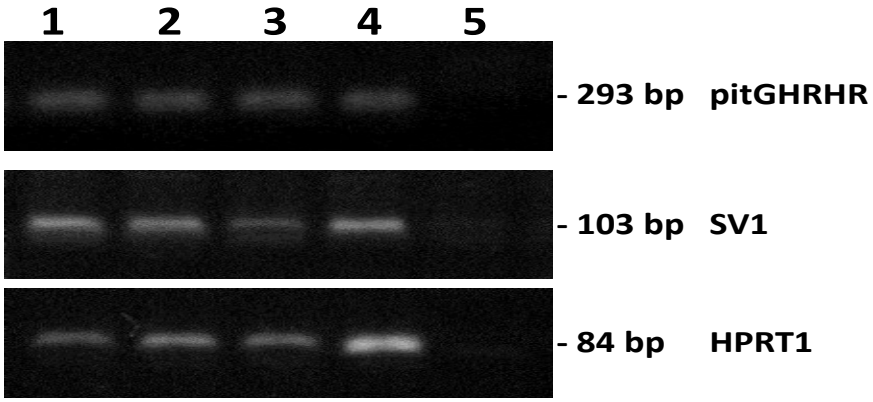

Figure 7: RT-PCR analysis of the pituitary GHRHR (pit-GHRHR) and the splice variant 1 (SV1) of the GHRHR. An aliquot of 2 µg RNA was reverse transcribed to cDNA. PCR for full-length GHRH receptor (pit-GHRHR) cDNA and SV1 cDNA were performed. PCR products were analyzed by electrophoresis on 2% agarose gel and photographed under UV-light. As expected, the amplified products corresponded to 293 bp for pit-GHRH-R, 103 bp for SV-1, and 84 bp for HPRT, which was amplified as control gene to ensure equal loading. mRNA expression of SV1 and pit-GHRHR could be detected in cell line HCC1806 (lane 1), JEG3 (lane 2), MDA-MB-231 (lane 3), and OVCAR-3 (lane 4). A negative control was run in lane 5.

To establish the detection of pit-GHRHR and SV1 protein, we analyzed the previously used cell lines on a western blot. The antibody used can identify both SV1 and GHRHR (Schulz S and Rocken C, 2006). Previous studies based on western blot assays have shown that bands at 47 kDa and 39 kDa are corresponding to pit-GHRHR and SV1 (Havt A *et al.,* 2005). We observed a signal for the

protein expression of SV1 and pit-GHRHR in all examined cell lines. In OVCAR-3, the protein expression of SV1 is stronger than that of pit-GHRHR (**Fig. 8**).

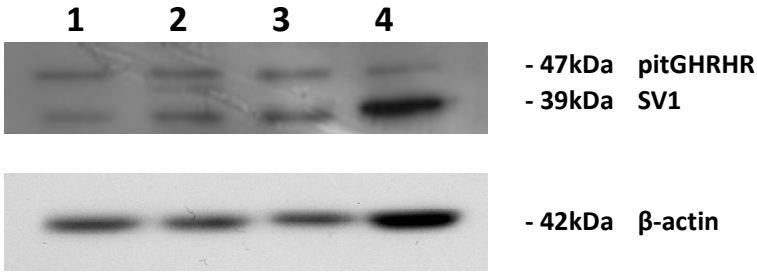

Figure 8: Western blot analysis of pit-GHRHR and SV1 expression in the cell lines HCC1806 (lane 1), JEG3 (lane 2), MDA-MB-231 (lane 3) and OVCAR-3 (lane 4).

3.1.2 Effect of GHRH antagonist MIA-602 on the proliferation of tumor cell lines

As is well known, the GHRH antagonists have an antiproliferative effect in a variety of cell types. In our study, we investigated the effects of the GHRH antagonist MIA-602 on proliferation by MTT-Proliferation Assay. The cells were incubated for 72 h in medium supplemented with graded concentrations of MIA-602 (from 1 µmol/L to 0.01 µmol/L). Controls were treated with medium only. The solvent group (Solv) was treated with medium containing 0.01% DMSO, which corresponds to the concentration of DMSO in 1 µmol/L MIA-602. Compared with the control group (medium only), a concentration of 1 µmol/L MIA-602 in the media significantly inhibited proliferation of all cell lines. When treated with 0.01 µmol/L MIA-602, a significant inhibition was only seen in HCC1806 cells. In the cell line JEG3, the solvent (DMSO) was also able to inhibit proliferation. The strongest inhibitory effect was observed at 1 µmol/L MIA-602 (**Fig. 9**).

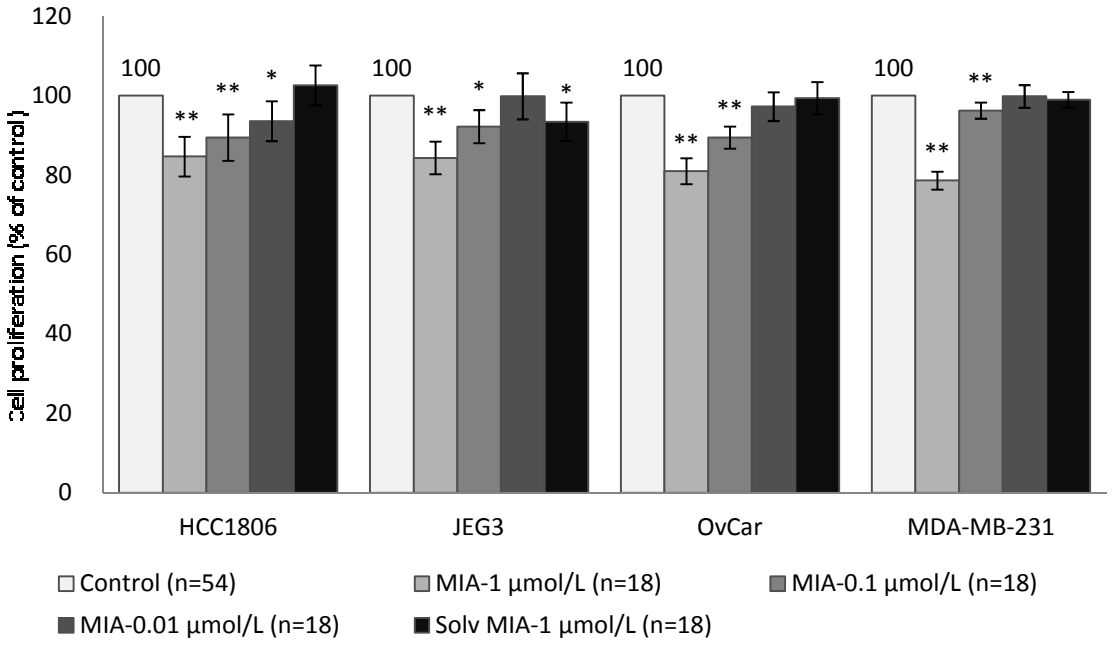

Figure 9: Effect of the GHRH antagonist MIA-602 on cell line proliferation, assessed by MTT-test. The cells were incubated for 72 h with serum replacement I and with graded concentrations of MIA-602 (from 1 µmol/L to 0.01 µmol/L) or with medium only (Control). The solvent group (Solv) was treated with 0.01% DMSO, corresponding to the concentration of DMSO in 1 µmol/L MIA-602. Data, expressed as percent of control, represent the mean ± SE of three independent experiments. Each experiment was performed in sextuplicate except controls, which were run three more times. *P<0.05; ∗∗P<0.01; vs. control.

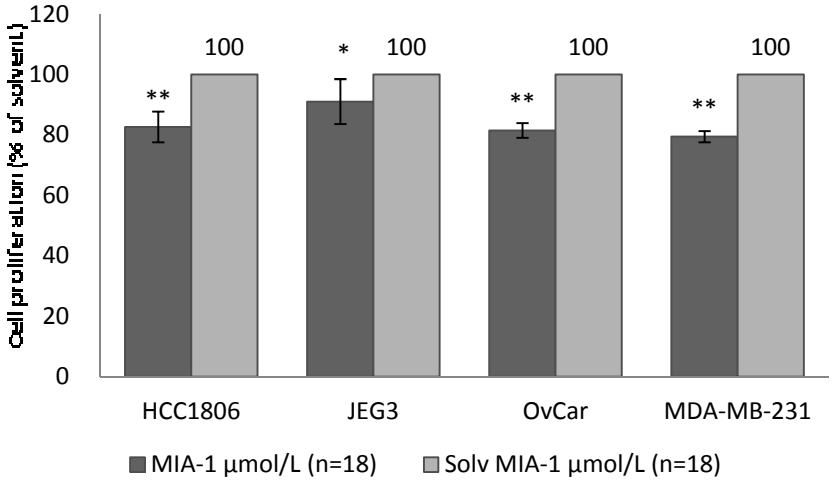

Figure 10: Comparison of the effect of 1 µmol/L MIA-602 to its solvent on the proliferation of cell lines, assessed by MTT-test. The solvent group (Solv) was treated with 0.01% DMSO, corresponding to the concentration in 1 µmol/L. Data, expressed as percent of control, represent the mean ± SE of three independent experiments. Each experiment was performed in sextuplicate except controls, which were run three more times. *P<0.05; ∗∗P<0.01; vs. control.

Compared with the solvent group (0.01% DMSO in incubation medium), 1 µmol/L MIA-601 showed significant antiproliferative action (**Fig. 10**).

3.1.3 Expression of GHRHR in endometrial tissues

We analysed the protein expression of pit-GHRHR and SV1 GHRHR in eutopic endometrial tissues from women with or without endometriosis in western blot and observed that SV1 and pit-GHRHR were expressed in endometrium from women both with and without endometriosis. Through all the samples the extent of GHRHR expression was differing very much, regardless of diagnosis. In nearly all samples SV1 exhibited stronger expression than pit-GHRHR (**Fig. 11**).

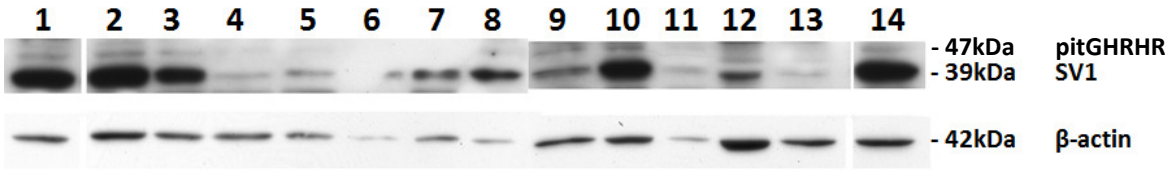

Figure 11: Western blot analysis of pit-GHRHR and SV1 expression in endometrium tissues. Lane 1-10: eutopic endometrium from different endometriosis patients; lane 11-14: eutopic endometrium tissue from women without endometiosis.

3.1.4 Expression of GHRHR in EESCs

We isolated and cultured the primary eutopic endometrial stromal cells (EESCs) of endometrium tissues from patients with endometriosis (**Fig. 12**).

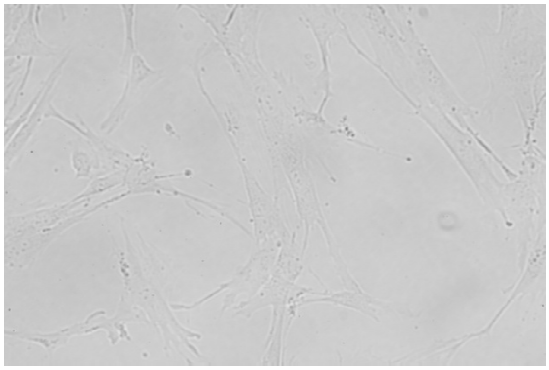

Figure 12: The primary endometrial stromal cells (EESCs) were isolated from the endometrium tissues from patients with endometriosis and incubated in RPMI medium supplemented with 10% FCS, penicillin G (100 U/ml), streptomycin (100 µg/ml) and gentamicin (100 µg/ml) in a humidified atmosphere at 37 °C in 5% CO_2.

Full-length GHRHR (pit-GHRHR) and SV1 mRNA expression was detected in primary EESCs isolated from eutopic endometrium from endometriosis patients (n=8) by RT-PCR. SV1 and pit-GHRHR mRNA was detected in all EESCs although, in some samples, pit-GHRHR was expressed at very low levels (**Fig. 13**).

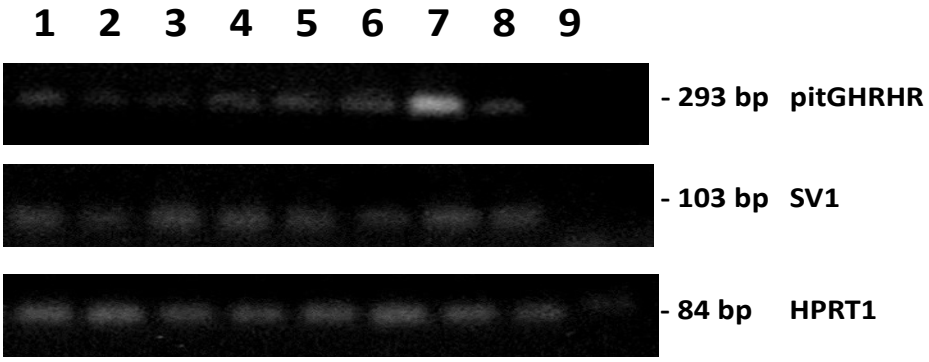

Figure 13: RT-PCR analysis of the expression of pit-GHRHR and the splice variant 1 (SV1) of the GHRHR gene in EESCs from eutopic endometrium of patients with endometriosis. An aliquot of 2 µg RNA was reverse transcribed to cDNA. Expression of Full-length GHRH-R (pit-GHRHR) cDNA and SV1 cDNA was analysed. PCR product size was verified by electrophoresis on 2%7 agarose gel. As expected, the amplified products corresponded to 293 bp for pit-GHRH-R, 103 bp for SV-1, and 84 bp for HPRT, which was amplified as control gene to ensure equal loading. Lanes 1-8: EESCs from patients of endometriosis; lane 9: negative control.

We analyzed the protein expression of pit-GHRHR and SV1 in the EESCs from eutopic endometrium of endometriosis patients by western blot and we observed that, in one case, SV1 was expressed stronger in tissue than in EESCs although the expression of pit-GHRHR is similar in these two samples (**Fig. 14**).

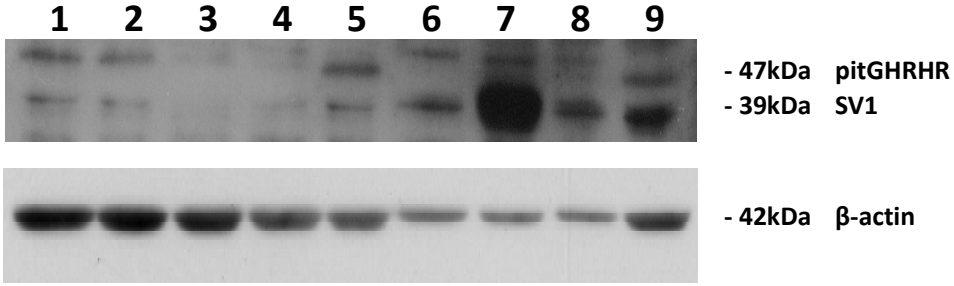

Figure 14: Western blot analysis of pit-GHRHR and SV1 expression. Lane 1-6, 8, 9: EESCs from eutopic endometrium from different endometriosis patients; lane 7: the eutopic endometrium tissue from the same patient of lane 6. SV1 is expressed stronger in tissue than in EESCs although the expression of pit-GHRHR is similar in these two samples.

3.1.5 Effect of GHRH antagonist MIA-602 on EESCs proliferation

Furthermore, we investigated the effects of the GHRH antagonist MIA-602 on the proliferation of EESCs from endometriosis patients by MTT-proliferation assay. The EESCs were incubated in the presence of serum (FCS) throughout the whole assay. For 72 h, the cells were treated with graded concentrations of 1 µmol/L to 0.01 µmol/L MIA-602 in culture medium and controls were incubated in plain medium. The solvent group (Solv) was treated with 0.01% DMSO, corresponding to the concentration of DMSO in 1 µmol/L MIA-602.

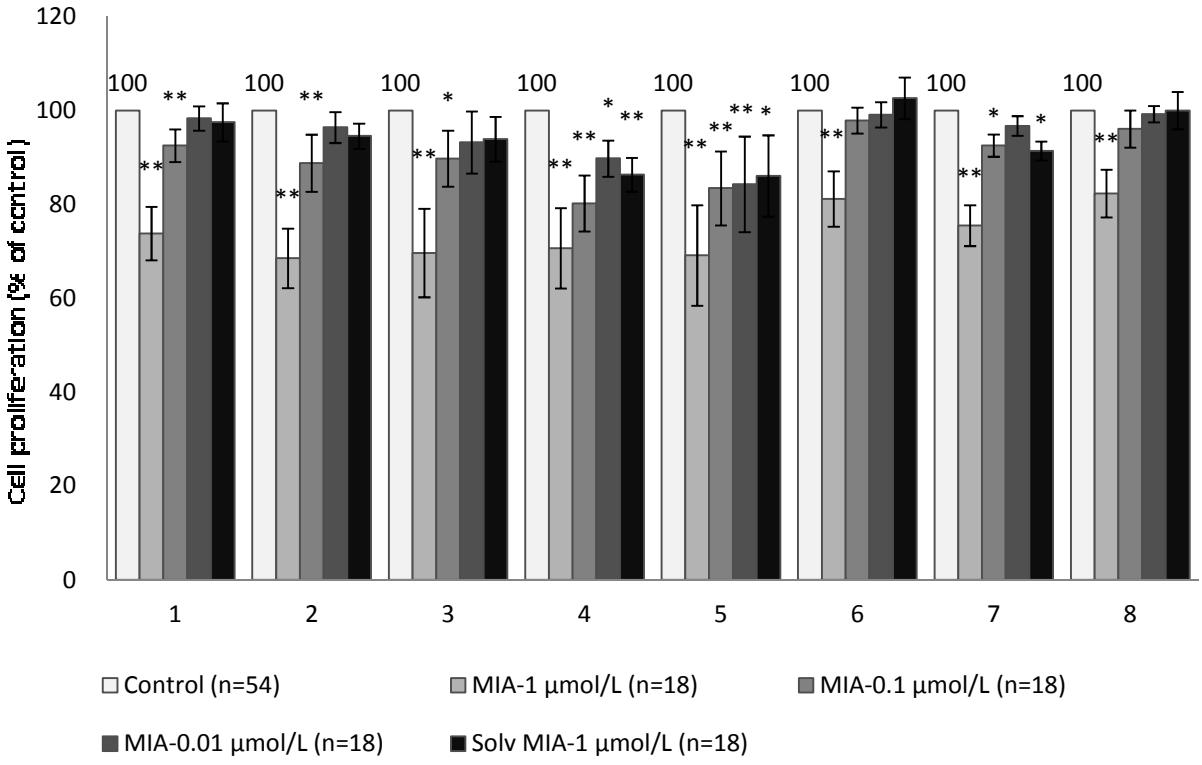

Figure 15: Effect of the GHRH antagonist MIA-602 on proliferation of EESCs from endometriosis patients, assessed by MTT-test. In contrast to cancer cell lines, EESCs were incubated in the presence of serum throughout the whole assay. The cells were incubated for 72 h in medium with graded concentrations of MIA-602 (1 µmol/L to 0.01 µmol/L,), and the solvent group (Solv) was treated with 0.01% DMSO, corresponding to the concentration of DMSO in 1 µmol/L MIA-602. Data, expressed as percent of control, represent the mean ± SE of three independent experiments. Each experiment was performed in sextuplicate except controls, which were run three more times. *P<0.05; ∗∗P<0.01; vs. control.

Compared with the control group, the proliferation of EESCs was inhibited at all concentrations, though not always significantly. In detail, 1 µmol/L MIA-602 significantly inhibited proliferation in all EESC cultures, 0.1 µmol/L MIA-602 significantly inhibited proliferation in 6 of 8 EESC cultures, and 0.01 µmol/L MIA-602 significantly inhibited proliferation in only 2 of 8 EESC cultures. The solvent DMSO significantly inhibited the proliferation in 3 of 8 EESC cultures. (**Fig. 15**).

Compared with the solvent group (incubation medium containing 0.01% DMSO), a concentration of 1 µmol/L MIA-602 exhibited significant antiproliferative activity (**Fig. 16**).

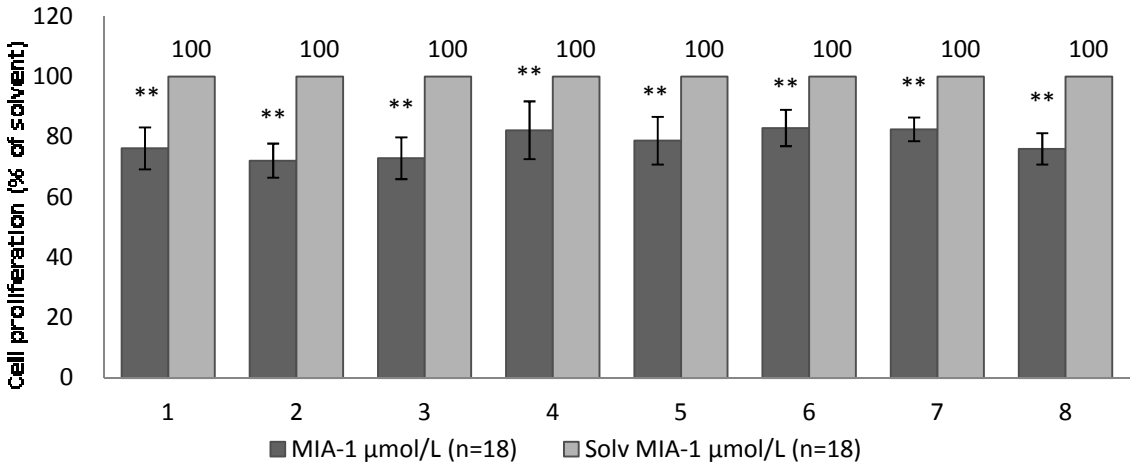

Figure 16: Comparison of the effect of 1 µmol/L MIA-602 with its solvent on the proliferation of EESCs from endometriosis patients, as assessed by MTT-test. The cells were incubated for 72 hours in culture medium containing FCS and graded concentrations of MIA-602 (1 µmol/L to 0.01 µmol/L), and the solvent group (Solv) was treated with culture medium containing FCS and 0.01% DMSO, corresponding to the concentration of DMSO in 1 µmol/L MIA-602. Data, expressed as percent of control, represent the mean ± SE of three independent experiments. Each experiment was performed in sextuplicate. *P<0.05; ∗∗P<0.01; vs. solvent.

3.2 *In vivo* study

3.2.1 Effect of GHRH antagonist MIA-602 on growth of endometrial implants

In this *in vivo* model, human endometrial tissue was implanted in nude mice and allowed to grow for 2 weeks before starting treatment. We sutured the human endometrial fragments to intraperitoneal sites to ensure equal number and size of the transplanted lesions recovered in each treated mouse (Grummer *et al.*, 2001).

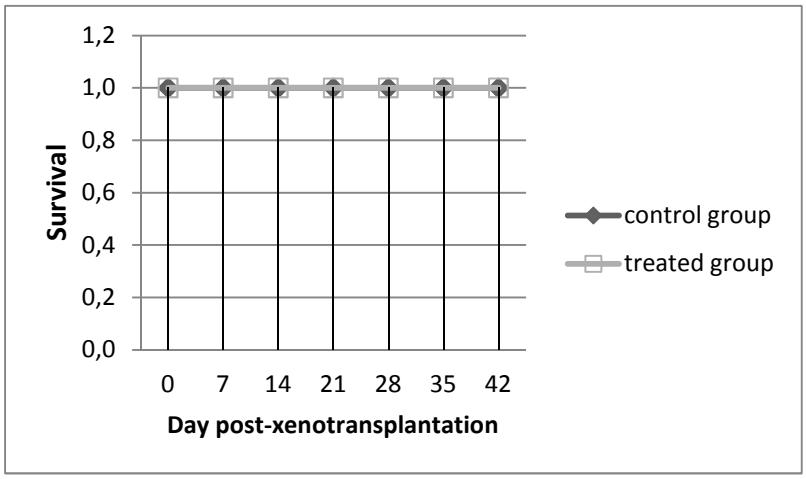

Figure 17: Effect of endometrial tissue implantation and treatment with MIA-602 on the survival rate of animals. All mice survived after surgery and treatment with 5 ug/day MIA-602 (treated group) and vehicle (control group). The treatment commenced on day 14 *post-xenotransplantationem*.

All mice survived throughout the whole *in vivo* study (**Fig. 17**) and no macroscopic changes of the heart, liver, spleen, intestines, or kidneys of tested animals were detected. After 28 days treatment, we sacrificed the mice and measured the lesions (**Fig. 18**).

Compared with control mice treated with the solvent alone, mice treated with MIA-602 had significantly smaller endometriotic lesions (**Fig. 19**). We have not implanted estrogen tablets in the first 18 mice. When we found some lesions to be rather small (≤0.5 mm^3) (**Fig 18** b and f) we decided to implant estrogen tablets in the last group of eight mice, as has been done in some previous *in vivo* studies on endometriosis to increase the intake rate and growth of the lesions (Fortin M *et al.* 2004). As a result, lesions were of greater size in those 8 mice which were subcutaneously implanted with estrogen tablets (**Fig. 18** c, d, g, h), however, the difference in lesions size between controls and treated mice was still significant **(Fig. 19** B).

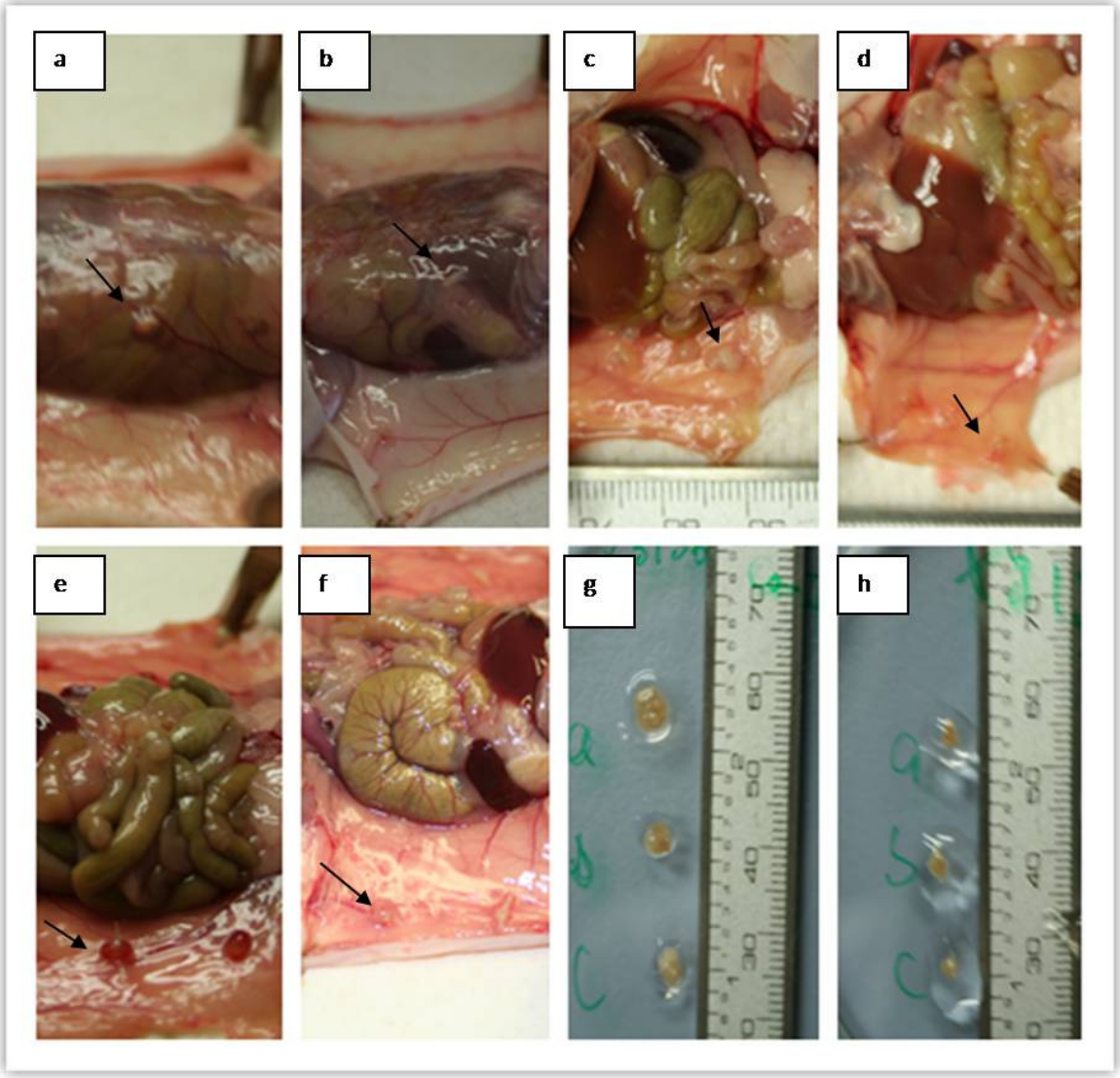

Figure 18: Exposure and measure of endometrial implants. a), b): observation of the lesions from outside the peritoneal cavity. c), d), e), f): Implants, as observed in mice treated with vehicle (c, e) or MIA-602 (d, f). Mice were implanted with (c, d) or without (e, f) estrogen. g), h): the size of the lesions in mice treated with vehicle (g) or MIA-602 (h). The black arrows are pointing to the endometrial implants.

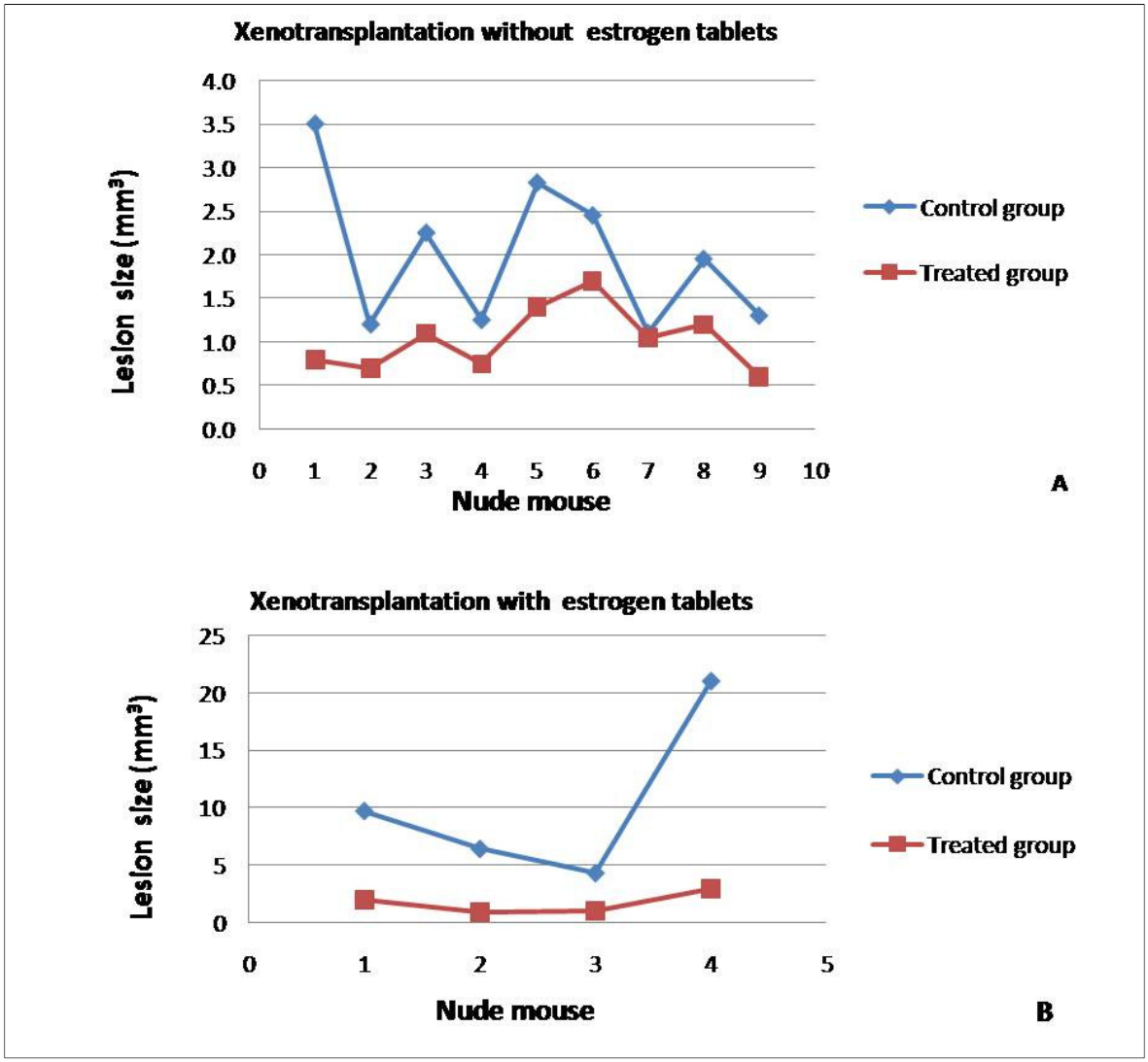

Figure 19: Effect of MIA-602 on endometrial implant size. A): lesions from mice without estrogen treatment. B): lesions from mice treated with estrogen. Statistical analyses showed, independent of estrogen treatment, a significant decrease in the lesion sizes in MIA-602-treated mice compared with respective vehicle-treated controls ($P<0.01$). It was also shown that lesion sizes of controls were bigger in estrogen-treated mice than estrogen-free mice.

3.2.2 Microscopy of sections from endometriotic implants in mice after treatment

Human endometriotic lesions after xenotransplantation and treatment in mice were fixed in paraformaldehyde and embedded in paraffine. Serial sections were stained with hematoxylin and eosin and morphologically evaluated. The implanted tissue displayed endometrial stromal and glandular structures surrounded by mouse tissue as shown in **Figure 20**.

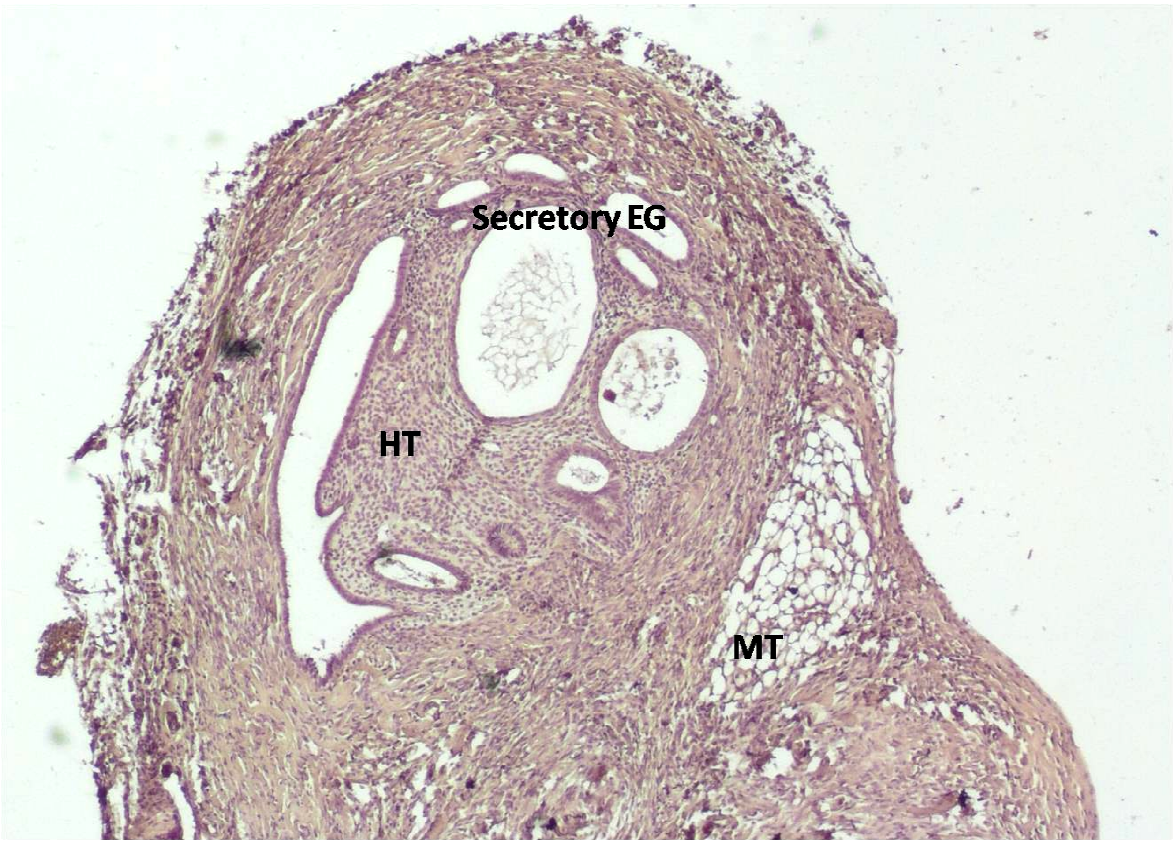

Figure 20: Microscopic observation and histological examination of endometrial implants. The epithelial glands were surrounded by stromal cells, and the human tissue was surrounded by mouse tissue. EG: endometrial gland; HT: human tissue; MT: mouse tissue.

A specific GHRHR antibody was applied to demonstrate immunoreactivity in the cytoplasm of stromal cells. In the epithelial cells surrounding the endometrial glands immunostaining was also found (**Figure 21** A**)**. No immunoreaction was observed in the negative controls without using the primary antibody, as shown in **Figure 21** B.

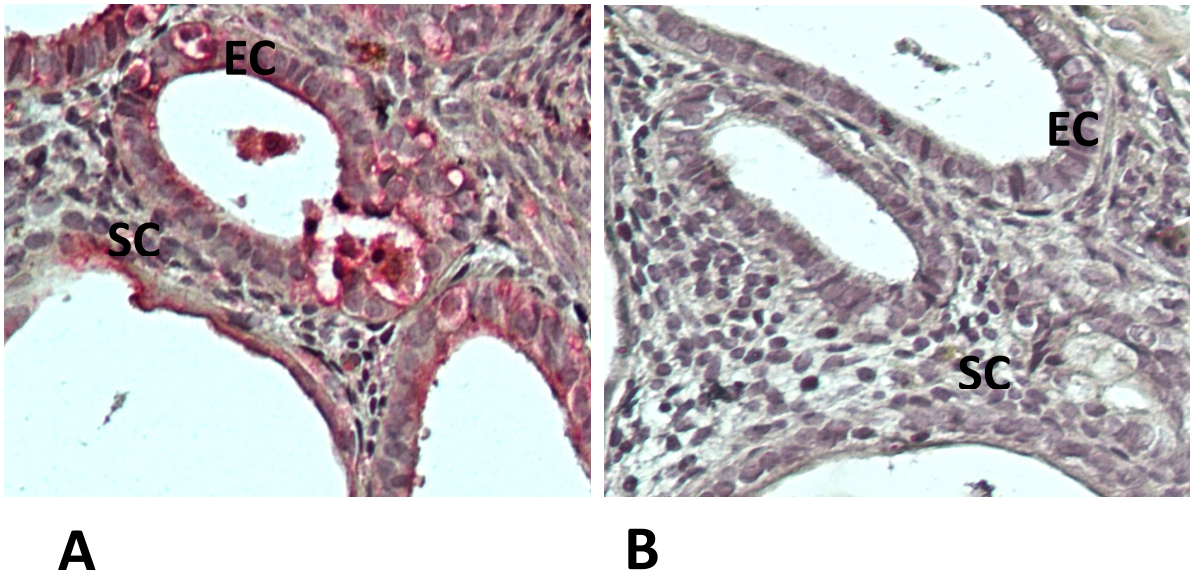

Figure 21: Expression of GHRHR detected in endometriotic lesions by immunohistochemistry. A) Positive control; B) Negative control. The epithelial cells (EC) encircling the endometrial glands are surrounded by stromal cells (SC). GHRHR expression was detected in epithelial cells and, with lesser intensity, in the stromal cells of the sections from endometrial lesions (A). No immunoreaction was observed in the negative control omitting the primary antibody (B).

4 Discussion

4.1 Overview of experimental results

In this study, we detected the protein expression of both SV1 and pit-GHRHR in endometrium from women with or without endometriosis. Further, we observed the expression of SV1 and pit-GHRHR in all tested EESCs not only by western blot analysis but also by RT-PCR. At last, GHRHR was detected in the endometrial lesions grown in xenografted mice. We were using MIA-602, a new and highly potent GHRH antagonist to investigate its inhibitory effect in endometriosis. Our results demonstrate that this antagonist of GHRH is able to inhibit endometrial growth both in vitro and in vivo at low doses of 1 μmol/L and 10 μg/day, respectively.

4.2 Detection of the expression of pit-GHRHR and SV1

We analysed the mRNA and protein expression of pit-GHRHR and SV1 of the GHRHR in the cell lines HCC1806, JEG3, MDA-MB-231, OVCAR-3 using RT-PCR and western blot methods in order to prove their suitability for the use in endometrial cells and tissues. We detected both mRNA and protein expression of SV1 and pit-GHRHR in all examined cell lines, thus confirming the results of previous studies (Schally AV *et al.*, 2008; Köster F *et al.*, 2009).

We isolated and cultured primary EESCs from endometrium of patients with endometriosis, in all of which we subsequently detected gene as well as protein expression of pit-GHRHR and SV1 (n = 8). Previously, Fu L *et al.* detected a low rate of SV1 mRNA in eutopic (26%) and ectopic (24%) endometrial tissues from patients with endometriosis (Fu L *et al.*, 2008), but the pit-GHRHR mRNA was not examined. In contrast, Annunziata M *et al.* detected pit-GHRHR and SV1 mRNA in ectopic ESCs (Annunziata M *et al.*, 2009). The GHRHR protein expression has been previously demonstrated in human endometrium (Gallego R *et al.*, 2005). In our study, both the expression of SV1 and pit-GHRHR were observed in all tested EESCs, although it was extremely weak in some samples. This very weak expression may be the reason, why others have been able to detect GHRH-R expression only in a small percentage of tissues. In our western blot assay using a highly

specific and sensitive antibody we were able to detect smallest amounts of GHRHR in different endometrium tissue samples.

4.3 Growth inhibitory effect of MIA-602 *in vitro*

Previous studies demonstrated that either SV1 or pit-GHRHR mediated the antiproliferative effects of GHRH antagonists with most of them examined in tumor cell lines or xenotransplanted human tumors in mouse models (Schally AV *et al.*, 2008). In regard to endometriosis Annunziata M *et al.* demonstrated that the GHRH antagonist JV-1-36 inhibited ectopic ESC proliferation and survival. However, they did not run a control with DMSO alone. In our study, we investigated the antiproliferative effect of the GHRH antagonist MIA-602 on cell lines and EESCs by MTT-Proliferation Assay. MIA-602 is a novel GHRH antagonist synthesized in Professor Schally's laboratory and shows more potent antitumor effect than previous analogues (Varga JL *et al.*, 2004; Klukovits A *et al.*, 2012). There is no previous study to show its inhibitory effect in endometriosis. Annunziata had demonstrated the inhibitory effect of the previous GHRH antagonist JV-1-36. Since MIA-602 exhibits a more potent antitumor effect in human tumor cell lines, it may also exert more potent inhibition in endometriosis. To corroborate this assumption, we conducted the in vitro and vivo study to demonstrate the inhibition of MIA-602 in endometriosis.

The MTT-proliferation assay formed part of the in vitro study. After 72h treatment with MIA-602, the proliferation of human tumor cell lines and primary EESCs from endometrium tissue of patients with endometriosis receded visibly. Compared with the control group (incubated in medium only), MIA-602 inhibited the proliferation of all cell types at 1 µmol/L concentration, and of all cell lines and the majority of EESCs at 0.1 µmol/L. This antiproliferative effect was also detected in a minority of cells treated with 0.01 µmol/L. The best inhibitory effect was observed at 1 µmol/L concentration which decreased the proliferation of all tested cells by more than 15%. This inhibitory effect of MIA-602 dissolved in DMSO on the proliferation of cell lines and EESCs is dose-dependent. However, we demonstrated that DMSO alone in a concentration equivalent to dissolve 1 µmol/L MIA-602 could suppress the proliferation of several sample EESCs equally well. Dimethyl sulfoxide (DMSO) is an organosulfuric compound with the formula (CH3)2SO. It is an

amphipathic molecule with a highly polar domain and two non-polar methyl groups, rendering it soluble in both aqueous and organic media. DMSO is one of the most common solvents for the in vivo and in vitro administration of several water-insoluble substances. Despite being frequently used as a solvent in biological studies and as a vehicle for drug therapy, DMSO by itself is of low toxicity. Some previous studies have demonstrated that DMSO exerted an inhibitory effect on the proliferation of a variety of cell types (Eter N *et al.*, 2002; Ece Simsek Öz *et al.*, 2012). Conversely, in some other studies a DMSO control was run showing no inhibition (Moreira AL *et al.*, 1999; Bauer KS *et al.*, 1998). The effect might be species specific and caused by the action of one of its metabolites. In our study, the antiproliferative effect of MIA-602 solution appears to be at least in part attributed to DMSO alone. To segregate the influence of DMSO, we compared the inhibitory effects of 1 μmol/L MIA-602 and 0.01% DMSO (the exact amount contained in 1 μmol/L MIA-602) and the difference is statistically significant in all cells. These data demonstrate that MIA-602 by itself has the antiproliferative effect.

GHRH antagonists affect some of the signaling mechanisms involved in cell proliferation, survival, and metastasis, and activate proapoptotic signaling mechanisms. GHRH antagonists could exert the inhibitory effect through indirect or direct pathways (Schally AV *et al.*, 1998). The indirect mechanism would operate through suppression of GHRH-evoked GH release from the pituitary and the resulting inhibition of the production of IGF-I. The antiproliferative effects of GHRH antagonists can be also mediated through direct mechanisms. One of these mechanisms is based upon the inhibition of the secretion of autocrine/paracrine IGF-I or IGF-II from the tumors, while probably the most influential pathway involves the blockade of action of autocrine GHRH. Endometriosis is one of the most common benign gynecologic disorders of women in reproductive age. Previous findings suggested that GHRH may play a role in the development of endometriosis (Khorram O *et al.*, 2001). GHRH binding to GHRHR results in GH production mainly by the cAMP dependent pathway, but also by the phospholipase C pathway (IP3/DAG pathway) (Frohman LA *et al.*, 1999; Silverman BL *et al.*, 1988; Vasilatos-Younken R *et al.*, 1992; Radecki SV *et al.*, 1994). The mitogenic effect of GHRH is also mediated by a protein encoded by splice variant 1 (SV1), a splice variant of the GHRH receptor, which displays greatt similarity to the pituitary GHRH receptor. Various previous findings (Barabutis N and Schally AV 2008a; Hohla F *et al.* 2008; Fu L *et al.* 2009) have enhanced the important role of the SV1 receptor.

4.4 Growth inhibitory effect of MIA-602 *in vivo*

There are several previous *in vitro* studies demonstrating the antiproliferative effect of GHRH antagonist, some of which are dealing with endometriosis. Inhibitory effects of GHRH antagonists have been evaluated in *in vitro* studies and in a variety of human cancer models in nude mice (Schally AV and Varga JL, 1999; Schally AV and Varga JL, 2006; Varga JL and Schally AV, 2006). As yet, there exists no *in vivo* study evaluating the inhibitory effect of GHRH antagonist in endometriosis mice models. Fortunately, models have been developed by implanting human endometrial tissue instead of autologous endometrium into immunodeficient mice because causal factors for the development and maintenance of endometriosis, which may not exist in the rodent endometrium, could originate in the human endometrium itself. Because it is based on human endometrium, the heterologous mouse model for endometriosis represents a promising tool for experimental approaches not only for evaluating the aetiology of this disease but also for therapeutic testing of pharmacological and hormonal modulations. Regulatory mechanisms, for example, after drug administration, can be evaluated in the human ectopic tissue in an *in vivo* situation and long-term studies that are not feasible in women can be performed (Grümmer R, 2006).

In this study, we used an *in vivo* model of experimentally induced endometriosis where human endometrial tissue was implanted into immunodeficient mice and allowed to grow prior to treatment. The development of endometriotic lesions involves critical steps including the capacity of the migrating endometrial tissue to survive and resist apoptosis, attach to and invade the host tissue, to proliferate and activate the host's angiogenic responses. We dissected endometrial tissues into five small pieces (~2×1×1 mm^3) per mouse and sutured the fragments to intraperitoneal sites to ensure complete recovery. This method is, with a view to drug testing, preferable to the inoculation of tissue as it improves the comparability of drug effects. (Grümmer R *et al.*, 2001). Once we found, upon recovery, some lesions to be rather small in size (≤0.5 mm^3), we tried to simultaneously improve growth conditions by subcutaneous implantation of estrogen tablets in 8 individuals. Beliard A *et al.* demonstrated that pretreatment of human endometrial cells with estrogen before injection into the peritoneal cavity of nude mice resulted in a higher rate of animals developing endometriotic-like lesions (Beliard A *et al.*, 2002). A significant

reduction in lesion size in estrogen-treated nude mice compared to estrogen-untreated individuals (both models ovariectomized) was observed using optical *in vivo* imaging (Fortin M *et al.*, 2004). Upon exposure, the estrogen-treated lesions appeared to be bigger than those untreated, but the sample size is too small for statistics. Our research group will continue this study and enlarge sample size to corroborate these findings. Otherwise, the lesions size in the treated group was compared to the control group, and a significant reduction was observed independent of estrogen supplementation

Our data show that MIA-602, an analogue of GHRH antagonist, inhibits human endometrial tissue growth in the peritoneal cavity of xenografted nude mice. Klukovits A *et al.* have tested MIA-602 at a dose of only 5 μg daily and it inhibited the growth of OVCAR-3 and SKOV-3 xenografts in nude mice by 70% and 77%, respectively (Klukovits A *et al.*, 2012). They treated the mice for 7 weeks, and there was no weight loss or macroscopic changes of the heart, liver, spleen, intestines, or kidneys in the animals tested. In our study, we treated the mice with a daily dose of 10μg for 28 days, and all mice survived treatment in good health. There were no macroscopic changes in the major organs of the mice tested. These results suggest that these new GHRH antagonists are not likely to be toxic and this further supports the possibility of clinic application of MIA-602 as a new therapy for endometriosis.

4.5 Immunostaining of GHRHR in endometrial lesions

In the following immunohistochemistry assay, we stained slides first with hematoxylin. Implanted tissue displayed endometrial stromal and glandular structures surround by mouse tissue. GHRHR immunoreactivity was detected in the cytoplasm of endometrial glands and weak immunostaining was found also in stromal cells. A specific polyclonal antibody was used and positive as well as negative controls were included in all assays in accordance with the manufacturer's recommendations. No positive immunoreaction was observed in the negative controls which fact confirmed the specificity of the primary antibody and the technique. These results support our theory that the inhibitory effect of the GHRH antagonist is due to its binding onto the GHRHR.

In conclusion, we detected the expression of both SV1 and pit-GHRHR in all tested EESCs. We also detected the expression of GHRHR in the endometrial lesions. Our results demonstrate that

MIA-602 inhibits endometrial growth both *in vitro* and *vivo* in low doses of 1 µmol/L and 10 µg/day, respectively. As GHRH antagonists operate in different pharmacological ways, they might be combined with other drugs for an enhanced antiproliferative effect. Further studies are required with increased sample size to further define the inhibitory action of GHRH antagonists in endometriosis.

4.6 Conclusion and prospects

In conclusion, the present study shows that the GHRH antagonist MIA-602 inhibits endometrial growth both *in vitro* and *in vivo* in low doses of 1 µmol/L and 10 µg/day, respectively. Both receptor types expected, the SV1 and the pit-GHRHR, were found to be expressed in all tested EESCs and endometrial tissues. GHRHR expression was also detected in xenotransplanted endometriotic lesions 6 weeks after implantation. GHRH has been shown to induce cAMP production in cells expressing GHRHR and SV1 (Fu L *et al.*, 2008). Other GHRH antagonists were found to decrease intracellular cAMP levels and to counteract GHRH-induced cAMP increase in cancer cells (Schally AV *et al.*, 2008). A recent study showed cAMP levels to be significantly reduced in a human cell line of immortalized ESCs after treatment with the GHRH antagonist JV-I-36 (Annunziata M *et al.*, 2009).

Because GHRH antagonists operate according to different pharmacologic principles, they might be combined with other drugs for an enhanced antiproliferative effect. Further studies are required with increased sample size and numbers of tested animals to ascertain this inhibitory activity of GHRH antagonists in endometriosis along with the underlying mechanisms. Furthermore, the possible toxicity of MIA-602 should be better evaluated to ensure its safety for potential clinical studies. These data would be of great importance with a view to the possible clinical application of MIA-602 as a new targeted therapy for endometriosis.

5 Summary

Endometriosis, a benign chronic gynecological disease, is prevalent in approximately 10% of women of childbearing age. It is defined by the presence of endometrial-like tissue outside the uterine cavity. Although many therapies have been successfully applied, endometriosis still remains an enigmatic disease. New promising agents are being designed to prevent or inhibit the development of endometriosis. Recently, some studies suggested that growth hormone-releasing hormone (GHRH) may play a role in the development of endometrial cancers and endometriosis. In consequence, GHRH antagonists might be useful as therapeutic reagents to target endometriotic cell growth. In our study, the expression of pituitary type (pit-GHRHR) and splice variant 1 (SV1) of GHRH receptor was detected by RT-PCR and western blot analysis in all tested primary eutopic endometrial stromal cells (EESCs) isolated from endometrium of endometriosis patients. We also detected the GHRHR immunoreactivity both in epithelial and stromal cells of endometriotic lesions growing in xenografted mice. These results suggest that the inhibitory effect of the GHRH antagonist could be due to its binding onto the GHRH-receptor (GHRHR). In MTT-proliferation assays, the proliferation of EESCs declined after 72 h treatment with increasing concentrations of MIA-602 from 0.01 µmol/L to 1 µmol/L in a dose-dependent manner. At 1 µmol/L the inhibitory effect of MIA-602 was statistically significant in all cells. These data demonstrate that MIA-602 by itself has an antiproliferative effect in endometrial stromal cells. We established the endometriosis mouse model by suturing five human endometrial fragments to intraperitoneal sites of nude mice (n=26). Estrogen tablets were subcutaneously implanted into 8 out of 26 mice to promote lesions growth. Starting on day 14 post-implantation, a daily intra-peritoneal injection of 10 µg MIA-602 over 28 days (treated group, n=13) significantly reduced the size of the lesions compared to the injection of placebo solvent (control group, n=13) over the same period. All mice survived surgical and therapeutical proceedings and were in good health throughout the whole study. This is the first time that the inhibitory effect of a GHRH antagonist was evaluated in a xenotransplantation mouse model. These results suggest the potential use of MIA-602 as a new therapeutic agent for endometriosis.

5.1 Zusammenfassung in deutsch (übersetzt von PD Dr. rer. nat. Frank Köster)

Die Endometriose ist eine benigne chronische gynäkologische Erkrankung, die bei ca. 10 % aller Frauen im Kinderwunschalter auftritt. Sie ist durch das Auftreten von endometrialem Gewebe außerhalb der Uterushöhle definiert. Obwohl einige erfolgreiche Therapieoptionen existieren, ist diese Erkrankung für die meisten Betroffenen weiterhin ein großes Problem. Neue, Erfolg versprechende Medikamente werden entwickelt, um die Entstehung und das Fortschreiten der Endometriose zu verhindern. In zwei aktuellen Studien wurde die mögliche Rolle des Growth Hormone-Releasing Hormons (GHRH), welches bei der Entwicklung einer Reihe von Tumoren involviert ist, bei Endometriose untersucht. Darin wurde gefolgert, dass der Einsatz von GHRH Antagonisten eine sinnvolle gezielte Therapieoption zur Verhinderung des ektopen Wachstums von endometrialem Gewebe sein könnte. In der hier vorliegenden Studie konnte mittels rT-PCR und Westernblot sowohl die Splicevariante SV1 als auch der hypophysäre Typ des GHRH-Rezeptors in allen getesteten primären eutopischen endometrialen Stromazellen (EESC) aus Endometrium von Patientinnen mit Endometriose nachgewiesen werden. Im MTT-Proliferationsassay konnte nach 72 h Inkubation von EESCs mit abgestuften Konzentrationen des GHRH Antagonisten MIA-602 von 1 µmol/L bis 0.01 µmol/L eine Dosis abhängige Wachstumsinhibition festgestellt werden. Bei einer Konzentration von 1 µmol/L MIA-602 war die Inhibition des Wachstums bei allen Zellen statistisch signifikant. Dies zeigt, dass MIA-602 einen direkten antiproliferativen Effekt auf endometriale Stromazellen besitzt, welcher wahrscheinlich über die nachgewiesenen GHRH-Rezeptoren erzielt wird. Für ein *in vivo*-Endometriosemodell wurden je fünf gleichgroße Stücke von humanem Endometrium in die Peritonealhöhle von immunsupprimierten Nacktmäusen implantiert. Um das Anwachsen des endometrialen Gewebes zu unterstützen, wurden acht der insgesamt 26 Mäuse zusätzlich subkutan Östrogendepot-Tabletten implantiert. Beginnend an Tag 14 nach der Xenotransplantation wurde 13 Tieren täglich 10 µg MIA-602 injiziert. Verglichen mit den 13 Tieren der Placebogruppe wurde bei den therapierten Mäusen nach 28 Tagen eine signifikante Verringerung der Läsionsgröße festgestellt. Weiterhin konnten wir in immunhistochemisch gefärbten Schnitten die Expression des GHRH Rezeptors in den Stromazellen der transplantierten Endometriosebiopsien nachweisen. Zusammenfassend konnte in dieser Studie zum ersten Mal in einem *in vivo*-Modell für Endometriose die Wirksamkeit eines GHRH Antagonisten gezeigt werden. Aufbauend auf diesen Ergebnissen könnte in weiteren Forschungsarbeiten die mögliche Einsetzbarkeit von GHRH Antagonisten in einer neuen gezielten Therapie bei der Endometriose untersucht werden.

6 References

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7 Attachments

7.1 Votum of the Ethics-Committee

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Klinik für Frauenheilkunde und Geburtshilfe

im Hause

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Direktor der Klinik für Frauenheilkunde und Geburtshilfe

Ethik-Kommission
Vorsitzender:
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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 (Sozialmedizin, Vorsitzender der EK)
Prof. Dr. Schweiger (Psychiatrie)
☒ Prof. Dr. Dendorfer (Pharmakologie)
☒ Frau Prof. E. Stubbe (Theologin)
☒ Prof. Dr. Borck (Medizin- und Wissenschaftsgeschichte
☒ Frau H. Müller (Pflege)
☒ Dr. Kaiser (Kinderchirurgie)
Herr Dr. Fieber (Richter am Amtsgericht Ahrensburg)
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☒ Herr Prof. Dr. Giesler (Medizinische Klinik I)
☒ Frau Prof. Dr. M. Schrader (Plastische Chirurgie, Stellv. Vors.)
☒ Herr PD Lauten (Kinder- und Jugendmedizin)
☒ Frau A. Farries (Richterin am Amtsgericht Lübeck)

7.2 Votum of the Animal Experiment-Committee

Ministerium für Landwirtschaft,
Umwelt und ländliche Räume
des Landes Schleswig-Holstein

Ministerium für Landwirtschaft, Umwelt und ländliche Räume | Postfach 71 51 | 24171 Kiel

Herrn
PD Dr. Dominique Finas
Klinik für Frauenheilkunde und Geburtshilfe
Universitätsklinikum Schleswig-Holstein
Campus Lübeck
Ratzeburger Allee 160
23538 Lübeck

hr Antrag vom: 22.06.2010
Mein Zeichen: V 312-72241.122-10 (82-7/10)
Herr Buttchereit
Telefon: 0431 988-7128
Telefax: 0431 988-7042

nachrichtlich:
Herrn Dr. R. Noël
Gemeinsame Tierhaltung
der Universität zu Lübeck
Ratzeburger Allee 160
23538 Lübeck

10. August 2010

Genehmigung zur Durchführung von Versuchen an Wirbeltieren

Sehr geehrter Herr Dr. Finas,

gemäß § 8 Abs. 1 des Tierschutzgesetzes in der Fassung der Bekanntmachung vom 18. Mai 2006 (BGBl. I S. 1206), zuletzt geändert durch Gesetz vom 15. Juli 2009 (BGBl. I S. 1950), erteile ich Ihnen die Genehmigung, im Rahmen des Versuchsvorhabens

Rezeptorvermittelte Tumortherapie mit Peptidantagonisten des Growth-Hormon-Releasing-Hormon (GHRH) im Endometriosemodell der Maus

zu Versuchszwecken Eingriffe und Behandlungen an Tieren vorzunehmen.

Die Genehmigung ist mit folgenden Nebenbestimmungen verbunden:

1. Es dürfen bis zu 24 Mäuse verwendet werden.

2. Die Genehmigung ist bis zum 31. August 2012 befristet.
   Sie kann auf formlosen, hinreichend begründeten Antrag um ein Jahr verlängert werden, sofern keine wesentlichen Änderungen der Genehmigungsvoraussetzungen eingetreten sind.

3. Leiter des Versuchsvorhabens:
   Herr PD Dr. Dominique Finas

4. Sein Stellvertreter:
   Herr PD Dr. Frank Köster

5. Wechselt der Leiter des Versuchsvorhabens oder sein Stellvertreter, so ist mir diese Änderung unverzüglich anzuzeigen; die Genehmigung gilt weiter, wenn sie nicht innerhalb eines Monats widerrufen wird.

6. Verantwortlicher Tierarzt nach § 9 Abs. 2 Nr. 8 Tierschutzgesetz:
   Herr Dr. R. Noël

7. Weitere Nebenbestimmungen:
   Die Genehmigung beschränkt sich zunächst auf das „Mensch-Maus-Endometriosemodell" mit einer Applikationsform. Danach ist ein Zwischenbericht vorzulegen. Darin ist hinsichtlich des „Maus-Maus-Modells" auch zu erläutern, aus welchen Gründen erwartet wird, dass sich Uterusgewebe peritoneal zu einem geeigneten Endometriosemodell entwickeln kann.

Die Bestimmungen der §§ 8, 9 und 9a Tierschutzgesetz sowie der §§ 116 und 117 des Landesverwaltungsgesetzes in der Fassung der Bekanntmachung vom 2. Juni 1992 (GVOBl. Schl.-H. S. 243) sind zu beachten.

Ich weise insbesondere darauf hin, dass Tierversuche nur durch den in § 9 Abs. 1 Tierschutzgesetz genannten Personenkreis durchgeführt werden dürfen.

Rechtsbehelfsbelehrung
Gegen diesen Bescheid kann innerhalb eines Monats nach Zustellung schriftlich oder zur Niederschrift des Urkundsbeamten der Geschäftsstelle Klage beim Verwaltungsgericht Schleswig-Holstein in 24837 Schleswig, Brockdorff-Rantzau-Straße 13, erhoben werden.

Mit freundlichen Grüßen

Dr. Sekulla

Ministerium für Landwirtschaft,
Umwelt und ländliche Räume
des Landes Schleswig-Holstein

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Herrn
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30. Dezember 2011

Durchführung eines Tierversuchsvorhabens

Sehr geehrter Herr Dr. Finas,

gegen die Mitwirkung von Fau Jin Li an dem genehmigten Tierversuchsvorhaben „Rezeptorvermittelte Tumortherapie mit Peptidantagonisten des Growth-Hormon-Releasing-Hormon (GHRH) im Endometriosemodell der Maus" erhebe ich keine Einwände.

Mit freundlichen Grüßen

Dr. Sekulla

8 Acknowledgements

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9 Curriculum Vitae

Personal Information：

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Education

09/2001-07/2003 **Master of Medicine**, specialized in Obstetrics and Gynecology, Zhejiang University, School of Medicine.

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Publications:

1. **Jin L**, Zhu XM, Luo Q, Qian Y, Jin F, Huang HF. A novel SNP at exon 17 of INSR is associated with decreased insulin sensitivity in Chinese women with PCOS. Mol Hum Reprod. 2006, 12(3):151-155.

2. **Jin Li**, Huang Hefeng, Jin Fan, Qian Yuli. The polymorphisms of insulin receptor exon 17 in patients with PCOS. Chin J Obstet Gynecol, 2005, 40(5): 323-326.

3. **Jin Li**, Huang Hefeng, Jin Fan, Genetic polymorphism in in patients with PCOS. Foreign Medical Sciences (Obstet Gynecol Fascicle), 2002,29 (6): 355-358.