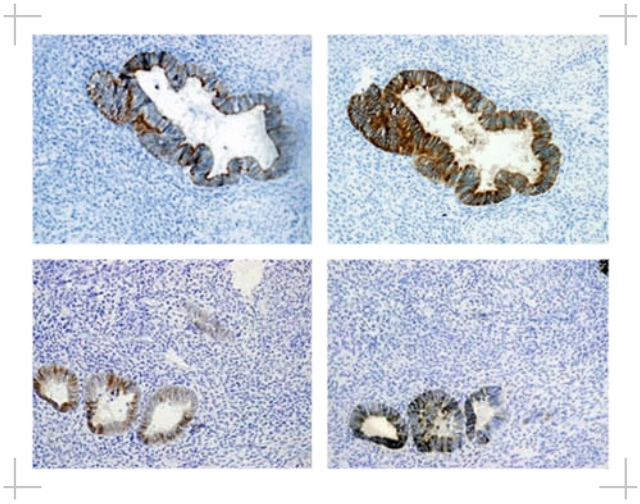
Characterization of the TGF-beta Signalosome and of TGF-beta-dependent Endometrial Cell Proliferation

Ezekiel Onyonka Mecha



INAUGURALDISSERTATION zur Erlangung des Grades eines **Doktors der Humanbiologie**
des Fachbereichs Medizin der Justus-Liebig-Universität Gießen

édition scientifique
VVB LAUFERSWEILER VERLAG

1. Auflage 2014

1st Edition 2014

Printed in Germany

édition scientifique
VVB LAUFERSWEILER VERLAG

STAUFENBERGRING 15, D-35396 GIESSEN
Tel: 0641-5599888 Fax: 0641-5599890
email: redaktion@doktorverlag.de

www.doktorverlag.de

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INAUGURAL-DISSERTATION
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vorgelegt von

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Gießen 2013

Aus dem
Zentrum für Frauenheilkunde
Universitätsklinikum Gießen und Marburg GmbH
Standort Gießen
Direktor: Prof. Dr. Dr. h. c. Hans-Rudolf Tinneberg

Gutachter: PD. Dr. Lutz Konrad

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Tag der Disputation: 04-06-2014

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Abbreviations

ActR	activin receptor
ALK	activin receptor-like kinase
Approx	approximately
APS	ammonium persulphate
BCA	bicinchonic acid
Bcl-2	B-cell lymphoma/Leukemia-2 protein
BMP	bone morphogenetic proteins
BMPR	bone morphogenetic protein receptor
BSA	bovine serum albumin
CA 125	cancer antigen 125
Caspase 3/7	cysteinyl-asparate-specific proteases 3/7
CD 10	cluster of differentiation
CK 18	cytokeratin18
cm^2	square centimeter
CO_2	carbon dioxide
Ctrl	control
DAB	3,3'-diaminobenzidine tetrahydrochloride
DMEM	Dulbecco's modified eagle's medium
DNA	deoxyribonucleic acid
DTT	Dithiothreitol
ECM	extracellular matrix
EDTA	ethylene diamine tetraacetic acid
ELISA	enzyme-linked immunosorbent assay
FasL	fas ligand
FBS	fetal bovine serum
FC	fragment crystallizable
FCS	fetal calf serum
Fig	figure
g	gravitational force
GDF	growth and differentiation factors
GnRH	gonadotropin-releasing hormone
g/L	grams/liter

HEPES	hydroxyethyl piperazineethanesulfonic acid
HRP	horseradish peroxidise
IFN-γ	interferon-gamma
IgG	immunoglobulin G
IHC	immunohistochemistry
IL	interleukin
JNK	c-Jun N-terminal kinases
LAP	latency associated peptide
LLC	large latent complex
LRPI	low-density lipoprotein receptor-related protein-1
LTBP	latent-TGF-β binding protein
Min	minutes
ml	mililiter
mM	millimolar
MMP	matrix metalloproteinases
mRNA	messenger ribonucleic acid
MUC1	mucin-1
NBF	neutral buffered formalin
ng	nanogram
NK	natural killer cells
nm	nanometer
OD	optical density
p38 MAPK	p38 mitogen-activated protein kinase
PAI-1	plasminogen activator inhibitor-1
PBS	Phosphate buffered saline
PDGF	platelet-derived growth factor
pen/strep	penicillin/streptomycin
PFA	paraformaldehyde
pg	picogram
PLA	proximity ligation assay
PMSF	phenylmethylsulfonyl fluoride
PVDF	polyvinylidene difluoride
rAFS	revised American Fertility Society System
rASRM	revised American Society for

	Reproductive Medicine
RCA	rolling cycle amplification
RFU	relative fluorescence units
rh	recombinant human
RT	room temperature
SBE	Smad binding elements
SERPIN	serine proteinase inhibitor
SCID	severe combined immune deficiency
SDS	sodium dodecyl sulfate
SiS3	specific inhibitor for Smad3
α-SMA	α-smooth muscle actin
SV40	Simian virus 40
TBST	Tris buffered saline Tween-20
TEMED	Tetramethylethyelenediamine
TGF-β	transforming growth factor betas
TIMP	tissue inhibitor of metalloproteinases
TMB	3,3',5,5'-Tetramethylbenzidine
tPA	tissue-type plasminogen activator
TβR	receptor of transforming growth factor-beta
Tris	tris (hydroxymethyl)-aminomethane
uPA	urokinase-type plasminogen activator
uPAR	urokinase receptor
UV light	ultraviolet light
µg	microgram
µl	microliter
µM	micromolar
v/v	volume/volume

List of Publications

a) **Mecha E,** Omwandho CA, Tinneberg HR, Konrad L (2013). Characterization of the TGF-beta signalosome and of TGF-beta dependent endometrial cell proliferation (2013). In preparation

b) Konrad L, Gronbach J, **Mecha EO**, Frank M, Berkes E, Gattenlöhner S, Omwandho COA, Oehmke F, Tinneberg HR (2013). Endometriosis is one entity. Submitted.

Abstracts from Conferences and Scientific Meetings

1) **Mecha E**, Omwandho CA, Sui C, Tinneberg HR, Konrad L (2013). TGF-betas induce smad-dependent signaling and apoptosis in endometrial and endometriotic cells. Abstract P005. 5th Dachverband Reproduktionsbiologie und medizin Kongress. Műnster, Germany. Dec 4-7, 2013.

2) **Mecha E**, Sui C, Kloeppels K, Omwandho CA, Tinneberg HR, Konrad L (2013). The TGF-betas in human endometrial and endometriotic cells. Abstract P45, 2nd European Congress on Endometriosis. Berlin, Germany. Nov 28-30, 2013.

3) Gronbach J, Kortum J, **Mecha E**, Berkes E, Omwandho CA, Tinneberg HR, Konrad L (2013)**.** Endometriosis – What do the neighbours think? Abstract SP11, 2nd European Congress on Endometriosis. Berlin, Germany. Nov 28-30, 2013.

4) **Mecha E,** Omwandho CA, Tinneberg HR, Konrad L (2013). The TGF-β signalosome in human endometrial and endometriotic cells. Abstract P99, 6th International Giessen Graduate School for life Sciences Conference, Giessen, Germany. Sept. 11-12, 2013.

5) **Mecha E,** Omwandho CA, Sui C, Tinneberg HR, Konrad L (2013). TGF-betas enhance secretion of MMP2/9 and PAI-1 of human endometrial and endometriotic cells. Abstract 68, 2nd International Scientific Conference, University of Nairobi/Kenyatta National Hospital, Nairobi, Kenya. June, 19-21. 2013.

6) Berkes E, Stammler A, **Mecha EO,** Tinneberg HR, Konrad L (2013). Sekretion von clusterin durch endometriale und endometriotische Zellen. 10th Endometriosis Congress, Linz, Austria, April 25-27, 2013.

7) Gronbach J, **Mecha E,** Berkes E, Stammler A, Omwandho CA, Tinneberg HR, Konrad L (2013). Stromal cells in the endometrium and endometriosis. 15th World Congress on Human Reproduction, Venezia, Italy, March 13-16, 2013.

8) **Mecha E,** Omwandho CA, Zoltan D, Tinneberg HR, Konrad L (2012). TGF-βs induce Smad-dependent signaling and apoptosis in human endometrial and endometriotic cells lines. Abstract SP 04, 1st European Congress on Endometriosis, Siena, Italy, 29th Nov- 1st Dec, 2012. J Endometriosis 4: 213-214.

9) **Mecha E,** Omwandho COA, Zoltan D, Tinneberg HR, Konrad L (2012). TGF-βs induce Smad-dependent signaling and apoptosis in primary human endometrial stromal cells. Abstract P99, 5th International Giessen Graduate School for life Sciences Conference, Giessen, Germany. Sept. 18-19, 2012

10) **Mecha E,** Omwandho CA, Zoltan D, Tinneberg HR, Konrad L (2011). Transforming growth factor betas strongly enhances secretion of plasminogen activator inhibitor 1 protein by endometrial and endometriotic cell lines. Abstract P100, 4th International Giessen Graduate School for life Sciences Conference, Giessen, Germany. August, 21-22. 2011.

1 Introduction

1.1 Endometriosis

Endometriosis is characterized by the presence of endometrial tissue outside the uterus most commonly in the ovary and the peritoneum but may also occur in pericardium, intestinal tract, lung, pleura or brain (Giudice and Kao, 2004). The prevalence is approximately 10% in the general female population in their reproductive age with 35-50% of the patients experiencing pain and infertility (Houston, 1984; Cramer, 1987; Rothman and Greenland, 1998; Rogers et al., 2009). Endometriosis is generally associated with inflammation, but, severe cases may result in extensive pelvic adhesions and distortion of pelvic anatomy, which could result in infertility (Giudice and Kao, 2004).

1.1.1 Pathogenesis, Diagnosis, Classification and Therapy of Endometriosis

The pathogenesis of endometriosis is still poorly understood and remains controversial. However, several theories including dissemination by retrograde menstruation, induction, coelomic metaplasia, altered cellular immunity, lymphatic and vascular metastasis, genetic causes and environmental causes have been presented to understand the pathogenesis and etiology of endometriosis (Giudice and Kao, 2004; Witz, 2005).

The most widely accepted hypothesis of the pathogenesis of endometriosis is dissemination and implantation after retrograde menstruation first proposed by Sampson (1922; 1927). According to Sampson, some of the endometrial tissue fragments passes through the fallopian tubes during menstruation, then attach and proliferate in the peritoneal cavity (Sampson, 1927). Recent data by Matsuzaki and Darcha (2012) and Konrad et al. (2013, submitted) provide evidence that the hypothesis by Sampson is probably correct.

However, Sampson's theory fails to explain why only a small percentage of women experiencing retrograde menstruation develop endometriosis (Koninckx and Martin, 1992). Thus, other factors like the immune system might be involved in the implantation of displaced endometrial cells which develop into endometriotic lesions (Donald and Dmowski, 1998; Kyama et al., 2003). In women with endometriosis, the immune system seems to be impaired, hence permitting endometrial cells to escape

immune surveillance and to grow in ectopic locations (Donald and Dmowski, 1998; Herington et al., 2011).

Diagnosis of endometriosis is by laparoscopy which is generally considered as the "gold standard", followed by histological confirmation of viable ectopic endometrial glands and stroma (Brosens, 1997; Kennedy et al., 2005). Sometimes, a non-invasive examination by auxiliary diagnostic methods such as pelvic ultrasound is used before performing surgery (Brosens, 1997; Champaneria et al., 2010). Peritoneal fluid, serum and tissue markers such as CD10 and CA125 have also been tested in some clinics but none are used routinely (Mihalyi et al., 2010).

The revised American Society for Reproductive Medicine (rASRM) score is currently the best known classification of endometriosis. According to rASRM, the staging is based on the location, amount, depth and size of endometrial implants. Four stages of endometriosis have been characterized

- Stage 1 (Minimal)
  - Findings restricted to superficial lesions only and possibly a few filmy adhesions
- Stage 2 (Mild)
  - Presence of deep lesions in the rectouterine pouch in addition to observations in stage1
- Stage 3 (Moderate)
  - Presence of endometriomas on the ovary and more adhesions in addition to observations in stage 2
- Stage 4 (Severe)
  - Presence of large endometriomas and extensive adhesions in addition to observations in stage 3

Treatment of endometriosis can be medical, surgical or a combination of both. Treatment options depend on the severity of the symptoms, pregnancy, age and therapeutic goals. Endometriosis is often treated surgically, but symptoms recur in 75% of cases within 2 years (Candiani et al., 1991). Medical treatment of endometriosis involves contraceptive steroids, progestagens, agonists of gonadotropin-releasing hormone (GnRH) analogues and non-steroidal anti-inflammatory agents (Lessey, 2000; Kennedy et al., 2005; Valle and Sciarra, 2008; Kappou et al., 2010). However, because of undesirable side-effects caused by most

of these drugs, they are useful only for a limited time period making it necessary to change or use additional medication (Kuohung et al., 2002; Kennedy et al., 2005).

1.1.2 In vivo and in vitro Models of Endometriosis

a) In vivo models

The most widely accepted hypothesis for the development of endometriosis is Sampson's theory of retrograde menstruation which implies the implantation of retrograde endometrial tissues in tissues outside the uterus. Thus, menstrual shedding is a requirement for spontaneous development of endometriosis which has been found to occur only in human, primates and some rats (Grümmer, 2006).

But owing to the species specificity, it is hard to compare human and primates. For example, spontaneous and induced endometriosis has been found in baboons to be as low as 4.8% and 27.6%, respectively, raising some doubts on the validity of the baboon model for endometriosis (Dehoux et al., 2011). Also, the very high costs of animal handling limit the use of baboons as an experimental model.

Recently, rodent models like severe combined immune deficiency (SCID) mice have been used also to study endometriosis. In these models, human endometrial and endometriotic tissue or cells were successfully engrafted into the peritoneal cavity of immunodeficient mice (Liu et al., 2010; Becker et al., 2011). The grafted tissues or cells were able to implant and develop into endometriotic lesions. The advantages of such models are: (1) animals are easily available, (2) low costs and (3) the peritoneal environment of the hosts can be altered to suit the study objectives. Mice xenotransplanted with human eutopic endometrial tissue developed endometriosis-like lesions in 63% and 68% in ovariectomized estrogen-supplemented TGF-β1-null mutant mice and wild-type control, respectively. With the aid of this model it was demonstrated that host derived TGF-β1 deficiency suppressed endometriotic lesion development (Hull et al., 2012).

The in vivo models of endometriosis have many advantages, but the limitations of such models is that they can hardly be used to study individual components and some biological functions like the involvement of intracellular proteins and pathways, These aspects of endometriosis can be addressed appropriately only by use of in vitro models.

b) In vitro models

In vitro models of endometriosis are build by obtaining endometrial and endometriotic tissues from patients after surgical treatment. Primary epithelial or stromal cells are isolated, cultured and then used for assays. Epithelial cells derived from endometriotic lesions were found to be as invasive as metastatic cancer cells using the matrigel invasion assay (Zeitvogel et al., 2001). Another model using both invasion and angiogenesis within a 3D fibrin matrix demonstrated the ability of ectopic endometrial fragments to proliferate and invade. The fragments generated in vitro appeared histologically similar to endometriotic implants in vivo (Fasciani et al., 2003). Therefore, in both models, there is a strong evidence of the possibility to study endometriosis in vitro. The limitations of using primary cells are that the cells are often heterogeneous and epithelial cells die at low passages (Starzinski-Powitz et al., 1998).

This problem has been sorted out by establishment of immortalized endometrial and endometriotic cells (Starzinski-Powitz et al. 1998; Boccellino et al. 2012). Konrad et al. (2010) and Sui. (2012) showed that the above established immortalized endometrial and endometriotic cell lines were able to secrete or to be stimulated by TGF-βs. Thus, the immortalized cell lines are a suitable model for studying endometriosis.

1.2 The Transforming Growth Factor Betas and TGF-β Receptors

TGF-βs are a member of a large superfamily including activins, inhibins, nodals, bone morphogenetic proteins (BMP), lefty A and B, growth and differentiation factors (GDFs) and anti-Mullerian hormones (Chang et al., 2002; Peng, 2003). TGF-βs comprise three isoforms namely TGF-β1, TGF-β2 and TGF-β3. Biologically, TGF-βs regulate cell motility, proliferation, apoptosis, gene expression and differentiation. In addition, TGF-βs tightly regulate production of the extracellular matrix (ECM) and are involved in wound healing and immunosupression (Roberts and Sporn, 1993; Roberts, 1998; Kaminska et al., 2005; Taylor, 2009). They are also involved in tumorigenesis and inflammation (Padua and Massagué, 2009; Santibañez et al., 2011).

Production and activation of the TGF-βs is triggered by cleavage of the inactive dimeric TGF-β precursor called latency associated peptide (LAP). LAP is bound by disulphide bonds to the latent-TGF-β binding protein (LTBP) resulting in the large

latent complex (LLC). The LLC is targeted either to the cell surface for activation or to the extracellular matrix for storage (Munger et al., 1997; Rifkin, 2005).

Gene knockout techniques have revealed the physiological roles of each of the TGF-β isoforms. TGF-β1 knockout mice die at gestation due to defective vasculature (Shull et al., 1992; Chen et al., 1996). TGF-β2 knockout mice exhibit perinatal mortality and a wide range of developmental defects (Sanford et al., 1997; Shi et al., 1999). TGF-β3 knockout mice die within 24 hours after birth due to abnormal lung development and feeding problems associated with a defective cleft palate (Kaartinen et al., 1995; Proetzel et al., 1995).

The TGF-β family receptors are divided into three groups, namely type I, type II and type III receptors. The three receptor types have distinct properties (Heldin et al., 1997; Chang et al., 2002). TGF-β receptor type I (TβRI) and type II (TβRII) are transmembrane serine/threonine kinases. The type III receptors, betaglycan (TβRIII) and endoglin are accessory receptors and have high affinity to all three TGF-β isoforms (Wrana et al., 1994; Gordon et al., 2008).

The general mechanism of TGF-β signaling starts by TGF-β binding either to TβRIII, which presents it to TβRII, or binding to TβRII directly, which then binds to and transphosphorylates TβRI. Then the activated TβRI phosphorylates Smad2 or Smad3, which bind to Smad4 in the cytoplasm or the nucleus forming a Smad complex. The Smad complex interacts with transcription factors in the nucleus to regulate TGF-β responsive genes (Chen et al., 2003; Guglielmo et al., 2003; Biondi et al., 2007; Wrighton et al., 2009).

1.2.1 The Smad-dependent and Smad-independent Pathways in TGF-β Signaling

a) Smad-dependent Pathways

Smads are intracellular proteins that transduce signals from the TGF-β receptors to the nucleus where they activate downstream gene transcription (Miyazono al., 2000). There are three classes of Smads, namely the receptor-regulated Smads (R-Smads) including Smad1, 2, 3, 5 and 8/9 (Wu et al., 2001). The common-mediator Smad (co-Smad) with only Smad4, which interacts with R-Smads to participate in signaling (Shi et al.,1997). The inhibitory Smads (I-Smads), Smad6 and Smad7, block the activation of R-Smads and co-Smads (Itoh et al., 2001). BMP type I receptor activates Smads1,

5 and 8 (Moustakas et al., 2001), while TβRI or activin receptors activate Smad2 and Smad3 (Chen et al., 2003).

A general Smad-dependent signaling pathway activated by TGF-β is shown in Figure 1 (Rebecca, 2000).

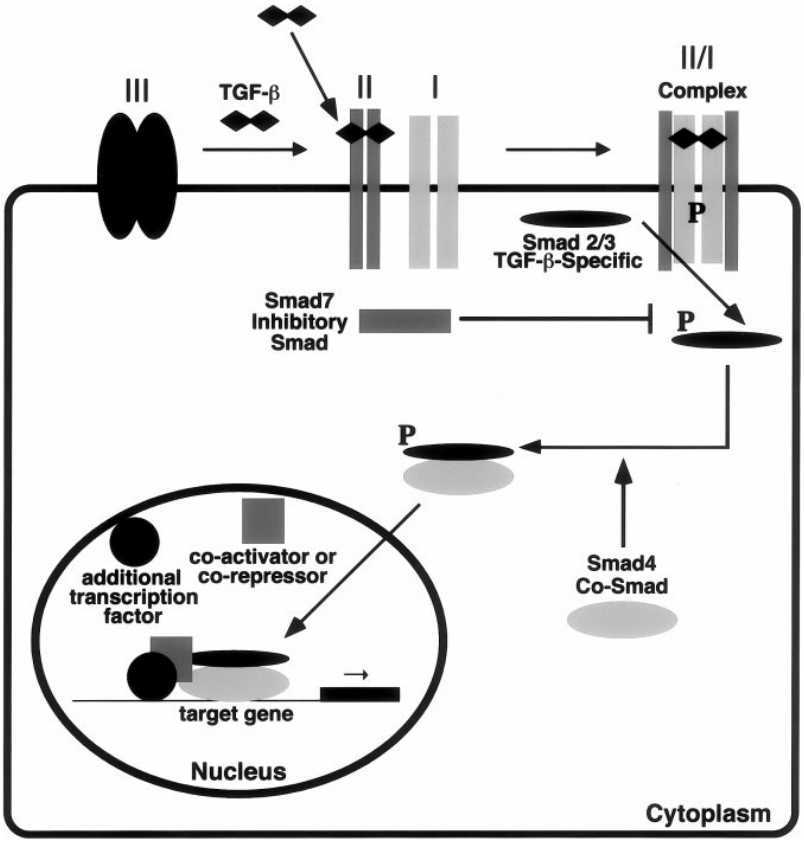


Figure 1. The TGF-β induced Smad signaling pathway (Rebecca, 2000). Binding of TGF-β results in the formation of the TβRI/TβRII complex and then the TβRII kinase phosphorylates and activates TβRI. The activated TβRI kinase phosphorylates Smad2/Smad3. The phospho-Smad2/Smad3 form complexes with the co-Smad (Smad4) and move into the nucleus, where they interact with other transcription factors to regulate transcription.

b) Smad-independent Pathways

Smad-independent pathways which reinforce the signal transduction of the TGF-βs were shown by Derynck and Zhang. (2003), Moustakas and Heldin. (2005) and Zhang (2009). The Smad-independent pathways provide alternative TGF-β signal transduction without the direct involvement of Smad proteins. For instance, the p38 substrate kinase, regulates the transcriptional activity of Smad3 by enhancing its association with p300 (Abécassis et al., 2004) and TGF-β-activated JNK was shown to phosphorylate Smad3 thus inducing its nuclear translocation (Engel et al. 1999).

1.2.2 TGF-βs in the Normal Endometrium and Endometriosis

a) Normal Endometrium

The TGF-βs are stage-specifically expressed in the human endometrium during the menstrual cycle (Fig. 2). TGF-β1 and TGF-β3 are highly expressed in stromal and glandular cells (Chegini et al., 1994). TGF-β2 was found to be strongly expressed in stromal cells compared to glandular cells (Gold et al., 1994; Gaide Chevronnay et al., 2008). This observation suggests the involvement of TGF-βs in the normal function of the human endometrium (Omwandho et al., 2010).

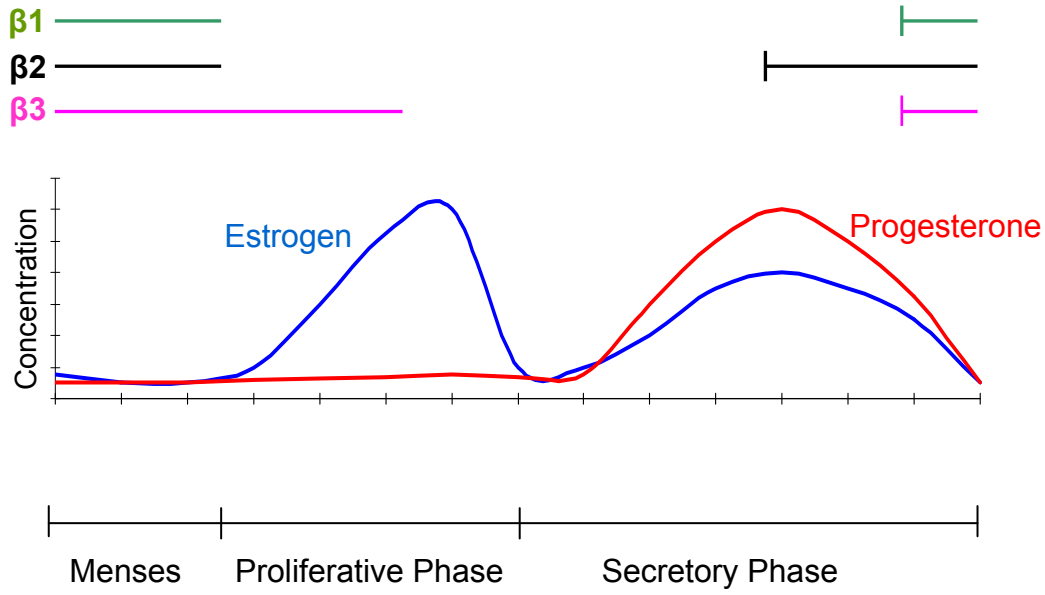


Figure 2. Levels of TGF-βs during the menstrual cycle in human endometrium. Only the start and endpoints of the strongest expression of the TGF-βs is shown. The three TGF-βs isoforms are differentially and highly expressed during the menstrual cycle (Omwandho et al., 2010).

b) TGF-βs in Endometriosis

TGF-βs were found to be expressed significantly higher in the serum and peritoneal fluid in women with endometriosis (Pizzo et al., 2002). Also, TGF-βs levels are enhanced markedly concomitant with the severity of the disease with high levels observed in stages III and IV, suggesting a possible role in the pathogenesis of endometriosis (Pizzo et al., 2002).

Endometriosis comprises six developmental stages namely cell shedding and refluxing, cell survival, immune suppression, cell adhesion and invasion, angiogenesis, and bleeding (Omwandho et al., 2010). TGF-βs have been shown to

be directly or indirectly involved in most of these stages (Fig. 3).

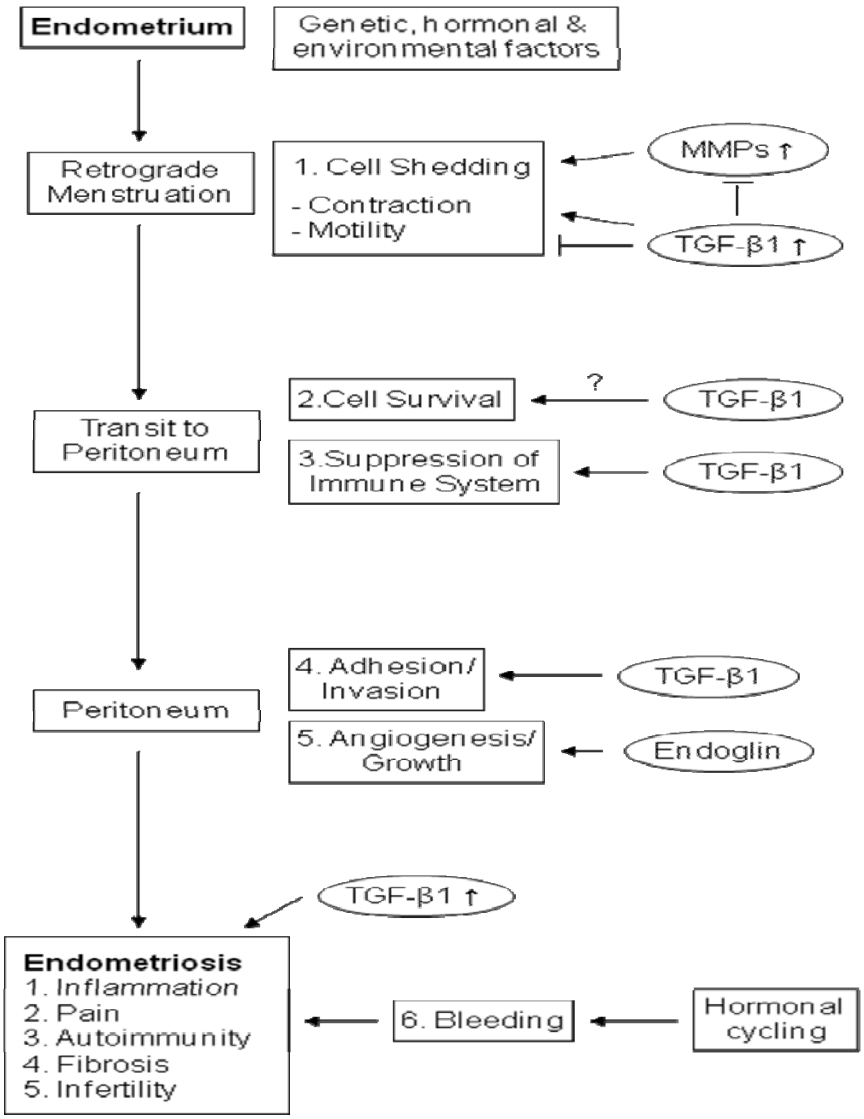


Figure 3. The six developmental stages leading to endometriosis. The involvement of the TGF-βs directly or indirectly in each stage has been emphasized (Omwandho et al., 2010).

Retrograde menstruation is the most widely accepted theory of pathogenesis of endometriosis (Sampson, 1927), thus for establishment of endometriosis to occur, shedding and refluxing of endometrial cells are prerequisites. Highest expression of the TGF-βs was detected during menstruation, suggesting a possible involvement of the TGF-βs in cell shedding (Gaide Chevronnay et al., 2008). Also, women with endometriosis were found to experience abnormal myometrial contractions with higher and a different frequency (Bulletti et al., 2002), which is possibly associated

with the dissemination of endometrial fragments.

TGF-βs regulate a wide variety of cellular responses including proliferation, apoptosis, gene expression, immune responses, cell motility, tumorigenesis, immune responses and extracellular matrix production (ECM) (Derynck et al., 2001). Interestingly, TGF-β effects can be extremely variable depending on cell types and stimulation context. For example, TGF-βs cause growth arrest in epithelial cells, but induce activation of fibroblasts (Rahimi and Leof, 2007). In endometrial cells, TGF-β1 was found to stimulate DNA synthesis of epithelial cells with lower cell number, but repressed it when the cell number was higher in women with and without endometriosis (Meresman et al., 2003).

Escape of endometrial fragments from apoptosis and immune attack as they enter and transit the peritoneal cavity is also very important for their survival. TGF-β1 can induce expression of FasL mRNA in endometrial stromal cells (Garcia-Velasco et al., 1999), possibly preventing apoptosis during transit of the peritoneal cavity. In addition, TGF-β1 can inhibit IFN-γ and IL-10 secretion by uterine NK cells from human endometrium but upon blocking of TGF-β1, secretion of the two cytokines was increased (Eriksson et al., 2004). In general, the high levels of the TGF-βs might have a direct or indirect effect on the immune escape by decreasing the response of NK cells to ectopic endometrial fragments hence increasing their survival rate.

TGF-βs might be regulating cell adhesion to the ECM indirectly through regulating the balance of matrix metalloproteinases (MMPs) and tissue inhibitor of metalloproteinases (TIMPs) which are involved in the normal fibrolytic processes. Also MMPs have been shown to convert the inactive membrane-bound FasL, to its active soluble form which induces apoptosis (Otsuki, 2001). Taken together, TGF-βs might be involved in most of the biological processes involved in the pathogenesis of endometriosis, although more studies are needed to clarify their exact participation.

1.3 TGF-β-induced Apoptosis in Human Endometrium and Endometriosis

1.3.1 Apoptosis

Apoptosis (programmed cell death) enables multicellular organisms to remove excessive and potentially dangerous cells. Balance and coordination between apoptosis and cell survival is important for homeostasis and development of multicellular organisms (Green, 1998). A defect in the control of the balance may cause diseases like cancer, neurodegenerative conditions and autoimmune diseases

(Kroemer et al., 2007). The morphological alteration of cells undergoing apoptosis is caused mainly by cysteine protease proteins, called caspases, which are mainly activated in cells undergoing apoptosis (Green, 1998).

The process of apoptosis is characterized by cell shrinkage, chromatin condensation, DNA fragmentation and formation of cytoplasmic blebs (Elmore, 2007). Two major apoptotic pathways exist in mammalian cells, the mitochondrial pathway (intrinsic) and the death-receptor pathway (extrinsic). The two pathways are initiated by different mechanisms but both cause activation of caspases which eventually result in apoptosis.

1.3.2 TGF-β-induced Apoptosis

Uterine cells have been shown to undergo apoptosis during blastocyst implantation, oestrous cycle and decidualization. The control of the coordinated blastocyst implantation processes presumably depends on secretion of various endometrial factors, among them TGF-βs (Pollard, 1990).

TGF-β1 and TGF-β2 mRNAs have been detected in decidual cells after the first week of pregnancy (Tamada et al., 1990; Manova et al., 1992). TGF-β1 has been reported to increase apoptosis in primary cell cultures of uterine epithelial cells (Rotello et al., 1989), also TGF-β1 and TGF-β2 stimulated the increase in nucleosome DNA fragmentation in endometrial stromal cells indicating stimulation of apoptosis (Bruce, 1994). Furthermore, neutralization of TGF-βs secreted by endometrial stromal cells completely inhibited apoptosis in vitro (Bruce, 1994). All above data strongly show that TGF-βs are an important component in apoptosis of stromal and epithelial cells.

1.3.3 TGF-β-induced Apoptosis in the Human Endometrium

Apoptosis occurs in normal endometrial tissue throughout the cycle and is important in maintaining the homeostasis of cells during the menstrual cycle. Specific nuclear DNA fragmentation related to apoptosis was reported in the human endometrium (Hopwood and Levinson 1995). The B-cell lymphoma/Leukemia-2 protein (Bcl-2), an anti-apoptotic protein, is expressed throughout the menstrual cycle with higher levels in the glandular component of the human epithelium (Gombel et al., 1994, Otsuki et al., 1994: McLaren et al., 1997).

The Bcl-2 immunoreactivity was maximal in the proliferative endometrium and the levels decreased to a minimum in the secretory endometrium (Tao et al., 1997). In

contrast, levels of the pro-apoptotic proteins, Bax and Fas/FasL, were increased in the secretory endometrium with minimal expression in the glandular epithelium (Watanabe et al., 1997; Meresman et al., 2000). Furthermore, in Bcl-2-deficient mice, many apoptotic cells and bodies were often observed in glands and myometrium (Daikoku et al., 1998), implying that Bcl-2 may be essential in survival of both endometrial glandular cells and myometrial smooth muscle cells (Meresman et al., 2000).

Coincidently, TGF-βs have been shown to be stage-specifically and abundantly expressed throughout the menstrual cycle with higher levels expressed in both stromal and glandular cells (Chegini et al., 1994; Gaide Chevronnay et al., 2008). The mRNA and protein expression of TGF-βs is high in the late secretory phase of the menstrual cycle (Omwandho et al., 2010). Also the levels of the pro-apoptotic proteins, Bax and Fas/FasL, are high in the secretory phase while the anti-apoptotic protein, Bcl-2 expression, is low in the secretory phase of the menstrual cycle (Tao et al., 1997; Watanabe et al., 1997; Garcia-Velasco et al., 1999; Meresman et al., 2000). Other studies have shown that TGF-β1 increased the apoptotic rate of endometrial stromal cells in vitro (Chatzaki et al., 2003).

We can only speculate that the stage-specific expression of TGF-βs, Bax, Fas/FasL (direct correlation) and Bcl-2 protein (inverse correlation) throughout the menstrual cycle indicates that TGF-βs might induce apoptosis of human endometrial stromal or epithelial cells in human endometrium during the secretory phase of the menstrual cycle. Further studies are needed to characterize the apoptotic effects of the TGF-βs and which apoptotic pathways are involved in the normal endometrium and endometriosis.

1.3.4 TGF-β-induced Apoptosis in Endometriosis

The role of TGF-β-induced apoptosis in endometriosis has not been well studied, however, the escape of endometrial fragments from apoptosis as they enter and transit the peritoneal cavity is very important for their survival (Garcia-Velasco et al., 1999). In addition, women who develop endometriosis showed greatly reduced apoptosis in sloughed endometrial cells, implying that a high number of surviving cells can enter the peritoneal cavity (Gebel et al., 1998).

Apoptosis was found to decrease as the severity of endometriosis increases (Dmowski et al., 2001). Also, Bcl-2 protein expression was found to be increased in

proliferative eutopic endometrium in women with endometriosis, suggesting a possible resistance to apoptosis (Meresman et al., 2000). Furthermore, FasL is highly expressed in endometriotic tissues which possibly contributes to their survival and thus to the development of endometriosis. Also higher levels of soluble FasL were present in serum and peritoneal fluid of women from endometriosis (Garcia-Velasco et al., 2002), which might contribute to increased apoptosis of Fas-expressing immune cells. Garcia-Velasco et al. (1999) showed that platelet-derived growth factor (PDGF) and TGF-βs are increased in peritoneal fluid of women with endometriosis. Also, PDGF and TGF-βs induced FasL expression by endometrial stromal cells (Garcia-Velasco et al.,1999). Increased FasL expression by the cells may protect the stromal cells from cytotoxic T-cells, hence ectopic endometrial cells are able to escape the immune system in the peritoneal cavity of women with endometriosis hence possibly contributing to the pathogenesis and maintenance of the disease.

Furthermore, FasL expression by endometriotic cells was induced after adhesion of the cells to ECM proteins of endometriotic patients (Selam et al., 2002), this possibly induces apoptosis of activated T-lymphocytes thereby lowering their ability to attack the endometrial cells. Thus, survival of endometriotic cells is promoted during initial attachment at ectopic sites.

Increased expression of FasL, Bcl-2 and TGF-βs in serum, peritoneal fluid and ectopic sites in women with endometriosis suggests the possible involvement of TGF-βs in apoptosis during entry and transit of endometrial cells in the peritoneal cavity, subsequent attachment of the cells at ectopic sites and eventual establishment and maintenance of endometriosis.

Although, the role of TGF-β-induced apoptosis in endometriosis has not been fully investigated, recently, Omwandho et al. (2010) showed that the stages of endometriosis are similar to those of cancer. Since TGF-βs have been shown to play an important role in suppression of apoptosis in various tumors (Lebrun, 2012), together with their increased expression in serum, peritoneal fluid and at endometriotic sites of women suffering from endometriosis, it will be of great importance to find out if the TGF-βs induce apoptosis in endometriotic cells and by which mechanisms.

1.4 Crosslink of TGF-β and Bone Morphogenetic Proteins (BMPs)

The BMPs are multifunctional proteins that regulate functions such as proliferation, apoptosis and differentiation of a large variety of cell types (Reddi, 1997). BMPs mediate their cellular functions through binding to a combination of type I and type II receptor serine/threonine kinases (Kawabata et al., 1998). The BMP ligands can bind to any of the three type II receptors (BMPRII, ActRIIa and ActRIIb) which then bind to one of the three type 1 receptors (ALK-2, ALK-3 and ALK-6). Upon binding, the constitutively active type II receptor phosphorylates type 1 receptor then phosphorylates the BMP-responsive Smad proteins namely Smad1, Smad5 and Smad 8. The activated Smads bind Smad4 either in the cytoplasm or in the nucleus for signaling (Yu et al., 2008). In addition, BMP signals have been found to activate other intracellular effectors like mitogen-activated protein kinase (MAPK) p38 via the Smad pathway (Nohe et al., 2004).

Although TGF-βs transduce their signals through activation of Smad2 and Smad3 (Chen et al., 2003), recent studies have indicated that they can also strongly but only transiently induce phosphorylation and activation of Smad1, Smad5 and Smad8 (BMP-responsive Smads) in endothelial cells, epithelial cells, fibroblasts and epithelium derived cancer cells (Bharathy et al., 2008; Daly et al., 2008; Liu et al., 2009). These observations have raised several questions of how the activation of Smads1/5/8 by TGF-βs affect BMP responses (Grönroos et al., 2012).

Several hypothesis have been put forward to explain the possible crosstalk of TGF-βs/BMP pathways, for example Grönroos et al., 2012 suggested the involvement of ALK-5 and formation of pSmad3-pSmad1/5 complexes. Recently, knockdown of Smad3 phosphorylation in mice abolished the ability of TGF-β to inhibit BMP-induced transcription (Grönroos et al., 2012), further supporting a possible crosstalk between TGF-β and BMP pathways.

Perturbations of both BMP and TGF-β signalling have been reported to cause distinct bone diseases (Jansens et al., 2000). In endothelial cells, ALK-1 together with ALK-5 can activate TGF-β-responsive Smads (Smad2/3) and also phosphorylate BMP-responsive Smads (Smad1/ 5/ 8; Miyazono and Kusanagi, 2001). Furthermore, Smad5-deficient mice exhibit defects in vascular tissues (Chang et al., 1999), which are similar to those observed in ALK-1 deficient mice. These observations suggest that Smad5 is a downstream target of ALK-1 (Miyazono and Kusanagi, 2001). In addition, molecules that repress both TGF-βs and BMPs have been found to be

involved in the pathogenesis of vascular diseases (Miyazono and Kusanagi, 2001), hence indicating a possible crosstalk of both pathways. What remains to be investigated is the exact location of cross-talk of the TGF-β and BMP pathways (e.g. at the extracellular membrane, in the cytoplasm or in the nucleus) and which molecules are involved (e.g. the individual BMP/TGF-β receptors or Plasminogen Activator Inhibitor 1 (PAI-1) secretion among other proteins. These will be helpful in determining the cross-talk of TGF-β and BMP pathways and understand their possible role in the pathophysiology of endometriosis.

1.5 Plasminogen Activator Inhibitor-1

PAI-1 is the major inhibitor of tissue-type plasminogen activator (tPA) and urokinase-type plasminogen activator (uPA) in vitro and is pivotal in fibrinolysis (Binder et al., 2002; Czekay and Loskutoff, 2004). The promoter region of PAI-1 contains three Smad binding elements (SBEs), which are the main response element of TGF-β signals (Binder et al., 2002).

Endothelial and stromal cells are the main producers of PAI-1 which is then released into the plasma (Bastelica et al., 2002; Vaughan, 2005). Expression of PAI-1 and uPA was shown to be much higher in endometriotic and endometrial tissues of women with endometriosis compared to tissue from women without endometriosis (Bruce et al., 2004). PAI-1 is able to regulate levels of cell surface integrins through triggering their internalization by binding to the low-density lipoprotein receptor-related protein-1 (LRP1) resulting in detachment of cells from various substrates (Akkawi et al., 2006; Pedroja et al., 2009; Czekay et al., 2011).

Recently, studies have shown that PAI-1 and TGF-βs are involved in fibrosis, wound healing and metastasis and are capable of inducing ECM remodeling through regulation of plasmin and MMP acitivity (Kortlever and Bernards, 2006). Furthermore, activity of TGF-βs is involved in the conversion of fibroblasts to myofibroblasts (Desmouliere et al., 2004), that are responsible for PAI-1 secretion at the edges of stromal tissue during cancer invasion (Dublin et al., 2000; Offersen et al., 2003). Given the fact that both TGF-β and PAI-1 are involved in fibrosis, wound healing, metastasis, induction of ECM remodeling and regulation of MMP activity and that their activities (TGF-β and PAI-1) are elevated in endometrial and ectopic tissues in women with endometriosis, thus we suppose that TGF-β/PAI-1 might play an important role in the pathogenesis of endometriosis. It will be interesting to

investigate whether or not, PAI-1 contributes to the pathophysiology of endometriosis and which pathways are involved.

1.6 Objectives

Despite the fact that TGF-βs have been demonstrated to play important roles in the pathogenesis of endometriosis, their precise function needs to be investigated in more depth. TGF-βs have been shown to mediate their functions via Smad molecules. Of note, cross-talk with other signaling pathways has been reported, but the exact molecules involved have not been fully established. Thus, it will be interesting to examine the distinct molecules involved (Smads and receptors) and the cross-talk with other pathways like BMPs to better understand the pathogenesis of endometriosis. Furthermore, PAI-1 expression has been found to be regulated by TGF-β isoforms in many tissues, thus it will be of great importance to elucidate how TGF-βs regulate the expression of this protein and the pathways utilized for its regulation.

Endometriosis has been studied by the use of immortalized endometriotic and endometrial cell lines (for comparison) both of which have been characterized well. Thus both endometrial and endometriotic cell lines together with primary endometrial stromal cells will enable us to establish an in vitro model which will allow a deeper understanding of the basic aspects and provide comparisons of endometriotic cells to normal endometrial cells.

The main objective of this study was to investigate the influence of TGF-βs on endometrial, endometriotic cell lines and primary endometrial stromal cells in regard to cell numbers, regulation of some proteins essential in endometriosis and TGF-β receptor interactions. The study will further investigate the signaling pathways of TGF-βs and possible cross-talks with other pathways in endometrial and endometriotic cell lines and primary endometrial stromal cells in vitro. In addition, the different characteristics of normal endometrial cells and endometriotic cells observed in all the above aspects will be reported.

2 Materials and Methods

2.1 Human Immortalized Endometrial, Endometriotic Cell Lines and Primary Endometrial Stromal cells

2.1.1 Endometrial Epithelial and Stromal Cells

The HES cells are a spontaneously immortalized human endometrial epithelial cell line (Fig. 4). The cells express E-cadherin, vimentin and cytokeratin (Desai et al., 1994) and estrogen receptor alpha (Banu et al., 2008).

The T-HESC cells (Fig. 4) are telomerase immortalized endometrial stromal cells (Krikun et al., 2004). T-HESC exhibit considerable and constitutive expression of progesterone receptor mRNA (Leila et al., 2011). The estrogen receptor beta and P-450 aromatase are strongly expressed also in T-HESC (Banu et al., 2008). The cells undergo decidualization after treatment with estrogen plus medroxyprogesterone 17-acetate (Krikun et al., 2004).

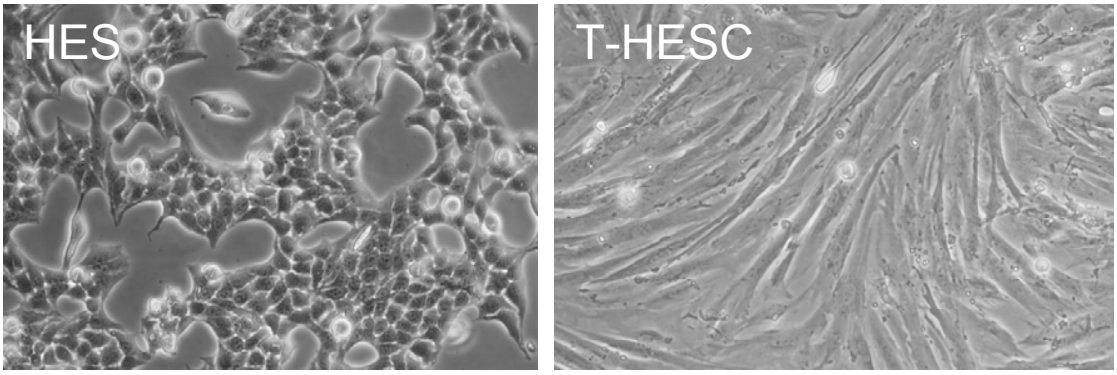


Figure 4. Human endometrial epithelial (HES) and stromal (T-HESC) cells. The HES cells are triangular, flat and strongly aggregate indicating epithelial cell-to-cell contacts. The T-HESC cells show a spindle-shaped, fibroblastoid morphology and are larger in size compared to HES. They are often scattered when their numbers are few, however, they often aggregate in parallel clusters when crowded.

2.1.2 Endometriotic Epithelial and Stromal Cells

12ZVK are epithelial-like endometriotic cells and are immortalized with SV40 (Fig. 5). They express cytokeratins, E-Cadherin and vimentin usually present in endometrial epithelial tissue (Zeitvogel et al., 2001). They express steroidogenic acute regulatory protein, the estrogen receptor α and β, progesterone receptor and steroidal stimulating factor-1 (Banu et al., 2008).

The 22B cells are stromal-like endometriotic cells and have been immortalized with SV40 (Fig. 5). They express cytochrome P-450 mRNA abundantly and estrogen receptor β. 22B are E-Cadherin-negative but they are vimentin-positive (Zeitvogel et al., 2001).

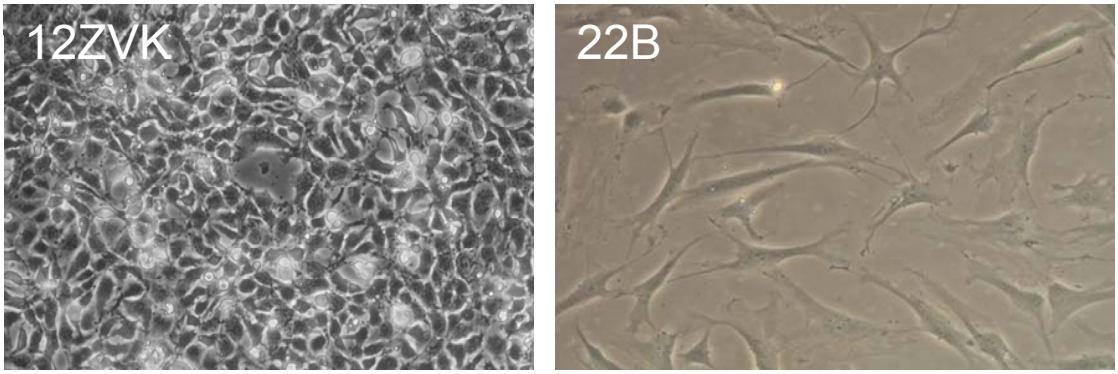
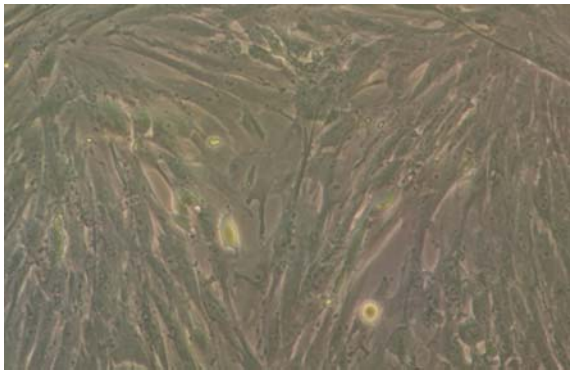


Figure 5. Human endometriotic epithelial (12ZVK) and stromal (22B) cells. The 12ZVK cells are triangular, flat and aggregate closely to each other suggestive of epithelial cell-to-cell contacts. The 22B cells show a spindle-shaped, elongated, fibroblastoid morphology and some of them have a branched cytoplasm. 22B cells often aggregate in parallel clusters when crowded and are scattered when their numbers are few.

2.1.3 Primary Endometrial Stromal Cells

Primary endometrial stromal cells which have been isolated earlier from endometrial tissue obtained from a fertile women in their reproductive age after laporoscopy were characterized using antibodies against cytokeratin 18 and α-smooth muscle actin. They are α-smooth muscle actin positive but cytokeratin 18 negative. Morphologically, they show similar properties like those of the T-HESC cell line (Fig. 6).



Primary Endometrial Stromal Cells

Figure 6. Human endometrial stromal cells show a spindle-shaped, fibroblastoid morphology in vitro. They are often scattered when their numbers are few, however, they often aggregate in parallel clusters when crowded.

2.2 Cell Culture with Endometrial, Endometriotic Cell Lines and Primary Endometrial Stromal Cells

2.2.1 Aseptic Techniques for Cell Culture

Equipments and reagents were either bought sterile or sterilized in our laboratory. Heat-labile solutions were sterilized with filters (pore size 0.22, µm; Millipore). The bench surface of the laminar flow was completely cleared and the surface was wiped with 70% ethanol. The UV light in the laminar was turned on for 30 minutes. Then the hood blower and lights were turned on and the air was allowed to circulate for 20 minutes before use. The items needed were wiped with 70% ethanol just before introducing them into the laminar flow. Latex surgical gloves were worn to avoid contaminations and desinfected with 70% ethanol before starting to work which was repeated in regular intervals.

Cell culture media used were supplemented with 1% pen/strep (100X, PAA, Austria) and 1% plasmocin (Amaxa) or mycokill (10µg/ml, PAA) in order to avoid microbial contaminations like fungi, mycoplasms and bacteria. Transfer and preparations of cell culture media in the laminar was done using sterile media flasks and disposable pipettes to minimize contamination. When adding (or replacing) medium, care was taken not to touch the neck of the culture flasks which were cleaned with sterile cotton swabs after medium transfer before putting the flasks into the incubator.

2.2.2 Cell Culture Media for Endometrial and Endometriotic Cells

Generally, media were supplemented with appropriate amounts of serum, glutamine, antibiotics and other nutrients. All sterile media were prepared before starting to work with cells to avoid contamination and cross-infection during cell culture. All procedures for media preparations were carried out in the laminar to ensure maximal sterility.

The purchased ready-to-use media were stored at 4°C and after reconstitution were stored at 4°C for a maximum of one month. Before cell culture, appropriate amounts of reconstituted media were pre-warmed to 37°C in a water bath for about 20 minutes.

- Cell culture medium for primary endometrial stromal cells, endometrial (HES) and endometriotic (12ZVK) epithelial cells:

  DMEM High Glucose (4.5 g/L) without phenol red (PAA, Austria)
  + 10% fetal calf serum (FCS) (PAA, Austria)
  + 1% 100x pen/strep (PAA, Austria) and 1% L-glutamine (PAA, Austria)

- Cell culture medium for endometrial stromal cells (T-HESC):

  DMEM F12 with L-glutamine without phenol red (Invitrogen, U.S.A.)
  + 10% fetal calf serum (FCS) (PAA, Austria)
  + 1% 100x pen/strep (PAA, Austria) and 1% Insulin Transferrin Selenium X (Invitrogen, U.S.A.)

- Cell culture medium for endometriotic stromal cells (22B):

  DMEM High Glucose (4.5 g/L) without phenol red
  + 10% Charcoal/Dextran Treated FCS (Thermo Scientific, U.S.A.)
  + 1% 100x pen/strep (PAA, Austria) and 1% L-glutamine (PAA, Austria)

2.2.3 Changing Cell Culture Medium

Cell culture flasks were removed from the CO_2 incubator and placed directly in the laminar flow. The medium in the cell culture flask was discarded completely and replaced with fresh pre-warmed medium every 2-3 days depending upon cell confluency. The cap was fixed loosely on the flask so that air can enter into the flask. The date of medium change was recorded on the flask and then returned back to the CO_2 incubator.

2.2.4 Passaging or Splitting of Cells

Cells were passaged regularly at 80% confluency to avoid senescence associated with prolonged high cell density. All old medium was discarded, 10 ml pre-warmed accutase added and the flask was rotated to achieve a complete coverage of the accutase, then the cells were incubated (37°C, 5% CO_2) for 5 to 10 minutes. After microscopic inspection to ensure complete detachment of cells from the flask, it was gently tapped at the sides to release remaining adherent cells. Cells were resuspended in 10 ml fresh serum-containing medium to inactivate accutase, and then transferred into a 50 ml falcon tube and centrifuged for 5 minutes at 1500 x g. The supernatant was discarded and 5 ml of medium was added to re-suspend cell pellets. 500 µl to 2000 µl of cell suspension was transferred into a new cell culture flask with 25 ml fresh medium. The cell passage number and date of passage were documented on the flask. The flask was shaken gently to achieve a uniform cell suspension and then taken back into the incubator.

2.2.5 Cryopreservation of Cells

Cell freezing is essential for long-term storage of cells. The cell pellet obtained by the process of passaging (2.2.4) was resuspended in medium Filoceth^{plus} (Procryoptect, Germany). The cryo-medium reduced the freezing point of media and also allowed a slower cooling rate, greatly reducing the risk of crystal formation which can damage the cells and cause cell death. Aliquots of 1 ml cell suspensions were transferred into cryotubes which were then stored at -80°C overnight for slow cooling before storage in liquid nitrogen.

2.2.6 Thawing Frozen Cells

Proper thawing of cryopreserved cells is crucial to ensure viability and functionality of the cells. The thawing process is done very quickly to ensure that a high proportion of the cells survive the procedure.

Cryotubes were taken out of the liquid nitrogen and immediately placed into a 37°C water bath. The contents of the cryotube were transferred to a 15 ml falcon tube with 9 ml pre-warmed fresh medium. The cell suspension was centrifuged (1500 x g, 5 min) to remove the Filoceth. After centrifugation, the liquid phase was aseptically discarded without disturbing the cell pellet. The remaining pellet was gently resuspended with 1 ml medium and then transferred into a 75 cm² or 150 cm² cell

culture flask containing 14 ml or 24 ml medium, respectively. Mycokill AB (PAA, Austria) was added into the flasks to protect cells from contamination by microorganisms. The cell culture flasks were placed in an incubator (37°C, 5% CO_2).

2.2.7 Counting and Seeding of Cells

Counting of cells was done by adding accutase to the plates (500 µl for 6-well plates, 200 µl for 24-well plates) and then incubated in the incubator for 5-10 minutes. After complete detachment, equal volumes of fresh medium were added and cells were resuspended thoroughly by pipetting up and down. 10 µl of the cell suspension was transferred to a CASY tube with 10 ml CASY-ton solution and mixed well. Measurement of the cells was done with the CASY-counter (Schaerfe System, Germany).

Cells were seeded onto cell culture plates. In my experiments, $2x10^4$ cells were seeded into each well of a 96-well plate (Corning, USA) with 100 µl media containing 10% FCS; $4x10^4$ cells were seeded into each well of a 24-well cell culture plate (TPP, Switzerland) with 1 ml medium containing 10% FCS, and $2x10^5$ to $4x10^5$ cells were seeded into each well of a 6-well plate (TPP, Switzerland) with 4 ml medium containing 10% FCS. The plates were incubated overnight (37°C, 5% CO_2).

2.2.8 Cell Starvation and Stimulation with Recombinant Human TGF-βs

After overnight culturing, the old medium was discarded from the plates. Fresh medium containing 1% FCS was added to starve the cells for an appropriate time (37°C, 5% CO_2).

Cell stimulation was done with rh-TGF-β1 and rh-TGF-β2 (Promokine, Germany). Media containing 1% FCS with 10 ng/ml TGF-β1 or TGF-β2, respectively, were prepared. From the plates with the starved cells, the old medium was removed and fresh medium with 10 ng/ml TGF-β1 or TGF-β2 was added into the appropriate wells, respectively. PBS was added into the control well and then cultured for an appropriate time (37°C, 5% CO_2).

2.3 Analysis of PAI-1 and Inhibin B Secretion

To analyze how TGF-β1 or TGF-β2 influence PAI-1 or inhibin B secretion, PAI-1 or inhibin B ELISAs were performed (Fig. 7) and repeated in three independent experiments.

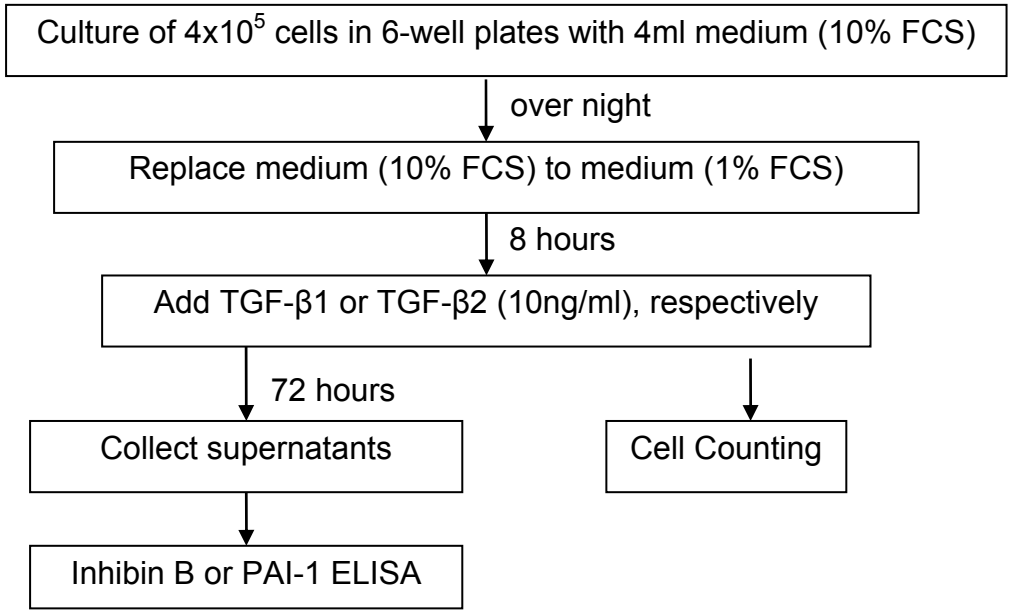


Figure 7. Scheme for analyzing secretion of inhibin B or PAI-1

2.3.1 Culturing of Cells with Specific Intracellular Pathway Inhibitors

Seven inhibitors targeting different pathways were used to investigate the pathways that might be involved in TGF-β signaling.

- Specific Inhibitor of Smad3: SiS3 (Calbiochem, USA; Masatoshi et al., 2005)
- TGF-β receptor type I inhibitor: LY364947 (Sigma-Aldrich, USA; Sawyer et al., 2003)
- BMP inhibitor: LDN 193189 (Stegment, USA; Yu et al., 2008)
- Activin R1A/ALK-2 inhibitor: FC Chimera (R&D Systems, USA; Wu and Hill, 2009)
- BMPR-1A/ALK-3 inhibitor: FC Chimera (R&D Systems, USA; Wu and Hill, 2009)
- BMPR-1B/ALK-6 inhibitor: FC Chimera (R&D Systems, USA; Kawabata et al., 1998)
- IgG1 inhibitor: FC Chimera (R&D Systems, USA)

Culture media with 1% FCS containing either 5 µM LY364947, 2µM SiS3, 5 µM BMP inhibitor, 10 µg/ml ALK-2 inhibitor, 4 µg/ml ALK-3 inhibitor, 6 µg/ml ALK-6 inhibitor and 2 µg/ml IgG1 inhibitor, respectively, were prepared. Media in 6-well plates was discarded. Then 2000 µl media with or without inhibitors was added into the corresponding wells, respectively. After cells were incubated for 2 hours (37°C, 5% CO_2), they were stimulated with 10 ng/ml TGF-β1 or TGF-β2 (Fig. 8).

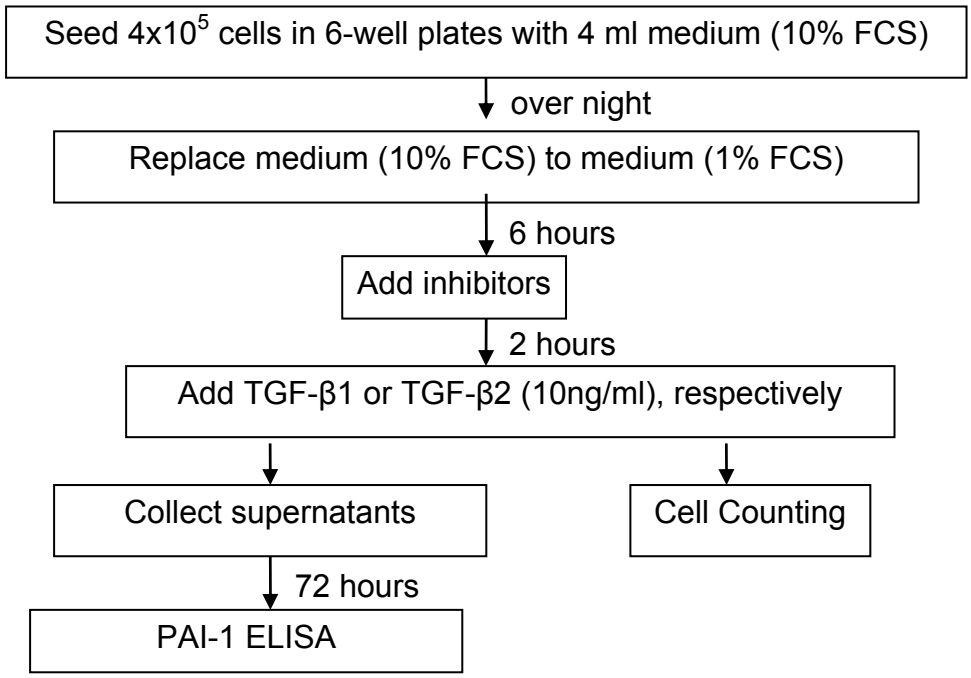


Figure 8. Scheme for analyzing secretion of PAI-1

2.3.2 Supernatants Collection and Cell Quantification

One ml of supernatant was collected from each well and 7 µl Protease Inhibitor cocktail (Sigma-Aldrich, USA) was added to protect against proteases. After vortexing and centrifugation (5000 x g for 10 min at 4°C), 750 µl of the supernatant from each tube was collected and stored as aliquots at -20°C. Quantification of cells was done as described (2.2.7).

2.3.3 PAI-1 ELISAs

The PAI-1 levels in the supernatants were quantitated by use of the TECHNOZYM® PAI-1 Antigen ELISA Reagent Kit (Technoclone, Germany).

The kit contains:

- **Coating Antibody** - a vial with 500µg lyophilized monoclonal anti-PAI-1
- **Conjugate monoclonal anti-PAI-1 POX, dyed blue.**

- **Calibrator-** 0.5ml lyophilized

Preparation of Reagents

Reagents were brought to room temperature before use.

- **Coating Buffer** -2.93 g $NaHCO_3$, 1.59 g Na_2CO_3 diluted with distilled water to a final volume of 1 liter, pH=9.6
- **Phosphate buffered saline (PBS, 1x)** - 8.0 g NaCl, 0.2 g KH_2PO_4, 1.44 g $Na_2HPO_4 \cdot 2H_2O$,0.2 g KCl, dissolved in distilled water to a final volume of 1 liter, pH=7.4
- **Washing Buffer** - 0.5% Tween 20 (Sigma, Germany) in 1x PBS
- **Incubation Buffer** - 1% bovine serum albumin (BSA, Roth, Germany) + 0.01% thimerosal in PBS
- **TMB substrate** (Calbiochem, Germany)
- **Stop Solution** - 2M sulfuric acid

Test Preparation

- **Coating Plate**

After reconstitution of the coating antibody (500 µl distilled water), 100 µl were mixed thoroughly with 10 ml coating buffer. Then 100 µl were pipetted into each well of a 96-well plate (Nunc Maxisorp, Denmark) and the sealed plate was incubated over night at 4°C for at least 16 hours.

- **Reconstituting Calibrator**

The calibrator was reconstituted with 500 µl distilled water (stock solution of 132.7 ng/ml), mixed for 10 seconds and then incubated at room temperature for at least 15 minutes with gentle agitation. 5 microcentrifuge tubes were labeled and 100 µl incubation buffer was added to each tube. Serial dilutions of the 100 µl stock solution were prepared (66.35 ng/ml, 33.18 ng/ml, 16.59 ng/ml, 8.29 ng/ml and 4.15 ng/ml). The last tube contained only incubation buffer which served as a blank (0 ng/ml).

- **Supernatant Samples**

Supernatants were removed from -20°C and put at room temperature. Supernatants from HES, 12ZVK, THESC, 22B and primary endometrial stromal cells were diluted 25-fold, 50-fold, 75-fold, 100-fold and 75-fold with incubation buffer, respectively.

• **Diluting Conjugate Solution**

The conjugate solution was stored as a 50x concentrated solution. One part of the conjugate solution (v/v) was diluted with 50 parts (v/v) of the incubation buffer.

Assay Procedure

All samples, standards and controls were assayed in duplicate.

1. The samples and the reagents were prepared as described above.
2. 100 µl incubation buffer was added into each well and the plate incubated at 37°C for 1 hour.
3. The incubation buffer was discarded and the plate washed with 200 µl wash buffer three times. The wash buffer was discarded.
4. 25 µl standard or sample diluted with 75 µl incubation buffer was added into each well and the plate incubated at 37°C for 1 hour.
5. All liquids were discarded and 100 µl conjugate working solution was added into each well. The plate was incubated at 37°C for 1 hour.
6. All wells were emptied thoroughly and washed with 200 µl wash buffer three times.
7. 100 µl TMB substrate was added into each well and the plate incubated at room temperature for 10 minutes protected from light.
8. 100 µl Stop Solution was added to each well.
9. OD values at 450 nm were measured on a microplate reader, with a reference wavelength set at 620 nm.

2.3.4 Inhibin B ELISAs

Inhibin B levels in the supernatant were measured by using inhibin B Enzyme immunoassay ELISA kits (RayBiotech, USA) detecting inhibin beta B, activin B and activin AB.

The kits contains:

• **Inhibin B Microplate** - A 96-well polystyrene microplate (12 strips of 8 wells) coated with anti-rabbit secondary antibody against human inhibin B

• **Anti-inhibin B polyclonal antibody** - A polyclonal antibody against human inhibin B with preservatives

• **Standard Inhibin B Peptide** - A buffered protein with preservatives

• **Biotinylated Inhibin B peptide** - A buffered protein with preservatives

- **Positive control** - A cell culture medium sample with an expected signal between 10% to 30% of total binding
- **Wash Buffer Concentrate** - A 20x solution of buffered surfactant with preservatives
- **Assay Diluent A** - Contains 0.09% sodium azide as preservative. Diluent for standards and serum or plasma samples
- **Assay Diluent B** - A 5x diluent buffer for standards, cell culture media or other sample types
- **HRP-Streptavidin Concentrate** - 400x HRP-conjugated Streptavidin
- **TMB One-Step Substrate Reagent** - 3, 3', 5,5'- tetramethylbenzidine (TMB) in buffered solution
- **Stop Solution** - 0.2 M sulfuric acid

Reagent Preparation

All kit reagents were kept on ice during reagent preparation. The plate was equilibrated to room temperature.

- **Assay Diluent B** - 15 ml of assay diluent B concentrate was diluted with 60 ml deionized water forming 1x assay diluent B.
- **Anti-inhibin B Polyclonal antibody** - 5 µl of anti-inhibin B polyclonal antibody was added to 50 µl assay diluent B (1x), mixed gently and placed on ice.
- **Biotinylated Inhibin B peptide** - 5 µl of biotinylated inhibin B peptide was added to 5 ml assay diluent B (1x) to a final concentration of 100 pg/ml, mixed gently and placed on ice.

- 10x biotinylated inhibin B peptide was prepared by adding 2 µl of biotinylated inhibin B peptide to 18 µl of assay diluent B.

- **Standards** - Six microcentrifuge tubes were labeled (10,000 pg/ml, 100 pg/ml, 10 pg/ml, 1 pg/ml and 0 pg/ml). Then 450 µl 1x biotinylated inhibin B peptide solution was added to each tube, except to the tube labeled 10,000 pg/ml. In this tube (10,000 pg/ml) 8 µl of standard inhibin B peptide was added with 792 µl of 1x biotinylated inhibin B peptide solution and mixed thoroughly. This was inhibin B stock solution (10,000 pg/ml inhibin B, 100 pg/ml biotinylated inhibin B) and served as the first standard. Then 50 µl of the first standard solution was used to produce dilution series (100 pg/ml, 10 pg/ml and 1 pg/ml). The final tube (0 pg/ml inhibin B, 100 pg/ml biotinylated inhibin B) served as the zero standard.

• **Positive Control** - 100 µl of the positive control was diluted with 101 µl of 1x Assay Diluent B. Also 2 µl of 10x biotinylated inhibin B peptide was added and mixed thoroughly.

• **Wash Buffer Concentrate** - 20 ml Wash Buffer Concentrate was diluted with 380 ml deionized water (1x Wash Buffer).

• **HRP-Streptavidin Concentrate** - The HRP-Streptavidin concentrate was diluted 400-fold with 1x Assay Diluent B.

• **Supernatant Sample** - Supernatants stored at -20°C were thawed at room temperature. 247.5 µl of each sample was added to 2.5 µl of 10x diluted biotinylated inhibin B peptide solution and mixed thoroughly before use.

Assay Procedure

All reagents and samples were kept on ice during reagent preparation steps.

All samples, standards and controls were assayed in duplicate.

1. Reagents and samples were prepared as described above.
2. 100 µl anti-inhibin B antibody was added to each well and the plate incubated overnight at 4°C.
3. Each well was aspirated and washed with 300 µl wash buffer four times.
4. 100 µl standard, control or sample was added to each well and the plate incubated for 2.5 hours on an orbit shaker at RT.
5. Each well was aspirated and washed with 300 µl wash buffer four times.
6. 100 µl of the prepared Streptavidin Solution was added and the plate incubated for 45 minutes on an orbit shaker at RT.
7. 100 µl TMB Substrate Solution was added to each well and the plate incubated for 30 minutes on an orbit shaker at RT protected from light.
8. 50 µl Stop Solution was added to each well.
9. OD values at 450 nm were measured on a microplate reader.

2.4 Phospho-Smad3 ELISAs

To analyze the affect of TGF-β1 or TGF-β2 on phosphorylation of Smad3, cell lysates were collected from all cell lines and primary endometrial stromal cells.

2.4.1 Collection of Cell Lysates for Phospho-Smad3 ELISAs

1. $4x10^5$ cells were seeded in 6-well plates with 4 ml medium (10% FCS) and the plate incubated overnight at 37°C.
2. The medium (10% FCS) was replaced with medium (1% FCS) and the plate incubated for 6 hours at 37°C.
3. SiS3 (3 μM) and Ly364947 (5 μM) inhibitors, respectively, were added and the plate incubated for 2 hours at 37°C.
4. TGF-β1 or TGF-β2 (10ng/ml), respectively, were added and the plate incubated for 30 minutes at 37°C.
5. Media were aspirated from each well and the plate washed once with 4 ml/well ice cold PBS.
6. 400 μl 1x Cell Lysis Buffer was added to each well and the plate incubated for 10 minutes on an orbit shaker at RT.
7. The cell lysates were collected.
8. Phospho-Smad3 ELISA was performed.

2.4.2 Phospho-Smad3 ELISA Assay Procedure

The phosphorylated human Smad3 (Ser423/425) in cell lysates was measured by using The InstantOne™ ELISA Reagent Kit (eBioscience, USA).

The kit contains:

- **InstantOne™ ELISA Assay Plate** - A 96-well polystyrene microplate
- **Phospho-Smad3 (Ser423/425) Capture Antibody Reagent** - Capture antibody reagent which will be mixed in equal parts with the Detection Antibody Reagent to yield the Antibody Cocktail (ELISA antibody sandwich pair).
- **Phospho-Smad3 (Ser423/425) Detection Antibody Reagent** - Detection antibody reagent which will be mixed in equal parts with the Capture Antibody Reagent to yield the Antibody Cocktail (ELISA antibody sandwich pair).
- **Positive Control Cell Lysate** - The Positive Control from various cell types is supplied lyophilized.
- **Enhancer Solution** - The Enhancer Solution is supplied as a concentrate.
- **Cell Lysis Buffer** - The Cell Lysis Buffer is a combination of the Cell Lysis Buffer and Enhancer Solution to yield a versatile Cell Lysis Mix that can be applied to many cells and tissues. The Cell lysis buffer (5x) contains a combination of detergents, phosphatase inhibitors, salt and buffers.

- **Wash Buffer** - The Wash Buffer is supplied as a 10x concentrate.
- **Detection Reagent** (TMB substrate)
- **Stop Solution** - The acidic Stop Solution is used for stopping the HRP-mediated colorimetric conversion.

Reagents Preparation

All reagents were brought to room temperature before use.

- **InstantOne™ ELISA Assay Plate** - Rinse plate with double distilled water prior performing the assay.
- **Antibody Cocktail** - The Antibody Cocktail was made by mixing 200 µl Capture Antibody Reagent with 200 µl Detection Antibody Reagent. Then 50 µl was added to each well.
- **Positive Control Cell Lysate** - The Positive Control Cell Lysate was reconstituted with 250 µl of double distilled water.
- **Wash Buffer** - The Wash Buffer (10x) was diluted to 1x with double distilled water.
- **Enhancer Solution** - The Enhancer Solution was supplied as a concentrate. It was warmed to 37°C and mixed prior to use.
- **Cell Lysis Buffer** - The Cell Lysis Buffer (5x) was prepared by mixing 900 µl Cell Lysis Buffer (5x) with 100 µl Enhancer Solution. The resulting solution was diluted with water to 1x Cell lysis buffer and was used to directly lyse the cells after aspiration of the medium.
- **Detection Reagent** - Warm to room temperature before use.
- **Sample preparation** - Cell lysates were collected earlier as described.

Assay Procedure

All reagents and samples were brought to room temperature before use. All samples and controls were assayed in duplicate. The procedure of the assay is described below

1. Reagents and samples were prepared as described earlier.
2. The desired number of InstantOne microplate strips was determined.
3. 50 µl sample lysates or controls were added into each well.
4. 50 µl of freshly prepared antibody cocktail was added to each of the test wells and the plate incubated for 1 hour on an orbit shaker at RT.
5. The liquid phase was discarded and wells were washed with 200 µl wash

buffer three times.

6. 100 µl Detection Reagent was added into each well and the plate was incubated for 30 minutes at RT protected from light.
7. 100 µl Stop Solution was added to each well.
8. The OD values at 450 nm were measured on a microplate reader, with the correction wavelength at 570 nm.

2.5 Cell Apoptosis Assays

Cell apoptosis assays were done to investigate whether or not the effect of TGF-β1 or TGF-β2 on cell numbers is through apoptosis and which apoptotic pathways might be involved.

2.5.1 Stimulation of Cells with TGF-β1 or TGF-β2

Cells were treated with or without TGF-β1 or TGF-β2 (10 ng/ml) and with or without the SiS3 (3 µM) and Ly364947 (5 µM) inhibitors, respectively. Staurosporine (0.1 µM, Promokine, USA) was used as a positive control while growth medium alone was used as blank. Cells were treated for 24 hours and then apoptosis assays were performed. The apoptotic parameters investigated included quantification of phosphatidylserine on the outer surface of the cell membrane, measurement of the inner mitochondrial membrane potential and quantification of Cysteinyl-asparate-specific proteases 3/7 (Caspase 3/7) levels (Fig. 9).

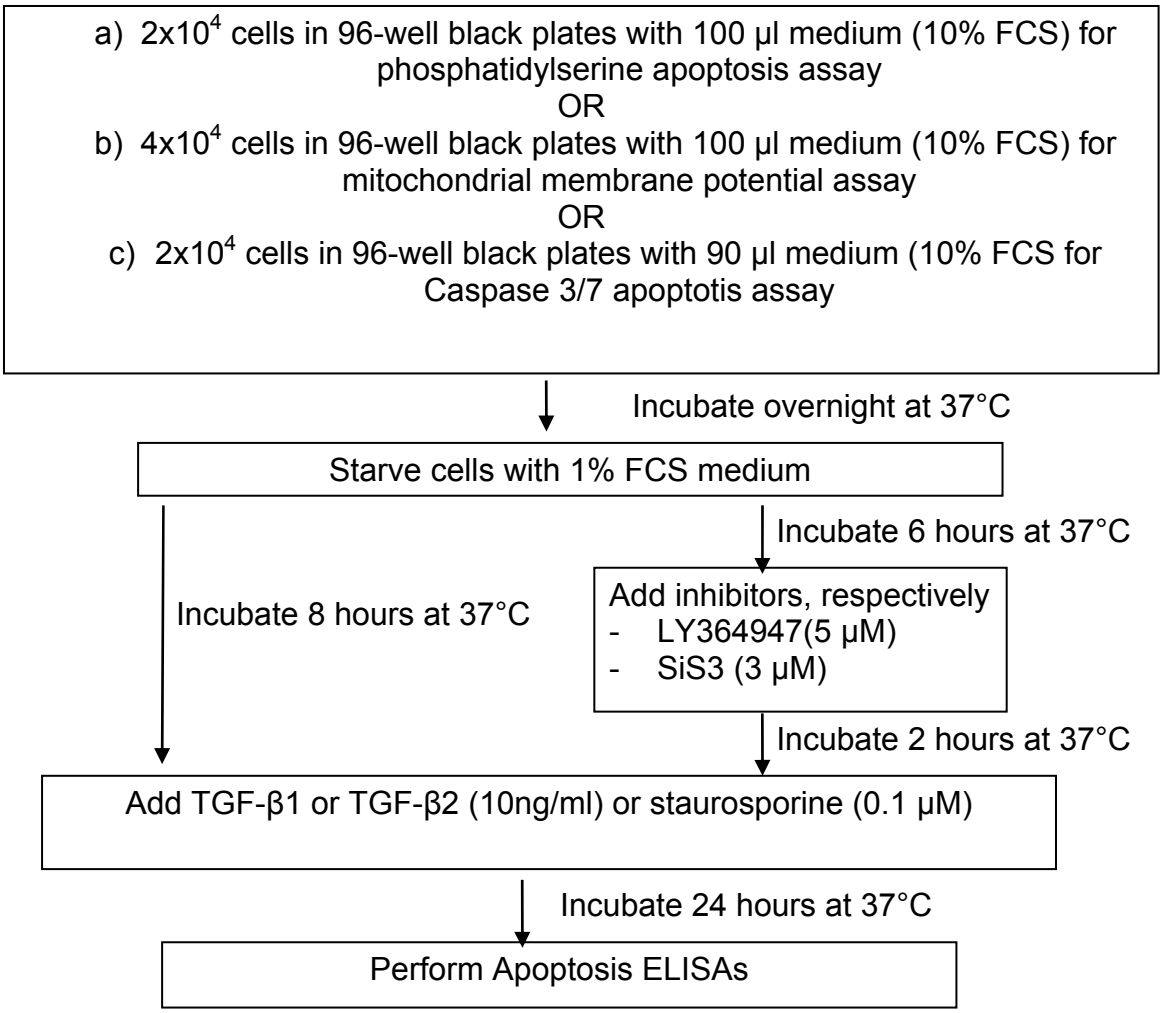


Figure 9. Scheme of treatment of cells for Apoptosis ELISAs

2.5.2 Phosphatidylserine Apoptosis ELISA

The effect of the TGF-βs on translocation of phosphatidylserine to the outer surface of the cell membrane was measured by the Cell meter™ Phosphatidylserine Apoptosis assay Kit (AAT Bioquest, USA).

The kit contains: **Apopxin™ Orange**(100X Stock solution); **Assay Buffer** (10ml).

Preparation of Reagents

All reagents were brought to room temperature before use.

- **Apopxin™ orange assay working solution** –10 µl of Apopxin™ Orange was added into 1 ml Assay Buffer and mixed well. The mixture was protected from light. Always a fresh mixture was prepared prior to use.

Assay Procedure

A 96-well plate (Corning, USA) with cells treated as indicated (2.5.1) was removed from the incubator after 24 hours incubation. All samples and controls were assayed in duplicate. The procedure is described below

1. Reagents and samples were prepared as described above.
2. The 96-well plate with cells treated was removed as indicated (2.5.1).
3. 100 µl of freshly prepared Apopxin™ orange assay working solution was added to each of the test wells and the plate incubated for 1 hour at RT protected from light.
4. The fluorescence intensity was measured at Excitation/Emission of 540/590 nm.

2.5.3 Mitochondrial Membrane Potential Apoptosis ELISA

The effect of the TGF-βs on the loss of the mitochondrial membrane potential by the cells was measured by the Cell meter™ Mitochondrial Membrane Potential Apoptosis assay Kit (AAT Bioquest, USA).

The kit contains: **MitoLite™ Orange** (200X Stock solution); **Assay Buffer A** (50 ml); **Assay Buffer B** (25 ml).

Preparation of Reagents

All reagents were brought to room temperature before use.

- **MitoLite™ Orange dye-loading solution** – 50 µl of MitoLite™ Orange was added to 10 ml Assay Buffer A and mixed well. The unused 200x MitoLite™ Orange was aliquoted and stored at -20°C.
- **Assay Buffer B** - Ready to use.

Assay Procedure

A 96-well plate (Corning, USA) with cells treated as indicated (2.5.1) was removed from the incubator after 24 hours of incubation. All samples and controls were assayed in duplicate. The procedure is described below

1. Reagents and samples were prepared as described above.
2. The 96-well plate with cells treated was removed as indicated (2.5.1).
3. The test wells were emptied and 100 µl of freshly prepared MitoLite™ Orange dye-loading solution was added to each of the test wells. The plate was incubated for 30 minutes at RT protected from light.

4. 50 µl of Assay Buffer B was added to each of the test wells.
5. The fluorescence intensity was measured at Excitation/Emission= 540/590 nm.

2.5.4 Caspase 3/7 Activity Apoptosis ELISA

The effect of the TGF-βs on Caspase 3/7 activation by the cells was measured by the Cell meter^{TM} Caspase 3/7 Activity Apoptosis assay Kit (AAT Bioquest, USA).

The kit contains: **Caspase 3/7 Substrate** (200x Stock solution); **Assay Buffer** (20ml).

Preparation of Reagents

All reagents were brought to room temperature before use.

- **Caspase 3/7 assay loading solution** - 50 µl of Caspase 3/7 Substrate was added to 10 ml Assay Buffer and mixed well. The unused 200x Caspase 3/7 substrate and assay buffer were aliquoted and stored at -20°C.

Assay Procedure

The 96-well plate (Corning, USA) with cells treated as indicated (2.5.1) was removed from the incubator after 24 hours of incubation. All samples and controls were assayed in duplicate. The procedure is described below

1. Reagents and samples were prepared as described above.
2. The 96-well plate with cells treated was removed as indicated (2.5.1).
3. 100 µl of freshly prepared Caspase 3/7 assay loading solution was added to each of the test wells. The plate was incubated for 1 hour at RT protected from light.
4. 50 µl of Assay Buffer B was added to all wells.
5. The fluorescence intensity was measured at Excitation/Emission of 350/450 nm.

2.6 Cell Surface ELISAs

The Cell surface ELISA was used to investigate the effects of TGF-β1 or TGF-β2 on cell surface expression of TβRIII in HES, T-HESC, 12ZVK, 22B and primary endometrial stromal cells.

2.6.1 Preparation of Cells

The cells were prepared as described below

1. A 24-well plate was coated with TGF-β1 or TGF-β2 (10 ng/ml), respectively and the plate incubated overnight at 4°C.
2. PBS was aspirated from each well and the plate washed once with ice cold PBS, 4 ml per well.
3. $4x10^4$ cells were seeded in a 24-well plate with 1 ml medium (10% FCS) and incubated for 48 hours at 37°C, 5% CO_2.
4. The Cell-Surface ELISA was performed.

2.6.2 Cell Surface ELISA Procedure

Preparation of Reagents

- **Phosphate buffered saline (PBS)** - 8.0 g NaCl, 0.2 g KH_2PO_4, 1.44 g $Na_2HPO_4 \cdot 2H_2O$, 0.2 g KCl, diluted with distilled water to a final volume of 1 liter, pH=7.4
- **4% paraformaldehyde** - 4.0 g paraformaldehyde (ROTH, Germany) were dissolved in 10ml PBS (10X) and diluted with distilled water to a final volume of 100ml, pH=7.3
- **Binding buffer** - 2.86 g HEPES, 8.18 g NaCl and 0.37 g KCl, diluted with distilled water to a final volume of 1 liter, pH=7.4
- **5% BSA in Binding buffer** - 2.5 g BSA in 50ml Binding buffer prepared above. Freshly made prior to the experiment.
- **Primary antibody** - Rabbit TβRIII monoclonal antibody (R&D Systems, USA). Diluted with 5% BSA binding buffer (1:200)
- **Secondary antibody** - Rabbit Anti-Goat IgG/HRP (DAKO, USA). Diluted with 5% BSA binding buffer (1:2000)
- **TMB substrate** (Calbiochem, Germany)
- **Stop Solution** - 0.18N sulfuric acid

Assay Procedure

All samples and controls were assayed in duplicate. The procedure of the assay is summarized below

1. Reagents and samples were prepared as described above.

2. A 24-well plate with cells treated as indicated (2.6.1) was put on ice.
3. The medium (10% FCS) was aspirated from each test well.
4. All wells were washed once with 1 ml ice cold binding buffer.
5. Cells were fixed with 500 µl ice cold 4% paraformaldehyde and the plate was incubated for 15 minutes at RT.
6. Wells were washed with 1 ml ice cold binding buffer three times.
7. Wells were blocked with 500 µl of 5% BSA binding buffer. The plate was incubated for 1 hour at RT.
8. 300 µl Primary antibody was added to each well. The plate was incubated for 1 hour at RT.
9. Wells were washed with 1 ml ice cold binding buffer three times.
10. 300 µl Secondary antibody was added to each well. The plate was incubated for 1 hour at RT protected from light.
11. Wells were washed with 1 ml ice cold binding buffer three times.
12. 300 µl TMB Substrate was added to each well. The plate was incubated for 1 hour at RT.
13. 300 µl Stop Solution was added to each well.
14. OD values at 450 nm were measured on a microplate reader, with the correction wavelength at 570 nm.

2.7 TGF-β1 and TGF-β2 ELISA

The TGF-β1 or TGF-β2 ELISA was performed to investigate the pathways after treatment of cells with TGF-β1 or TGF-β2. All cell lines and primary endometrial stromal cells were treated with TGF-β1 or TGF-β2 (10 ng/ml), respectively. Then cell supernatants were collected after 48 hours upon TGF-β1 or TGF-β2 treatment to quantitate TGF-β1 or TGF-β2 (Fig. 10).

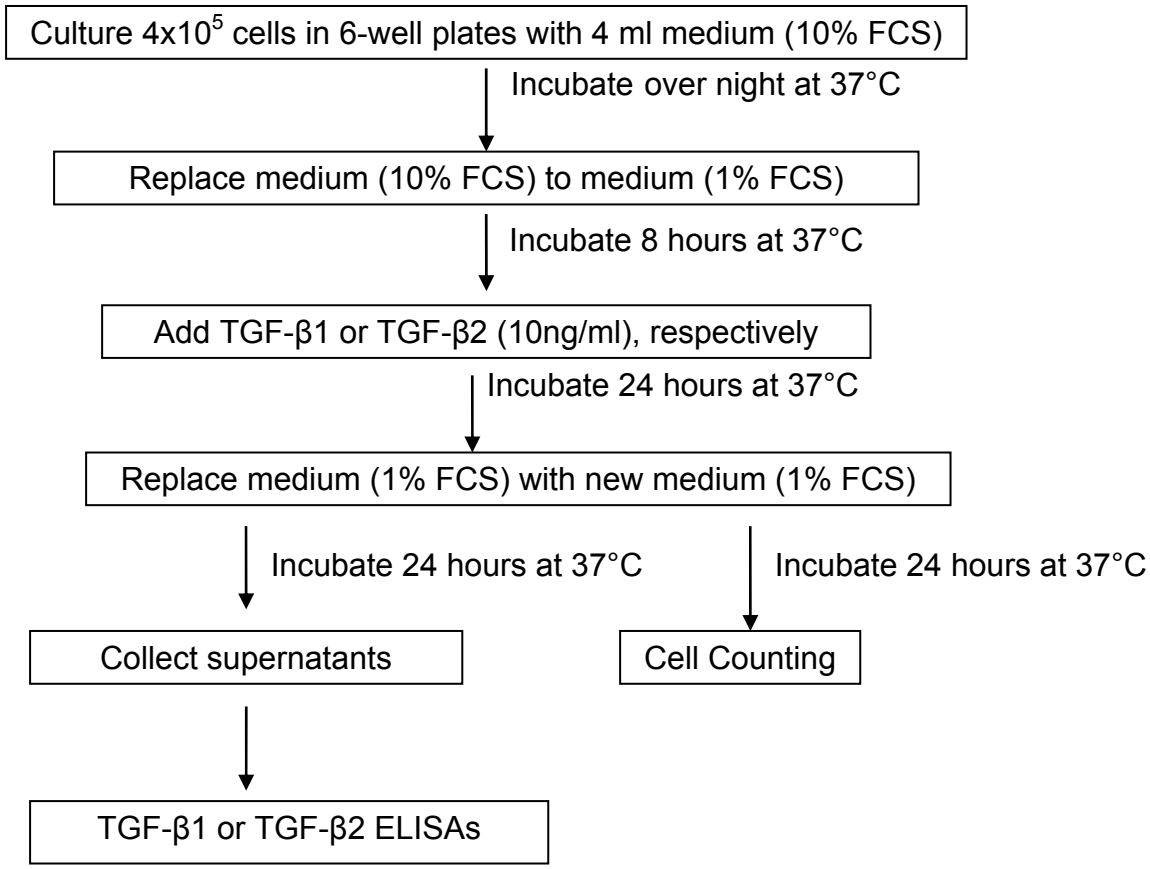


Figure 10. Scheme for analyzing the collected supernatants for TGF-β1 or TGF-β2 ELISAs

2.7.1 TGF-β1 or TGF-β2 ELISA Reagent Preparation

The TGF-β1 and TGF-β2 levels in the supernatants was measured by use of the TGF-β1 and TGF-β2 DuoSet Development Kits (R&D Systems, USA).

a) TGF-β1 DuoSet Development Kit contains:

- **Capture Antibody** - 360 µg/ml of mouse anti-TGF-β1 when reconstituted with 1 ml PBS
- **Detection Antibody** - 54 µg/ml of biotinylated chicken anti-human TGF-β1 when reconstituted with 1ml Reagent Diluent
- **Standard** - 140 ng/ml of recombinant human TGF-β1 when reconstituted with 0.5 ml Reagent Diluent
- **Streptavidin-HRP** - 1.0 ml of streptavidin conjugated to horseradish-peroxidase

Preparation of Reagents

All reagents were brought to room temperature before use.

- **Phosphate buffered saline (PBS)** - 137mM NaCl, 1.5mM KH_2PO_4, 8.1mM $Na_2HPO_4 \cdot 2H_2O$, 2.7mM KCl, diluted with distilled water to a final volume of 1 liter, pH=7.4, 0.2µm filtered
- **Wash Buffer** - 0.05% Tween 20 (Sigma, Germany) in PBS, pH=7.2-7.4
- **Block Buffer** - 5% Tween 20 (Sigma, Germany) in PBS
- **Reagent Diluent** - 1.4% delipidized BSA, 0.05 % Tween 20 (Sigma, Germany) in PBS pH=7.2-7.4, 0.2 µm filtered
- **TMB substrate** (Calbiochem, Germany), **Stop Solution** (2 N sulfuric acid), **1N HCl** (Roth, Germany), **1N NaOH** (Roth, Germany) and **0.5M HEPES** (Roth, Germany)

Test Preparation

- **Reconstituting Capture Antibody**

Reconstitution of the capture antibody was reconstituted with 1 ml PBS, aliquoted and stored at -80°C. One aliquot was diluted to a working concentration of 2.0 µg/ml in PBS and 100 µl of this dilution was pipetted into each well of a 96-well ELISA plate (Nunc Maxisorp, Denmark). Then the plate was incubated over night (at least 16 hours) at 4°C.

- **Reconstituting Detection Antibody**

The detection antibody was reconstituted with 1 ml Reagent Diluent, aliquoted and stored at -80°C. One aliquot was diluted to a working concentration of 300 ng/ml in Reagent Diluent and 100 µl of this dilution was pipetted into each well of a 96-well ELISA plate (Nunc Maxisorp, Denmark).

- **Reconstituting Standard**

The standard was reconstituted by mixing for 10 seconds with 0.5 ml Reagent Diluent (stock solution of 140 ng/ml), and kept at room temperature for at least 15 minutes with gentle agitation prior making dilutions. Aliquots were made and stored at -80°C. One aliquot was diluted to a working concentration of 2000 pg/ml in Reagent Diluent. Meanwhile, 7 microcentrifuge tubes were labeled and then 500 µl Reagent Diluent was added to each tube. Then 500µl stock solution was used to make serial dilutions

(1000 pg/ml, 500 pg/ml, 250 pg/ml, 125 pg/ml, 62.5 pg/ml and 31.25 pg/ml). The last tube contained only Reagent Diluent served as a standard of 0 pg/ml.

b) TGF-β2 DuoSet Development Kit contains:

- **Capture Antibody** - 360 µg/ml of mouse anti-TGF- β2 after reconstitution with 1 ml PBS
- **Detection Antibody** - 27 µg/ml of biotinylated goat anti-human TGF-β2 when reconstituted with 1 ml PBS
- **Standard** - 70 ng/ml of recombinant human TGF-β2 when reconstituted with 0.5 ml Reagent Diluent
- **Streptavidin-HRP** - 1 ml streptavidin conjugated to horseradish-peroxidase

Preparation of Reagents

All reagents were brought to room temperature before use.

- **Phosphate buffered saline (PBS)** - 137 mM NaCl, 1.5 mM KH_2PO_4, 8.1 mM $Na_2HPO_4 \cdot 2H_2O$, 2.7 mM KCl, diluted with distilled water to a final volume of 1 liter, pH=7.4, 0.2 µm filtered
- **Wash Buffer** - 0.05% Tween 20 (Sigma, Germany) in PBS, pH=7.2-7.4
- **Block Buffer** - 5% Tween 20 (Sigma, Germany) in PBS
- **Reagent Diluent** - 1% BSA in PBS, pH=7.2-7.4, 0.2 µm filtered
- **TMB substrate** (Calbiochem, Germany), **Stop Solution** (1N sulfuric acid), **1N HCl** (Roth, Germany), **1N NaoH** (Roth, Germany) and **0.5M HEPES** (Roth, Germany)

Test Preparation

- **Reconstituting Capture Antibody**

The capture antibody was reconstituted with 1 ml PBS, aliquoted and stored at -80°C. One aliquot was diluted to a working concentration of 2.0 µg/ml in PBS and 100 µl of this dilution was pipetted into each well of a 96-well ELISA plate (Nunc Maxisorp, Denmark). Then the plate was incubated over night (at least 16 hours) at 4°C.

- **Reconstituting Detection Antibody**

The detection antibody was reconstituted with 1 ml Reagent Diluent, aliquoted and stored at -80°C. One aliquot was diluted to a working concentration of 150 ng/ml in

Reagent Diluent and 100 µl of this dilution was pipetted into each well of a 96-well ELISA plate (Nunc Maxisorp, Denmark).

- **Reconstituting Standard**

The standard was reconstituted by mixing for 10 seconds with 0.5 ml Reagent Diluent (stock solution of 70 ng/ml), and kept at room temperature for at least 15 minutes with gentle agitation prior making dilutions. Aliquots were made and stored at -80°C. One aliquot was diluted to a working concentration of 2000 pg/ml in reagent diluent. Meanwhile, 7 microcentrifuge tubes were labeled and then 500 µl reagent diluent was added to each tube. Then 500 µl stock solution was used to make serial dilutions (1000 pg/ml, 500 pg/ml, 250 pg/ml, 125 pg/ml, 62.5 pg/ml and 31.25 pg/ml). The last tube contained only reagent diluent served as a standard of 0 pg/ml.

- **Reconstituting Streptavidin-HRP for TGF-β1 and TGF-β2 ELISAs**

The Streptavidin-HRP was diluted in Reagent Diluent (1:200).

- **Activation of latent TGF-βs for Both TGF-β1 and TGF-β2 ELISAs**

Supernatants from HES, 12ZVK, T-HESC, 22B and primary endometrial stromal cells (Fig. 10) were activated by HCl at room temperature. 100 µl supernatant sample and 3.5 µl HCl was added into each microcentrifuge tube, vortexed, placed on ice and incubated for 1 hour at 4°C. Neutralization was done by adding 7 µl NaOH and 9 µl HEPES, after vortexing thoroughly, 1 µl was taken from each tube for pH testing using a pH paper.

2.7.2 TGF-β1 and TGF-β2 ELISAs Assay Procedure

The same assay procedure was used for TGF-β1 and TGF-β2 ELISAs after respective reagent preparations as described.

All samples, standards and controls were assayed in duplicate. The procedure of the assay is summarized below

1. Reagents and samples were prepared as described above.
2. The Capture Antibody was also prepared as described above.
3. 100 µl diluted Capture Antibody was added into each well. The plate was incubated overnight at 4°C.
4. Test wells were emptied and washed with 400 µl wash buffer three times.

5. 300 µl block buffer was added into each well. The plate was incubated for 1 hour at RT.
6. Test wells were emptied and washed with 400 µl wash buffer three times.
7. 100 µl standard or sample or controls was added and the plate incubated for 2 hours at RT.
8. Test wells were emptied and washed with 400 µl wash buffer three times.
9. 100 µl diluted Detection Antibody was added into each well. The plate was incubated for 2 hours at RT.
10. Test wells were emptied and washed with 400 µl wash buffer three times.
11. 100 µl diluted Streptavidin-HRP was added into each well. The plate was incubated for 20 minutes at RT protected from light.
12. Test wells were emptied and washed with 400 µl wash buffer three times.
13. 100 µl TMB substrate was added into each well. The plate was incubated for 20 minutes at RT protected from light.
14. 100 µl Stop Solution was added to each well.
15. OD values at 450 nm were measured on a microplate reader with the reference wavelength at 570 nm.

2.8 Characterization of Endometrial and Endometriotic Cells

Purity of endometrial and endometriotic cells during the cell culture was assessed by morphological analysis and by expression analysis with cytokeratin 18 and α-smooth muscle actin monoclonal antibodies using immunofluorescence and Western blotting assays. Characterization was done to assess the purity of stromal or epithelial cells

2.8.1 Characterization of Endometrial and Endometriotic Cells by Immunofluorescence

The purity of endometrial and endometriotic epithelial cell cultures was characterized by indirect immunofluorescence using cytokeratin18 monoclonal antibody (Epitomics; Fig. 11).

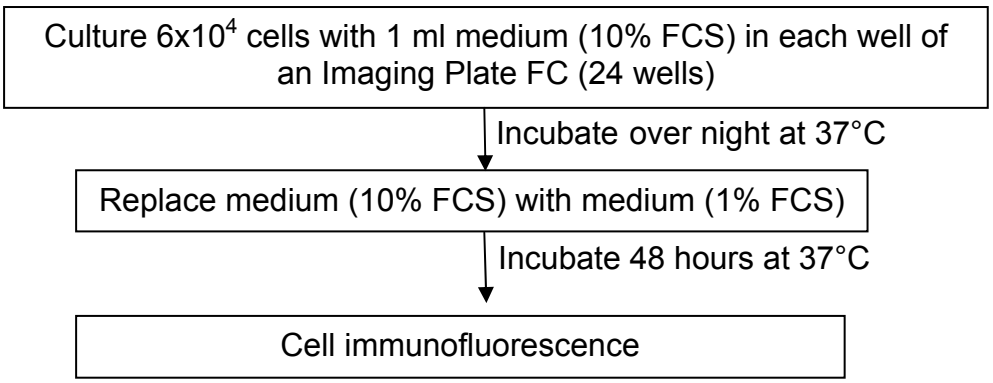


Figure 11. Procedure of preparation of cells for immunofluorescence

2.8.1.1 Preparation of Cells for Immunofluorescence Assay

After counting, $6x10^4$ cells were seeded into each well of a 24-well Imaging Plate FC (Zell-Kontact, Germany), designed specially for immunofluorescence, with 1 ml medium containing 10% FCS. After culturing overnight (37°C, 5% CO_2), cells were starved (2.2.9) for 48 hours.

2.8.1.2 Cell Immunofluorescence Assay Procedure

After an incubation of 48 hours (2.8.1.1) medium was removed and the immunofluorescence assay performed. Images were evaluated under a microscope (Olympus, Germany).

Preparation of Reagents:

- **Blocking buffer** - 3% bovine serum albumin (BSA) in TBST
- **Diluting buffer** - 1% bovine serum albumin (BSA) in TBST
- **Phosphate buffered saline (PBS)** - 0.2 g KCl, 0.2 g KH_2PO_4, 8.0 g NaCl, 1.44 g $Na_2HPO_4 \cdot 2H_2O$ diluted with distilled water to a final volume of 1 liter, pH=7.4
- **Fixation buffer** - Ice cold acetone and methanol mixture (1:1)
- **Tris buffered saline Tween-20 (TBST)** - 8.0 g NaCl, 0.2 g KCl, 3 g Tris-base, 500 µl Tween-20 diluted with distilled water to a final volume of 1 liter, pH=7.4
- **First antibody** – Cytokeratin 18 (Epitomics) from rabbit, 1:200 diluted in diluting buffer
- **Second antibody** - Goat anti-rabbit IgG labelled with Alexa Fluor® 546 dye (Invitrogen), 1:200 diluted in diluting buffer

- **Nuclei staining buffer** - Hoechst dye (Invitrogen), 1:1000 diluted in PBS

Cell Immunofluorescence Assay

1. After 48 hours (2.8.1), all wells were emptied and washed with PBS for 2 times.
2. 200 µl fixation buffer was added to each well. The plate was incubated for 10 minutes on an orbit shaker at RT.
3. Wells were emptied and then washed with PBS for 3 times.
4. 200 µl blocking buffer was added into each well. The plate was incubated for 1 hour on an orbit shaker at RT.
5. The blocking buffer was removed from each well.
6. Immediately, 200 µl first antibody was added into each well. The plate was incubated overnight at 4°C.
7. Wells were emptied and then washed with PBS for 3 times.
8. 200 µl second antibody was added into each well. The plate was incubated for 1 hour at RT protected from light.
9. Wells were emptied and then washed with PBS for 3 times protected from light.
10. 200 µl nuclei staining buffer was added into each well, protected from light and incubated for 5 minutes on an orbit shaker at RT.
11. Wells were emptied and then washed with PBS for 3 times.
12. 500 µl PBS was added into each well and the plate was protected from light.
13. Observation was done under a confocal microscope.

2.8.2 Characterization of Endometrial and Endometriotic Cells by Western Blotting

The CK18 and α-smooth muscle actin monoclonal antibodies were used to analyse the purity of the lysates from the cell lines by Western blotting assay comparable to the immunofluorescence analysis.

2.8.2.1 Preparation of Cells for Western Blot Assay

The procedure for preparation of endometrial and endometriotic cells for Western blotting assay is described below

1. Cells were cultured with 10% FCS medium and incubated overnight at 37°C,

5% CO_2.

2. Medium was removed and cells washed once with 15 ml ice-cold PBS on ice.
3. The ice-cold PBS was removed and 10 ml ice-cold PBS added on ice.
4. Cells were scrapped and transferred to labeled and weighed 50 ml falcon tube.
5. The cell culture culture flasks were rinsed with 5 ml ice cold PBS and cell contents transferred into appropriate 50 ml falcon tube.
6. The falcon tube were centrifuged for 5 minutes at 1200x g at 4°C.
7. Supernatants were discarded and the 50 ml falcon tube together with cell pellets was weighed.
8. The 50 ml falcon tube containing the pellets was transferred into the liquid nitrogen tank for 5 minutes.
9. The 50ml falcon tube with the cell pellet was stored at -80°C.

2.8.2.2 Isolation of Proteins from Cells

Cells stored in -80°C (2.8.2.1) were removed and proteins were isolated.

Reagents preparation:

• **Homogenization Buffer** - 7.88g Tris-HCl, 0.416 g EDTA, 1 ml of 10mM DTT, 1 ml of 100 mM PMSF diluted with distilled water to a final volume of 1 liter, pH=7.4

Protein Isolation Procedure

1. Stored cells were removed from -80°C (2.8.2.1) and put on ice.
2. 0.1g of cells were resuspended in 1 ml ice-cold homogenization buffer.
3. The mixture was put in a Potter-Elvehjem Tissue Grinder (Homogenizer).
4. The pellet was crushed with a pestle and the mixture transferred into a 15 ml falcon tube.
5. The Potter-Elvehjem Homogenizer was rinsed with 1 ml ice-cold homogenization buffer and the cell contents transferred into a 15 ml falcon tube.
6. Centrifugation was done for 8 minutes at 3000g at 4°C.
7. The liquid phase was collected and the pellet was discarded.
8. The proteins in the liquid phase were quantified using the BCA method.

2.8.2.3 Protein Quantification

Protein quantification was performed to ensure equivalent loading of samples on the gels. The proteins isolated from cells (2.8.2.2) were quantified with the Pierce bicinchonic acid (BCA) protein assay (Thermo Scientific, USA).

The kit contains:

- **BCA Reagent A** - 500 ml of sodium carbonate, sodium bicarbonate, bicinchonic acid and sodium tartrate in 0.1M NaOH.
- **BCA Reagent B** - 25 ml of 4% cupric sulfate
- **BCA Working Reagent** - 50 parts of BCA Reagent A with 1 part of BCA Reagent B (50:1).
- **Albumin Standard Ampules** - 2 mg/ml in 1 ml ampules, containing bovine serum albumin (BSA) at 2 mg/ml in 0.9% saline and 0.05% sodium azide

Preparation of reagents:

- **Diluted Albumin (BSA) Standards** - The BSA standards were prepared in BCA working reagent to get serial dilutions (25-2,000 µg/ml). The BCA working Reagent alone was used as a blank.

Assay procedure.

1. Reagents and samples were prepared as described above.
2. 25 µl standards or sample were added to each well of 96-well plate.
3. 200 µl working reagent was added to each well. The plate was mixed thoroughly on a shaker for 30 seconds and incubated for 30 minutes at 37°C.
4. The OD values were measured at 562 nm on a microplate reader.
5. Aliquots of quantified samples were stored at -80 °C until use in Western blot experiments.

2.8.2.4 Preparation of Gels

10% gels were prepared for separation.

Preparation of reagents:

- **Separating buffer -** 1.5 M Tris Base, 0.4% SDS, diluted with double distilled water to a final volume of 500 ml, the pH was adjusted to 8.8 with HCl and stored at 4 °C until use.

• **Stacking buffer -** 1 M Tris Base, 0.4% SDS, diluted with double distilled water to a final volume of 200 ml, the pH was adjusted to 6.8 with HCl and stored at 4 °C until use.

• **Ammonium persulphate (APS) -** 10% APS in double distilled water, aliquoted and stored at -20°C until use.

•**TEMED** (Roth, Germany)

• **Acrylamide/ Bisacrylamide solution** (AppliChem, Germany)

Assay Procedure (one gel)

All reagents were brought to room temperature before use. The gel cast was assembled with 1.5 mm spacers and tested with double distilled water for leaks. First the separation gel (10%) was prepared with 3.1 ml separation buffer, 4.1 ml acrylamide/bisacrylamide solution and 8.5 µl TEMED in 5.2 ml double distilled water. 85 µl ammonium persulphate was added to start the crosslinking reaction. This solution was immediately transferred into the gel cast with a plastic Pasteur pipette and overlaid with isobutanol, to stop interference of air with the crosslinking reaction. The remaining solution was used to confirm polymerization, which takes approximately 20 minutes. The isobutanol was poured off slowly, the top of the gel rinsed with double distilled water and dried with a blotting paper.

The stacking gel (5%) was prepared with 1.25 ml stacking buffer, 825 µl acrylamide/bisacrylamide solution and 5 µl TEMED in 2.85 ml double distilled water. 50 µl ammonium persulphate was added to start the crosslinking reaction. This solution was immediately overlaid on the separating gel and the gel comb inserted carefully to avoid bubbles. The remaining solution was used to confirm polymerization, which takes approximately 20 minutes.

2.8.2.5 SDS-polyacrylamide Gel Electrophoresis and Protein Transfer

Preparation of reagents:

• **5x SDS running buffer -** 0.125 M Tris Base, 1.25 M Glycine, 10% SDS, diluted with double distilled water to a final volume of 2000 ml and stored at 4 °C until use.

• **Blotting buffer -** 1.0 M Tris Base, 1.25 M Glycine, 1000 ml methanol, diluted with double distilled water to a final volume of 2000 ml. The pH was adjusted to 8.1-8.4 and the buffer stored at 4 °C until use.

- **4x Sample buffer - 12.5ml stacking buffer,** 5% SDS, 40% Glycerin, 0.004% bromophenol blue, diluted with double distilled water to a final volume of 50 ml. The pH was adjusted to 6.8 with HCl and the buffer stored at room temperature until use.
- **Human Uterus Protein Medley (Clontech, USA)** - 10 mg/ml protein solution in Laemmli buffer.
- **Spectra Multicolour Broad Range Protein Ladder** (Thermo Scientific, USA).

Assay Procedure

All reagents were brought to room temperature before use. The gel assembly was taken out of the casting apparatus and put into the gel tank. 5x SDS running buffer was diluted to 1x and then poured into the tank so that the buffer level was above the top of the gel (approximately 900 ml). The comb was removed and the wells rinsed with the running buffer with the gel still in the gel tank. Aliquots of the cell lysates (2.8.2.3) were removed from -80°C, appropriate volumes were transferred to eppendorf tubes, sample buffer was added at $1/6^{th}$ of the volume of the lysate and then boiled (100°C) for 5 minutes. 30 µg of the lysates were loaded into the wells using gel loading tips, run against a protein standard (Spectra Multicolour Broad Range Protein Ladder) and a positive control (Human Uterus Protein Medley). Gels were electrophoresed at 80V for two hours.

A PVDF membrane (Millipore, USA) was cut to the exact size of the gel and soaked in methanol (ten seconds), double distilled water (two minutes) followed by full immersion in blotting buffer. Blotting paper and sponges were also soaked in blotting buffer. The gels were removed from the electrophoresis tank and carefully removed from the glass plates. The gels were sandwiched in a transfer cassette (Fig. 12). The transfer cassette was loaded into the gel tank and an ice pack was added before adding blotting buffer. Electrophoresis was performed at 100V for 90 minutes.

White side of transfer cassette
Sponge
Blotting paper
PVDF membrane
Gel
Blotting paper
Sponge
Black side of cassette

Figure 12. Scheme for assembly of SDS-PAGE transfer cassette

2.8.2.6 Immunoblotting

Immunoblotting of the PVDF membrane was performed after protein transfer.

Preparation of reagents:

• **Tris buffered saline Tween-20 (TBST) tablets** – One tablet of TBST (Medicago, Sweden) diluted with double distilled water to a final volume of 500 ml.

• **Blocking buffer** – 5 % Skim Milk Powder (Fluka BioChemika, Zwitzerland), in TBST

• **Primary antibodies** – Cytokeratin 18 (Epitomics), from rabbit, 1:1000 diluted in blocking buffer

– α-smooth muscle actin (DAKO), from mouse, 1:500 diluted in blocking buffer

• **Secondary antibodies** – Anti-rabbit Alkaline Phosphatase (Perkin Elmer, USA), 1:2000 diluted in blocking buffer for the Cytokeratin 18 antibody

– Anti-mouse Alkaline Phosphatase (Perkin Elmer, USA), 1:20000 diluted in blocking buffer for α-smooth muscle actin

• **Western LightningTM CDP**-Star Chemiluminescence Reagent (Perkin Elmer, USA).

Immunoblotting assay procedure

Following protein transfer, the membrane was blocked with blocking buffer (5 ml) in a 50 ml falcon tube for one hour gently on a roller. After discarding the blocking buffer, the membrane was incubated with Cytokeratin 18 or α-smooth muscle actin primary antibody diluted in blocking buffer overnight at 4 °C on a roller. Thereafter, membranes were washed 3x 5 min in TBST solution followed by incubation with

either Anti-rabbit Alkaline Phosphatase or Anti-mouse Alkaline Phosphatase secondary antibody for Cytokeratin 18 or α-smooth muscle actin primary antibody, respectively. Incubation was done gently for one hour at room temperature on a roller protected from light. Membranes were washed 4x 10 min and then detection was done in the dark by addition of Western Lightning™ CDP-Star Chemiluminescence Reagent to the membranes for two minutes before the membrane was sandwiched between Saran wrap (Dow Company, Germany). The membrane was placed into an X-ray cassette and an X-ray film (Fuji) was placed on the membrane. The X-ray cassette was closed and then exposure done for various times. The X-ray film was developed in a developer (AGFA Healthcare, Belgium) until a good signal was observed. The film was rinsed once with distilled water and fixed in a rapid fixer (AGFA Healthcare, Belgium) for 5 minutes. Then the film was rinsed in running tap water and allowed to dry.

2.9 Characterization of Endometrial and Endometriotic Tissues by Immunohistochemistry

Endometrial and endometriotic glands were examined in vivo by immunohistochemistry for cytokeratin 18 (CK 18) and Mucin-1 (MUC1) proteins.

2.9.1 Patient Recruitment

The study included women undergoing laparoscopy who had provided a written informed consent under the study protocol 95/09 approved by the Ethics Committee of the Medical Faculty of the Justus-Liebig-University, Giessen, Germany. Endometrial and ovarian tissues were obtained by laparoscopy from endometriotic patients. Endometriosis was staged according to the revised American Fertility Society System (rAFS). Also, tissues were obtained from patients who did not show endometriosis after laparoscopy and were used as control.

2.9.2 Preparation of Tissue Samples

Immediately after collecting the tissue samples, they were fixed in Bouins solution and embedded in paraffin. The tissues were cut to a thickness of 5 µm.

2.9.3 Immunohistochemistry of Endometrial and Ovary Tissues

1. Slides were deparaffined and rehydrated with consecutive simmering in Neoclear and ethanol in the following way:
   - Three times for 20 min in Neoclear (Baker, Netherlands)
   - Two times for 5 min in ethanol 100% (Baker, Netherlands)
   - Two times for 5 min in ethanol 96% (Baker, Netherlands)
   - Two times for 5 min in ethanol 70% (Baker, Netherlands)
2. Slides were washed for 5 min in distilled water on a shaker.
3. Unmasking of the antigens on the sections was done by heating the slides in gourmetgarer (Braun, Germany) with citrate buffer, pH 6.0 at 100 °C for 20 minutes.
4. Slides were removed from citrate buffer and allowed to cool at room temperature for 20 minutes.
5. Slides were washed three times for 5 min in PBS on a shaker.
6. To inhibit unspecific protein binding, the slides were incubated with 3% H_2O_2 in methanol at room temperature for 30 minutes.
7. Slides were washed three times for 5 min in PBS on a shaker and then put in 1.5% BSA (Roth, USA) for 20 min on a shaker.
8. Primary CK 18 (Epitomics; USA) and MUC1 (Epitomics, USA) antibodies were diluted in antibody diluent (Dako, USA) at a dilution of 1:300 and 1:200, respectively and then applied to the tissue sections in a moist chamber and incubated overnight at 4 °C.
9. Slides were washed three times for 5 min in PBS on a shaker.
10. Secondary antibody (Dako, USA) was added until it covered the tissue section and slides incubated at room temperature for 30 minutes.
11. Slides were washed three times for 5 min in PBS on a shaker.
12. Slides were incubated with the substrate chromogen system solution DAB (Dako, USA) and microscopically controled.
13. Slides were washed three times for 5 min in distilled water on a shaker.
14. The tissue sections were counterstained with haematoxylin (Waldeck, USA) for 45 seconds.
15. The haematoxylin was rinsed off by putting the slides in running tap water for at least 10 minutes.
16. The slides were mounted with Eukitt quick-hardening mounting medium (Fluka

Analytical, Germany).

2.10 Proximity Ligation Assay (PLA)

The influence of TGF-β1 or TGF-β2 on interaction of TβRI, TβRII and TβRIII receptors on endometrial and endometriotic cells *in vitro* (Signalosome analysis) was investigated by use of Duolink^{R} In Situ kit (Olink Bioscience, Sweden).
The kit contains:

- **PLA probe Anti-Goat PLUS (5x)** - Donkey anti-goat secondary antibody conjugated to oligonucleotide PLUS enough for 30 reactions. Each reaction is based on 40 µl of the total reaction mixture covering 1cm^2.
- **PLA probe Anti-Rabbit MINUS (5x)** - Donkey anti-rabbit secondary antibody conjugated to oligonucleotide MINUS (30 reactions). Each reaction is based on 40 µl of the total reaction mixture covering 1cm^2.
- **Blocking solution (4 ml)** - for 30 reactions.
- **Antibody Diluent (2.5 ml)** - For dilution of PLA probes and primary antibodies (30 reactions).
- **Ligation (5x)** - Contains oligonucleotides that hybridize to the PLA probes (30 reactions).
- **Ligase (1 unit/µl)** - Enough for 30 reactions.
- **Amplification Red (5x)** - Contains all components needed for Rolling Circle Amplification (RCA). It also contains oligonucleotide probes labeled with a fluorophore that hybridizes to the RCA product (30 reactions).
- **Polymerase (10 units/µl)** - Enough for 30 reactions.
- **Wash buffer A** - Contains 3 pouches. For washes after the ligation, incubation with primary antibodies and PLA probes.
- **Wash buffer B** - Contains 1 pouch. For washes after incubation with the amplification reagents.
- **Mounting Medium with DAPI** - For preserving fluorescence signals.

2.10.1 Preparation of cells for PLA

Cells were cultured in 8-well chamber slides, treated with TGF-βs, fixed and then permeabilized as described (Fig. 13).

Reagent Preparation

- **Phosphate buffered saline (PBS)** - 0.2 g KCl, 0.2 g KH_2PO_4, 8.0 g NaCl, 1.44 g $Na_2HPO_4 \cdot 2H_2O$ diluted with distilled water to a final volume of 1 liter, pH=7.4
- **Tris buffered saline (TBS) (1x)** - 3.025 g Tris Base, 4.38 g NaCl, diluted with distilled water to a final volume of 500 ml, pH=7.6
- **10% Neutral Buffered Formalin** - 2 g NaH_2POH_2O, 3.25g $NaHPO_4$ 50ml Formaldehyde (37-40%) diluted with double distilled water to a final volume of 500ml, pH=6.8
- **10% Triton X-100** -Triton X-100 stock diluted with double distilled water (1:10)
- **0.25% Triton X-100** - 10 % Triton X-100 in TBS (1:40)

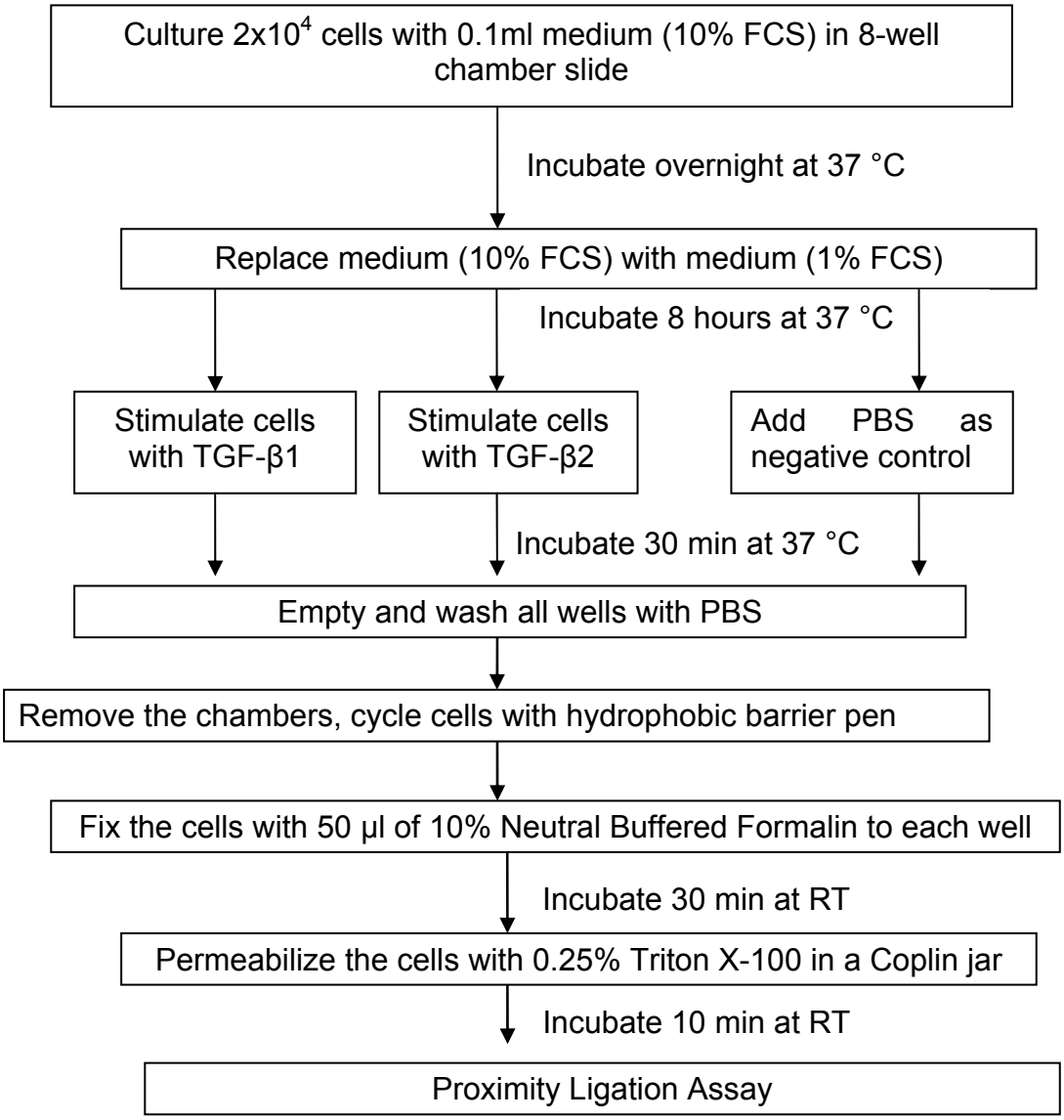


Figure 13. Scheme for preparation of cells for PLA

2.10.2 Proximity Ligation Assay procedure

The fixed and permeabilized cells as described in 2.9.1 were used for PLA.

Preparation of Reagents

- **PLA probe Anti-Goat PLUS (5x)** - After vortexing, it was diluted 1:5 in antibody diluent and vortexed again. Equal volumes of diluted PLA probe Anti-Goat PLUS and PLA probe Anti-Rabbit MINUS were mixed and vortexed immediately before addition to the slide.
- **PLA probe Anti-Rabbit MINUS (5x)** - After vortexing, it was diluted 1:5 in antibody diluent and vortexed again. Equal volumes of diluted PLA probe Anti-Rabbit MINUS and PLA probe Anti-Goat PLUS were mixed and vortexed immediately before addition to the slide.
- **Blocking solution (4 ml)** - Vortexed before use. Ready-to-use.
- **Antibody Diluent (2.5 ml)** - Vortexed before use. Ready-to-use.
- **Ligation (5x)** - Thawed at room temperature and diluted 1:5 in double distilled water immediately before use.
- **Ligase (1 unit/µl)** - The ligase was kept in a freezing block (-20°C) when diluted 1:40 in the ligation mixture prepared above. It was thoroughly mixed and then added to the slide.
- **Amplification Red (5x)** - Thawed at room temperature and diluted 1:5 in double distilled water immediately before use.
- **Polymerase (10 units/µl)** - The polymerase was kept in a freezing block (-20°C) when diluted 1:80 in the amplification mixture prepared above. It was thoroughly mixed and then added to the slide.
- **Wash buffer A**- To prepare 1x buffer, the content of 1 pouch was dissolved in double distilled water to a final volume of 1L.
- **Wash buffer B** - To prepare 1x buffer, the content of 1 pouch was dissolved in double distilled water to a final volume of 1L.
- **Mounting Medium with DAPI** - It was vortexed before use. Ready-to-use.
- **Primary antibodies**
    - **TGFβ RI antibody** (Santa Cruz, USA) from goat, 1:200 diluted in Duolink antibody diluent. Equal volumes of diluted TGFβRI and TGFβ

RII antibodies were mixed and vortexed immediately before addition to the sample.

- **TGFβ RII antibody** (Abcam, UK) from rabbit, 1:350 diluted in Duolink antibody diluent. Equal volumes of diluted TGFβRII and TGFβ RI or TGFβRIII antibodies were mixed and vortexed immediately before addition to the sample.
- **TGFβRIII antibody** (R&D Systems, USA) from goat, 1:200 diluted in Duolink antibody diluent. Equal volumes of diluted TGFβRIII and TGFβRII antibodies were mixed and vortexed immediately before addition to the sample.

PLA Assay Procedure

1. Cells were washed with PBS for 3 x 5 minutes in a Coplin jar with agitation at RT.
2. One drop of Duolink II blocking solution was added to each well and the slides incubated for 1 hour at 37°C.
3. 40 µl of the antibody pair was added into each well. In some wells, the primary antibody was omitted and instead 40 µl antibody diluent was added (Control). The slides were incubated over night at 4°C.
4. The slides were washed with 1x wash buffer A for 3x5 minutes in a Coplin jar with agitation at RT.
5. 40 µl of mixed and diluted PLA probes (MINUS+PLUS) were added into each well followed by an incubation for 1 hour at 37°C.
6. The slides were washed with 1x wash buffer A for 2x5 minutes in a Coplin jar with agitation at RT.
7. 40 µl of ligation-ligase solution was added to each well. The slides were incubated for 30 minutes at 37°C.
8. The slides were washed with 1x wash buffer A for 2x2 minutes in a Coplin jar with agitation at RT.
9. 40 µl of Amplification-Polymerase solution was added to each well, protected from light, incubated for 100 minutes at 37°C.
10. The slides were washed with 1x wash buffer B for 2x10 minutes in a Coplin jar with agitation at RT and protected from light.
11. The slides were dipped in 0.1x wash buffer B for 1 minute in a Coplin jar.

12. 40 µl mounting medium with DAPI was added on the cover slip and gently placed over the slide.
13. After 15 minutes, the samples were analyzed under the olympus fluorescence microscope.
14. The signals on the images were counted to obtain quantification of the signals.

2.11 Statistical Methods

Statistical analysis was performed to analyse experimental data. Each experiment was done at least three times in duplicate. The experimental data are expressed as means±SEM. The significance of the data was analysed by use of Instat Graphpad® statistics software and we performed the Kruskal-Wallis test which is a non-parametric test for all experimental data.

3 Results

3.1 Characterization of Endometrial and Endometriotic Tissues and Cells

3.1.1 Characterization of Endometrial and Endometriotic Cells

Endometrial and endometriotic cells were characterised by immunofluorescence and Western blotting with cytokeratin 18 and α-smooth muscle actin monoclonal antibodies to assess their purity. Cytokeratin 18 is a member of intermediate filament proteins that are expressed preferentially as pairs mainly in epithelial tissue (Pekny and Lane, 2007). Cytokeratins are critical in differentiation, tissue specialization and function to maintain the overall structure and integrity of epithelial cells. In contrast, stromal cells were characterised by antibodies against α-smooth muscle actin which is expressed mainly in smooth muscle cells, myofibroblasts and myoepithelial cells (Ogawa, 2003).

Cells were grown overnight and then fixed with ice-cold acetone/methanol (1:1). A high affinity cytokeratin 18 antibody was used as primary antibody. A goat anti-rabbit IgG labelled with Alexa Fluor® 546 dye was used as secondary antibody; nuclei were counterstained with Hoechst dye (Fig. 14).

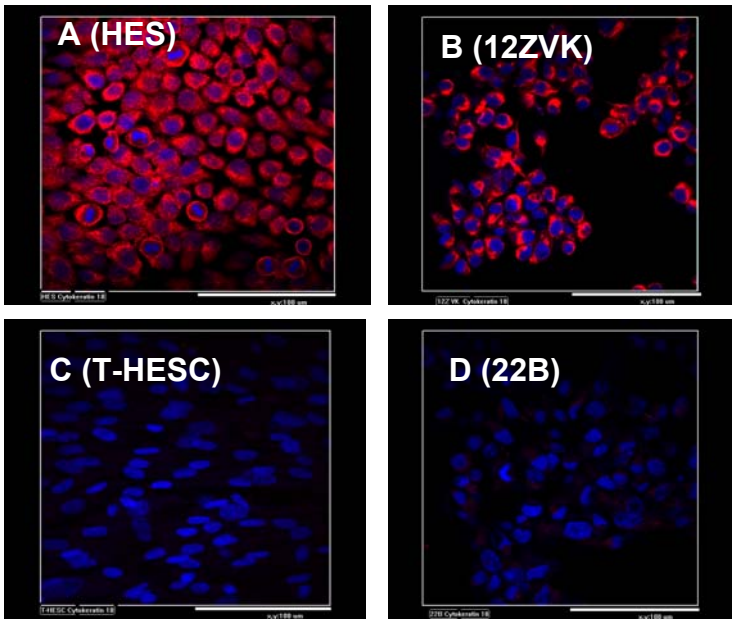


Figure 14. The epithelial HES **(A)** and 12ZVK **(B)** cells which represent endometrial and endometriotic epithelial cells, respectively, were strongly positive for cytokeratin 18. No staining for cytokeratin 18 was observed in T-HESC **(C)** and 22B **(D)** cells which represent endometrial and endometriotic stromal cells, respectively.

In addition to the immunofluorescence, the CK 18 and anti-α-smooth muscle actin antibodies were further used to analyse the lysates from the cell lines by Western blotting (Fig. 15).

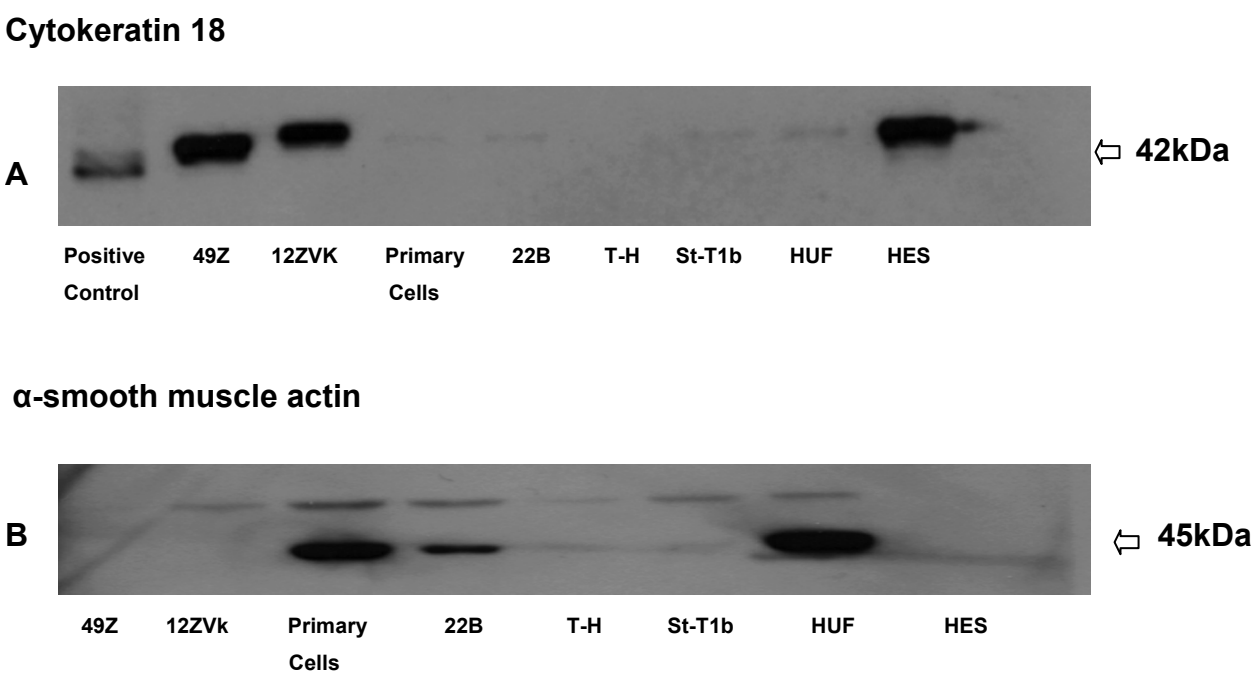


Figure 15. Cytokeratin 18 **(A)** and α-smooth muscle actin (**B)** expression in endometrial and endometriotic cell lysates. Cytokeratin 18 expression (42kDa) was found in HES cells and 12ZVK cells which represent endometrial and endometriotic epithelial cells, respectively, but not in T- HESC (T-H) cells and 22B cells, which represent endometrial and endometriotic stromal cells. In addition, there was no cytokeratin 18 expression in primary endometrial stromal cells. Alpha-smooth muscle actin (45kDa) was expressed in endometrial and endometriotic cell lysates of T- HESC (T-H) cells, 22B cells and primary endometrial stromal cells but not in HES or 12ZVK cells.

3.1.2 Characterization of Endometrial and Endometriotic Tissues

Endometrial and endometriotic glands were examined by immunohistochemistry to assess their characteristics. Endometrial biopsies were obtained from patients with or without endometriosis and then assessed for expression of cytokeratin 18 (CK 18) and Mucin-1 (MUC1). MUC1 is a member of the mucin family, is normally expressed on polarised epithelial cells and is also a component of glandular secretions (Thathiah and Carson, 2004; Hattrup and Gendler, 2008). Abnormal MUC1 expression has been observed in over 80% of all cancers and is often associated with poor prognosis (Hattrup and Gendler, 2008).

Both CK 18 and MUC1 were expressed in all endometrial glands of the endometrium. There were no differences observed in staining in patients with endometriosis (Fig.

16A and 16C) or without endometriosis (Fig. 16B and 16D). Further analysis of the expression of both proteins in the ovary was performed. In the ovaries, all endometriotic glands stained positive with CK 18 (Fig. 17A and 17B) as well as with MUC1 (Fig. 17C and 17D).

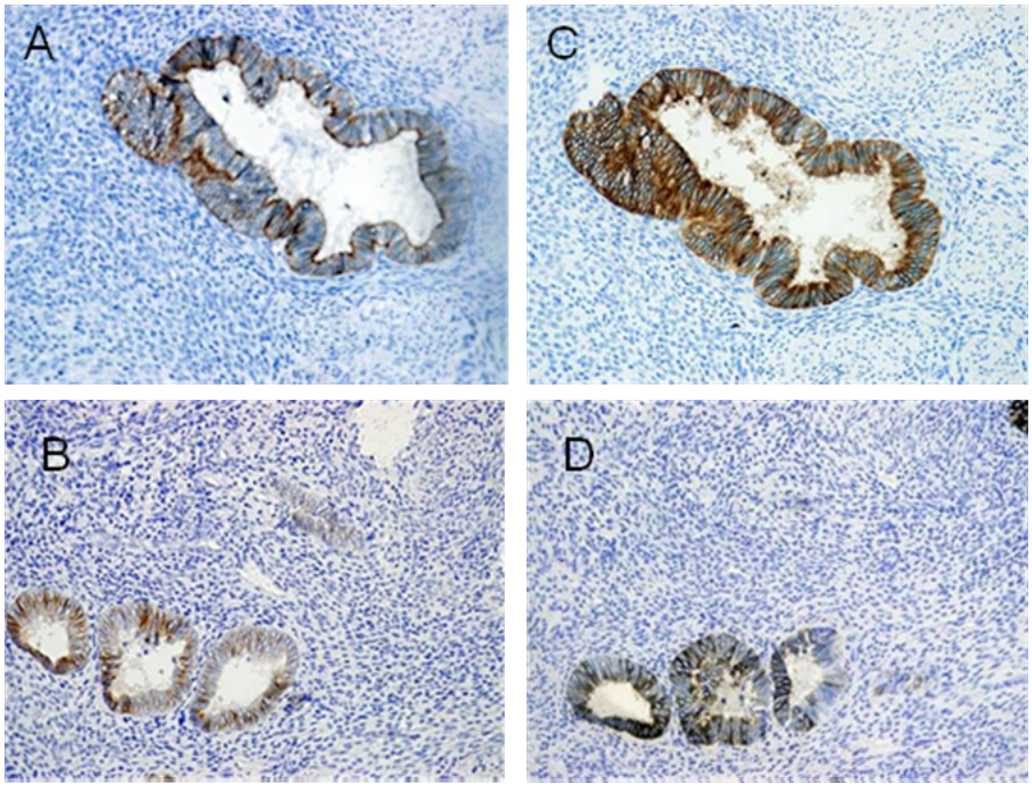


Figure 16. Immunohistochemical detection of CK 18 (A, B) and MUC 1 (C, D) in the endometrium of patients without endometriosis (B, D) and patients with endometriosis (A, C). Magnification 200X.

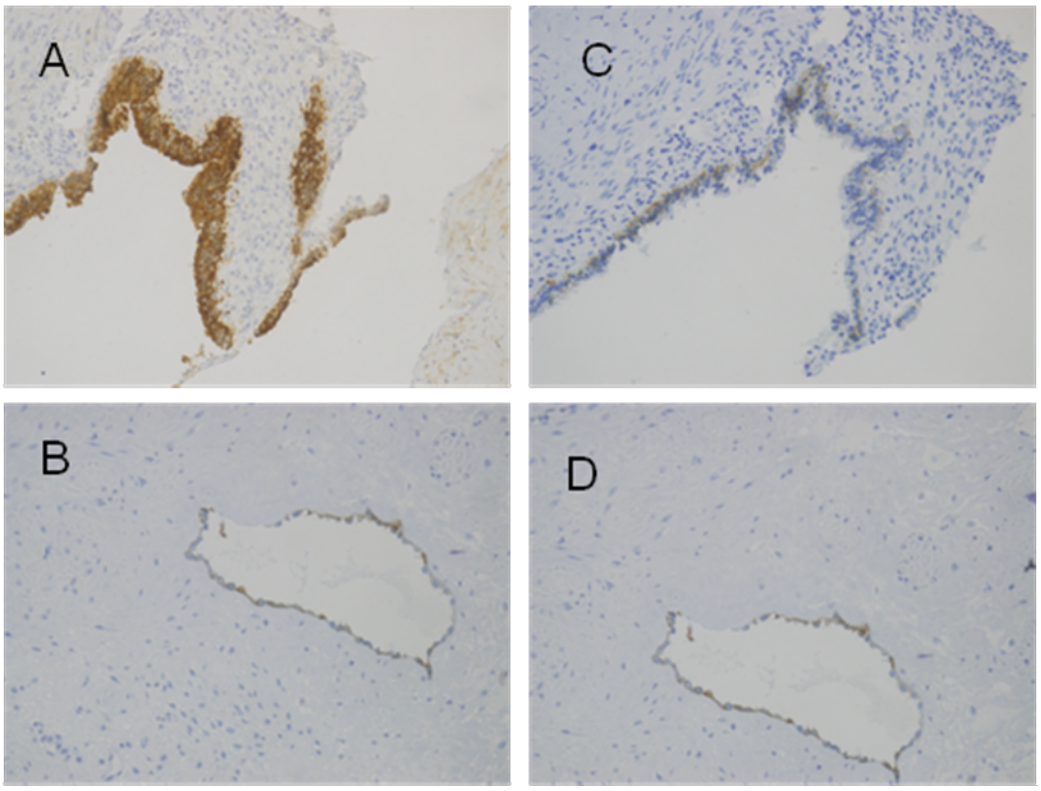


Figure 17. Immunohistochemical detection of CK 18 (A, B) and MUC 1 (C, D) in the ovaries of patients without endometriosis (B, D) and patients with endometriosis (A, C). Magnification 200X.

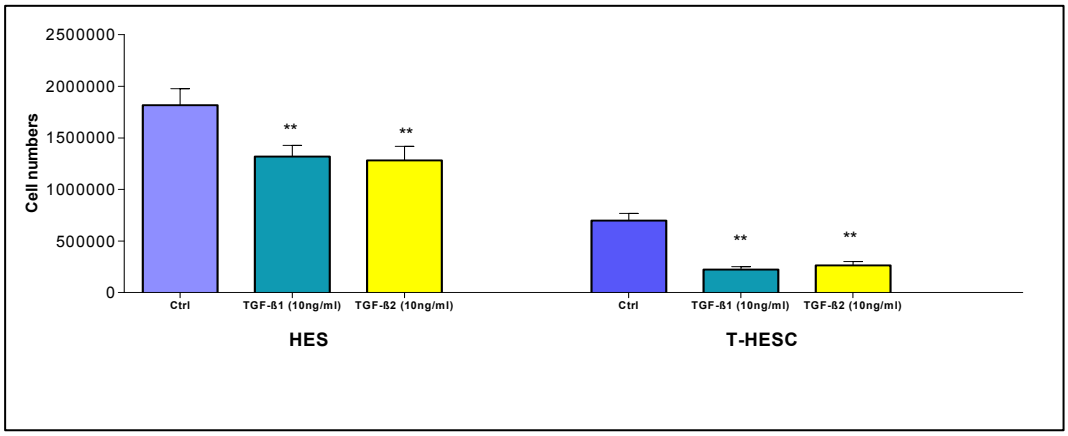
In summary, MUC 1 was expressed in all endometrial and endometriotic glands of all tissues. CK 18 was expressed in endometrial and endometriotic glands of endometrium and ovary. In addition, the surface epithelium of the ovary expressed CK 18.

3.2 Influence of TGF-β1 or TGF-β2 on Cell Numbers

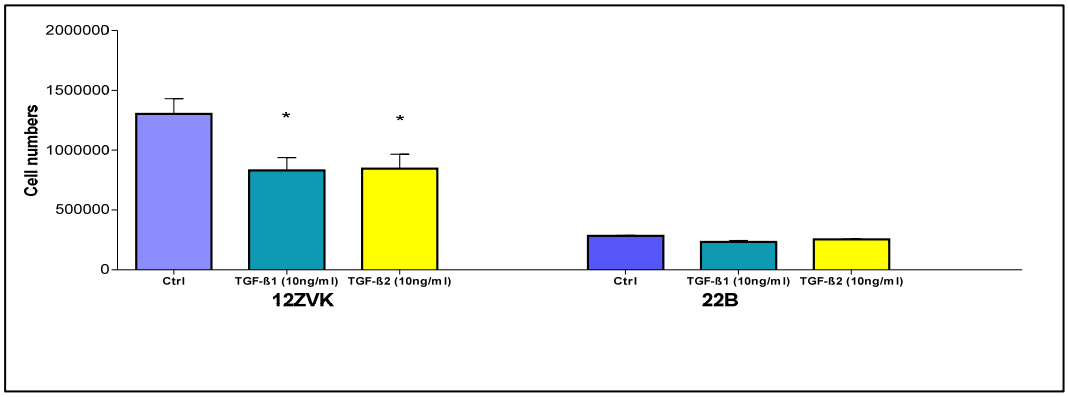
In order to find out how TGF-βs affect cell numbers, we treated endometrial, endometriotic cell lines and primary endometrial stromal cells with TGF-β1 or TGF-β2 (10ng/ml), respectively.

The results showed that TGF-β1 or TGF-β2 reduced cell numbers of both endometrial and endometriotic cell lines and primary endometrial stromal cells within 72 hours (Fig. 18). Reduction was higher in endometrial cells in comparison with endometriotic cells.

A



B



C

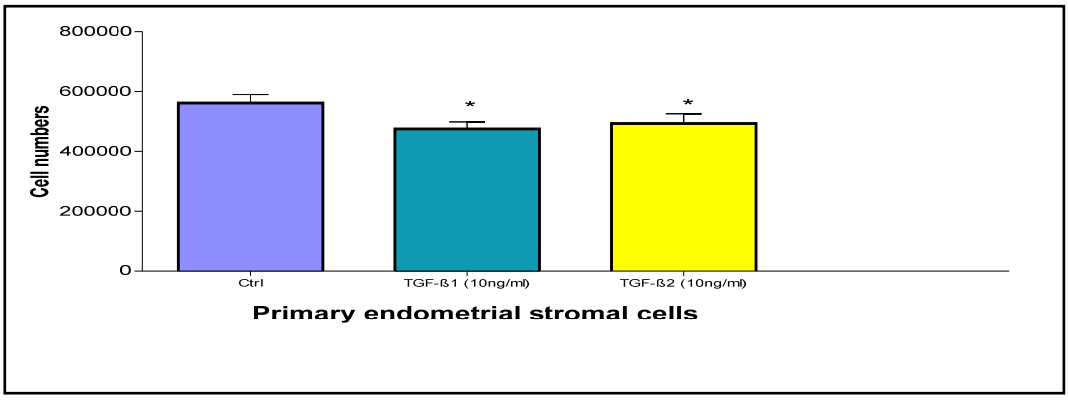
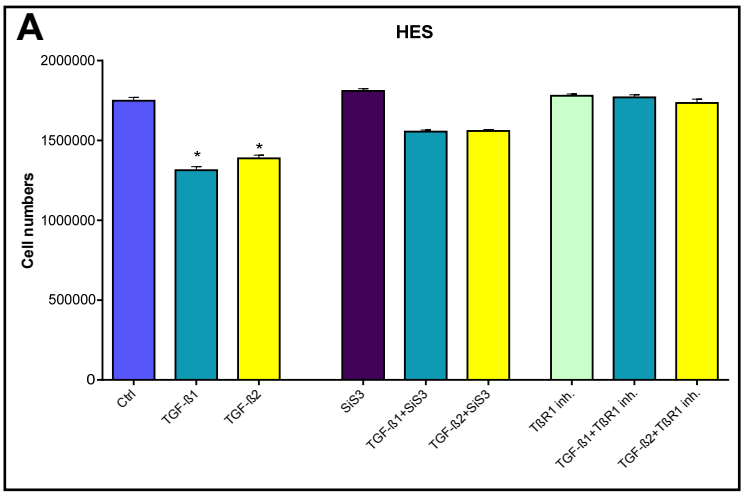


Figure 18. Quantification of cell numbers of endometrial cells **(A)**, endometriotic cells **(B)** and primary endometrial stromal cells **(C)** with or without treatment with TGF-βs (10ng/ml). The quantity of endometrial epithelial cells **(HES)** was reduced by approximately 39% and that of endometrial stromal cells **(T-HESC)** was reduced by approximately 51% **(A)**. Endometriotic epithelial cell numbers **(12ZVK)** were reduced by approximately 21% and of endometriotic stromal cells **(22B)** by approximately 13% **(B)**. Primary endometrial stromal cell numbers were reduced by approximately 19% **(C)**. (**=P<0.01, *=P<0.05, n=9).

3.3 Analysis of the Smad Pathway in Endometrial and Endometriotic Cells

The inhibitors LY364947 and SiS3 were used to investigate the role of the Smad-dependent pathway in cell proliferation. LY364947 and SiS3 inhibitors block the phosphorylation of Smad2/3 by selectively inhibiting TβR1 or Smad3, respectively. LY364947 (5 μM) or SiS3 (2 μM) were added two hours before adding the TGF-βs (10ng/ml). Cells were cultured for 72 hours after stimulation with the TGF-βs. The appropriate negative controls were also performed.

The results showed that TGF-β1 and TGF-β2 reduced cell numbers in all cell lines studied (Figs. 19-21). LY364947 was able to block completely the TGF-β-induced reduction in cell numbers to control levels in all cell lines studied, whereas SiS3 had only a partial but strongly reducing effect (70%) in blocking the TGF-β-induced reduction in cell numbers.



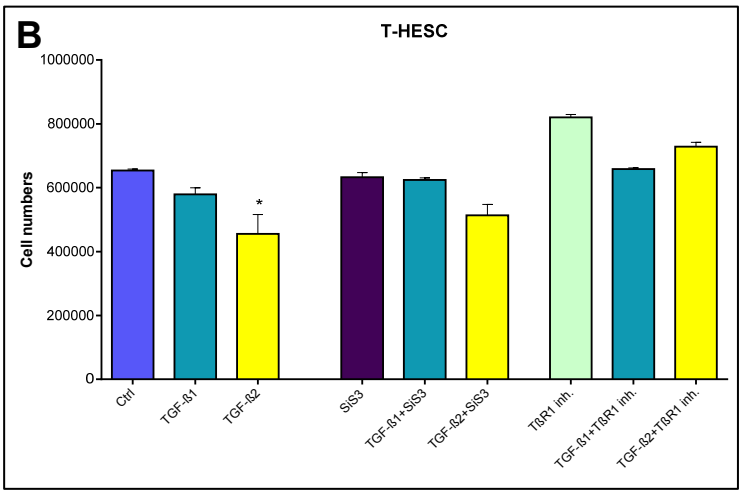


Figure 19. The TβR1 inhibitor LY364947 completely inhibited the TGF-β1- or TGF-β2-induced reduction of endometrial cell numbers of both endometrial epithelial cells, HES **(A)**, and endometrial stromal cells, T-HESC **(B)**. The Smad3 inhibitor SiS3 partially inhibited the TGF-β1- or TGF-β2-induced reduction of endometrial cell numbers of both endometrial epithelial cells, HES **(A)**, and endometrial stromal cells, T-HESC **(B)**, by approximately 60% and 80%, respectively (*=P<0.05, n=9).

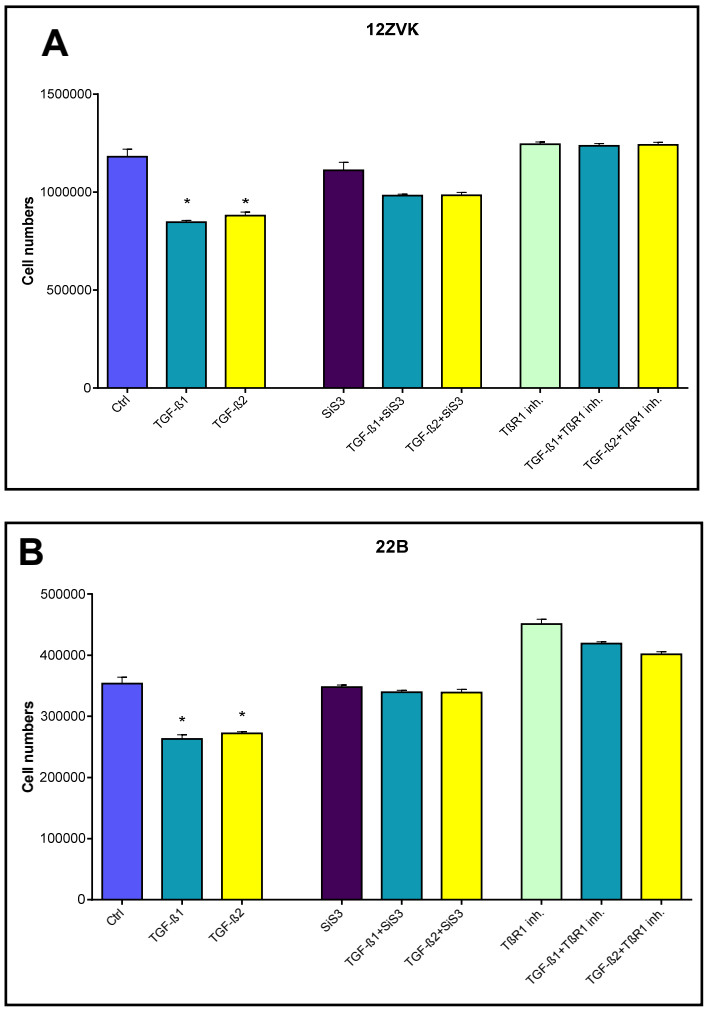


Figure 20. The TβR1 inhibitor (LY364947) completely inhibited the TGF-β1- or TGF-β2-induced reduction of endometriotic cell numbers of both endometriotic epithelial cells, 12ZVK **(A)**, and endometriotic stromal cells, 22B **(B)**. The Smad3 inhibitor SiS3 partially inhibited the TGF-β1- or TGF-β2-induced reduction of endometriotic cell numbers of both endometriotic epithelial cells, 12ZVK **(A)**, and endometriotic stromal cells, 22B **(B)**, by approximately 70% and 90%, respectively (*=P<0.05, n=9).

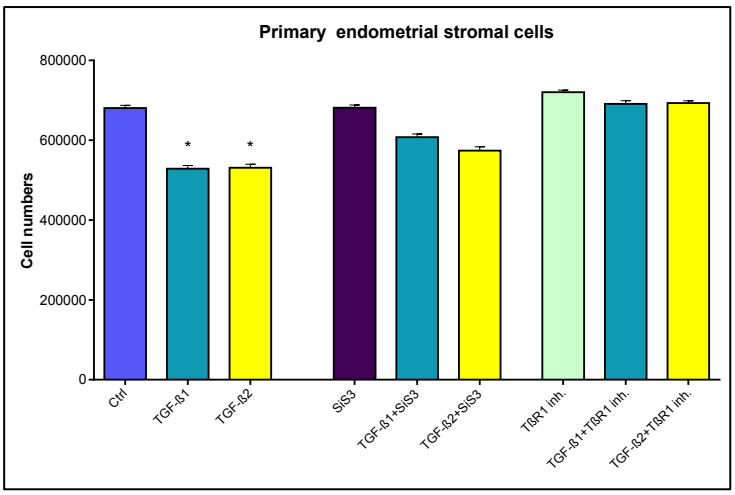


Figure 21. The TβR1 inhibitor (LY364947) completely inhibited the TGF-β1- or TGF-β2-induced reduction of cell numbers, whereas the Smad3 inhibitor SiS3 only partially inhibited the TGF-β1- or TGF-β2-induced reduction of cell numbers of primary endometrial stromal cells (*=P<0.05, n=9). These results were comparable to the results obtained with the endometrial stromal cell line, T-HESC (Fig. 19B).

3.4 Influence of TGF-β1 or TGF-β2 on Smad3 Phosphorylation of Endometrial and Endometriotic Cells in vitro

Smad3 is a member of a transcription factor family involved in mediating signaling of growth factors such as TGF-βs and activins. Smad3 belongs to a class of SMAD proteins that are activated via phosphorylation by specific receptors in response to ligand binding (Derynk et al., 1998). Upon TGF-β binding, Smad3 is phosphorylated by the TGF-β receptor then dissociates from the TGF-β receptor and interacts with Smad4 either in the cytoplasm or nucleus. This Smad complex modulate the TGF-β-induced transcription of genes (Attisano and Wrana, 2002). Smad3 is one of the standard proteins for studying the Smad-dependent effects of TGF-βs.

In our experiments, endometrial, endometriotic cell lines and primary endometrial stromal cells were treated with or without TGF-β1 or TGF-β2 (10ng/ml) for 30 minutes and then cell lysates were collected for the phospho-Smad3 ELISA to quantitate phosphorylated human Smad3.

Treatment of cells with TGF-β1 or TGF-β2, respectively, stimulated phosphorylation of Smad3 in endometrial, endometriotic cell lines (Fig. 22A and B), and primary endometrial stromal cells (Fig. 22C). Stimulation was higher in stromal cells compared to epithelial cells in both endometrial and endometriotic cells.

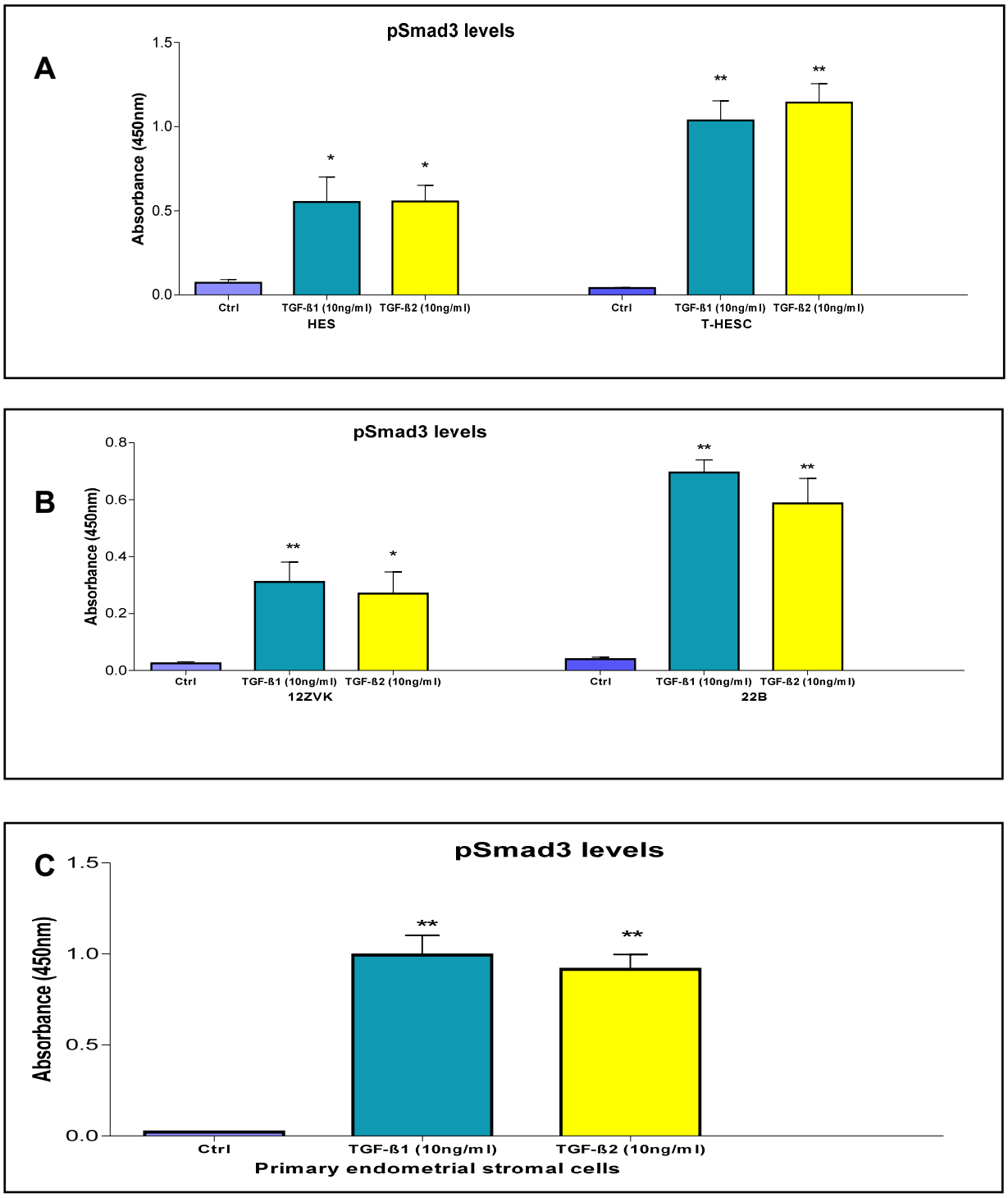
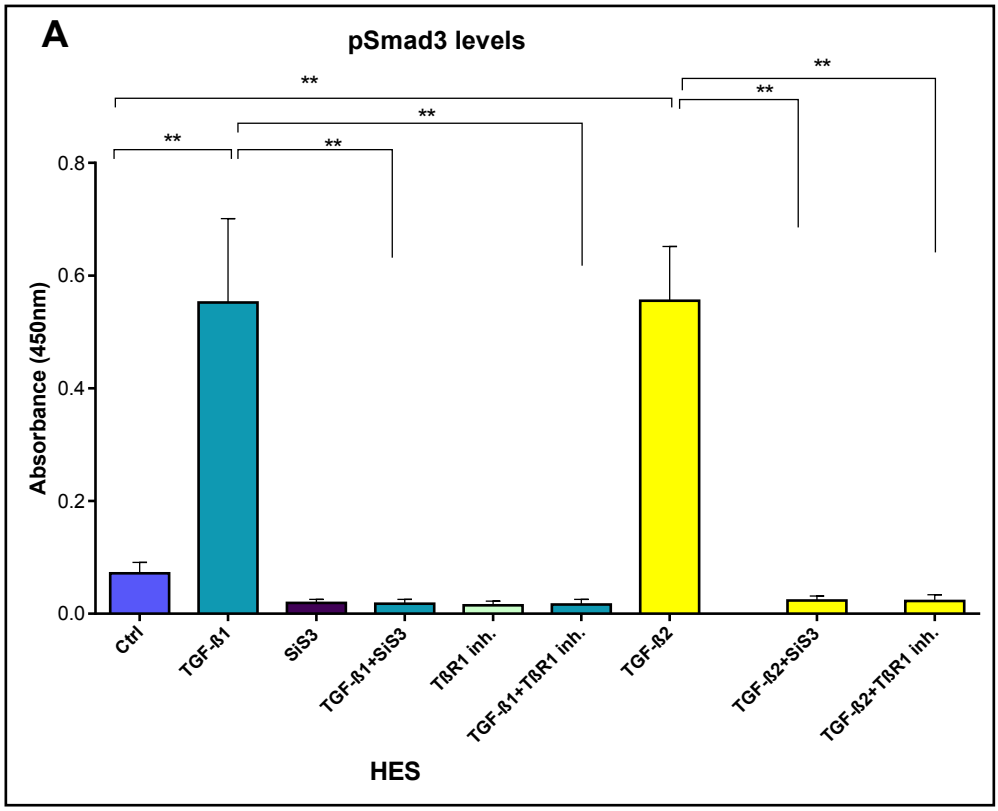


Figure 22. Treatment of cells with TGF-β1 or TGF-β2, respectively, stimulated phosphorylation of Smad3. Smad3 phosphorylation was 2-fold higher in endometrial stromal cells, T-HESC, compared to endometrial epithelial cells, HES **(A)**. In endometriotic stromal cells, 22B, 2-fold more Smad3 phosphorylation was observed compared to endometriotic epithelial cells, 12ZVK **(B)**. Interestingly, in endometrial cells more Smad3 phosphorylation occured compared to endometriotic cells. Primary endometrial stromal cells showed similar Smad3 phosphorylation levels compared to endometrial stromal cells, T-HESC **(C)** (**=P<0.01, *=P<0.05, n=9).

Analysis of the Smad3 Pathway in Endometrial and Endometriotic Cells

The inhibitors LY364947 and SiS3 were used to investigate the role of the Smad-dependent pathway in Smad3 phosphorylation. The inhibitors block phosphorylation of Smad2/3 by selectively inhibiting TβR1 and Smad3, respectively. LY364947 (5 μM) or SiS3 (2 μM) was added two hours before adding the TGF-βs (10ng/ml). Cells were incubated for 30 minutes after stimulation with the TGF-βs. Then cell lysates were collected to quantitate the amounts of phosphorylated Smad3 by phospho-Smad3 ELISAs.

The results showed that treatment of cells with TGF-β1 or TGF-β2, respectively, resulted in Smad3 phosphorylation in all cell lines. The LY364947 and SiS3 inhibitors were able to block completely the TGF-β-induced Smad3 phosphorylation in all cell lines studied (Figs. 23-25).



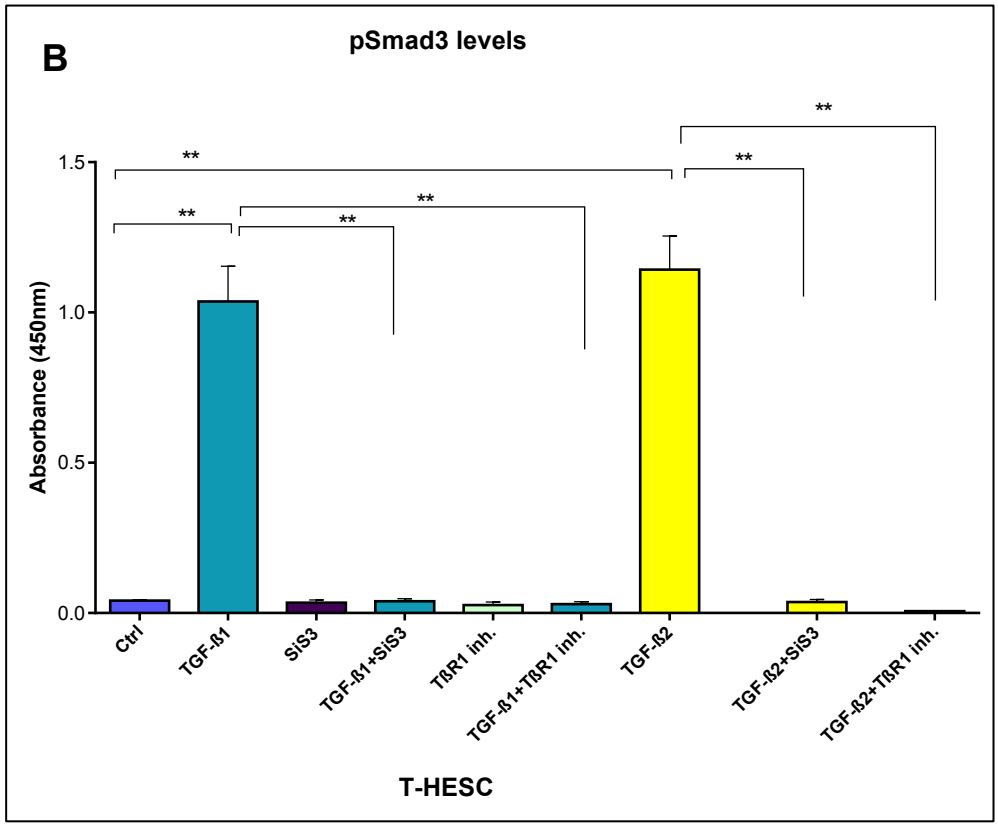
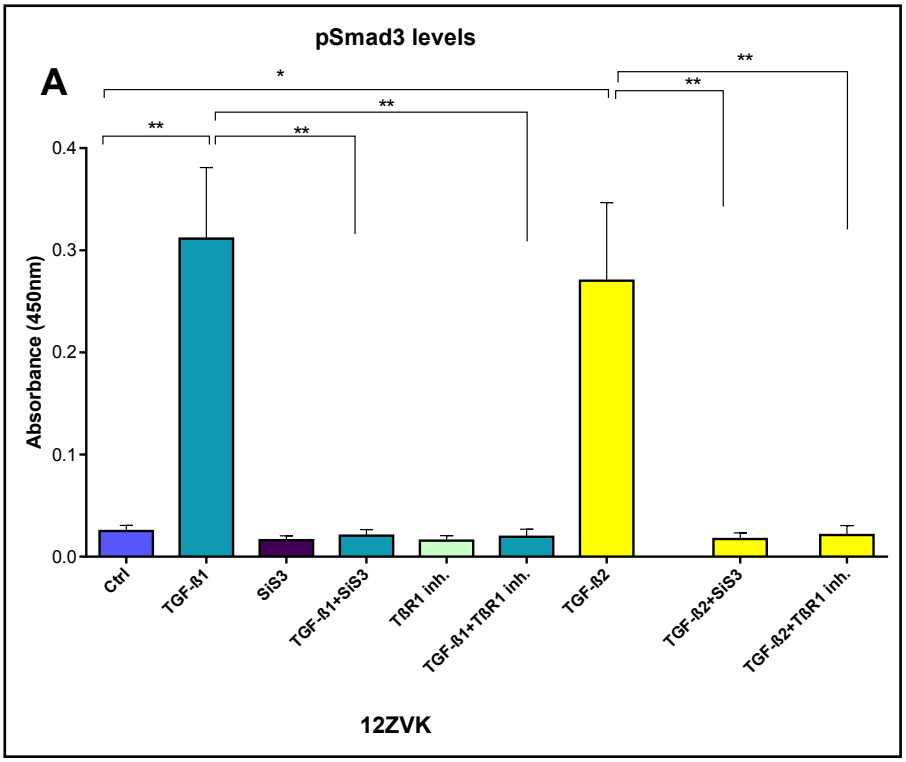


Figure 23. Treatment of endometrial cells with TGF-β1 or TGF-β2, respectively, stimulated phosphorylation of Smad3. Smad3 phosphorylation was 2-fold higher in endometrial stromal cells, T-HESC **(B)**, compared to endometrial epithelial cells, HES **(A)**. LY364947 and SiS3 inhibitors completely blocked the TGF-β-induced Smad3 phosphorylation in endometrial cells (**=P<0.01, n=6).



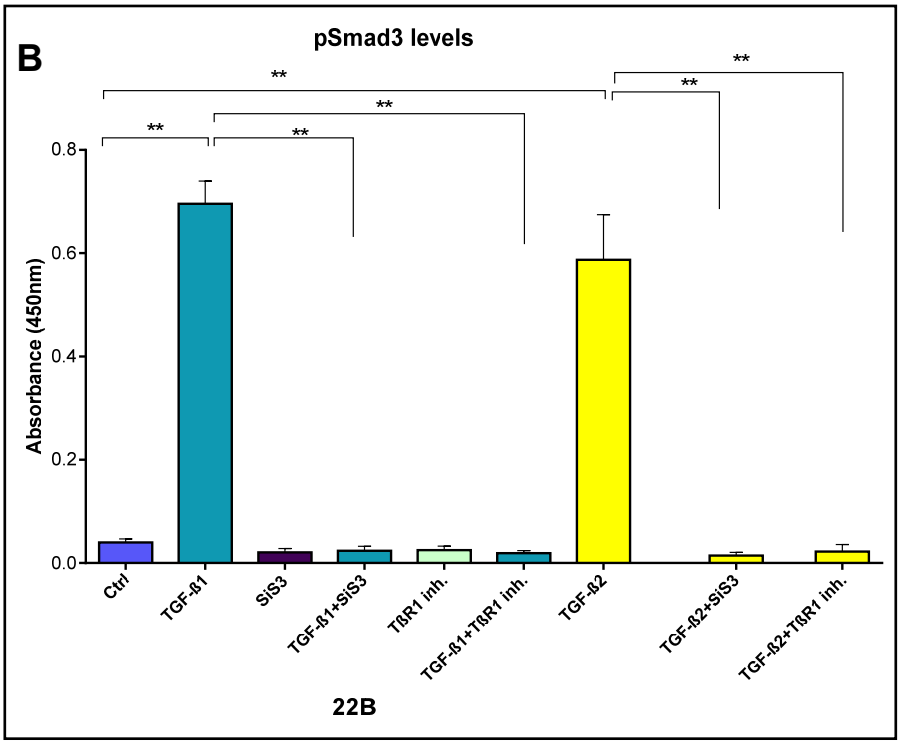


Figure 24. Treatment of endometriotic cells with TGF-β1 or TGF-β2, respectively, stimulated phosphorylation of Smad3. Smad3 phosphorylation was 2-fold higher in endometriotic stromal cells, 22B **(B)**, compared to endometriotic epithelial cells, 12ZVK **(A)**. LY364947 and SiS3 inhibitors completely blocked the TGF-β-induced Smad3 phosphorylation in endometriotic cells (*=P<0.05, **=P<0.01, n=6).

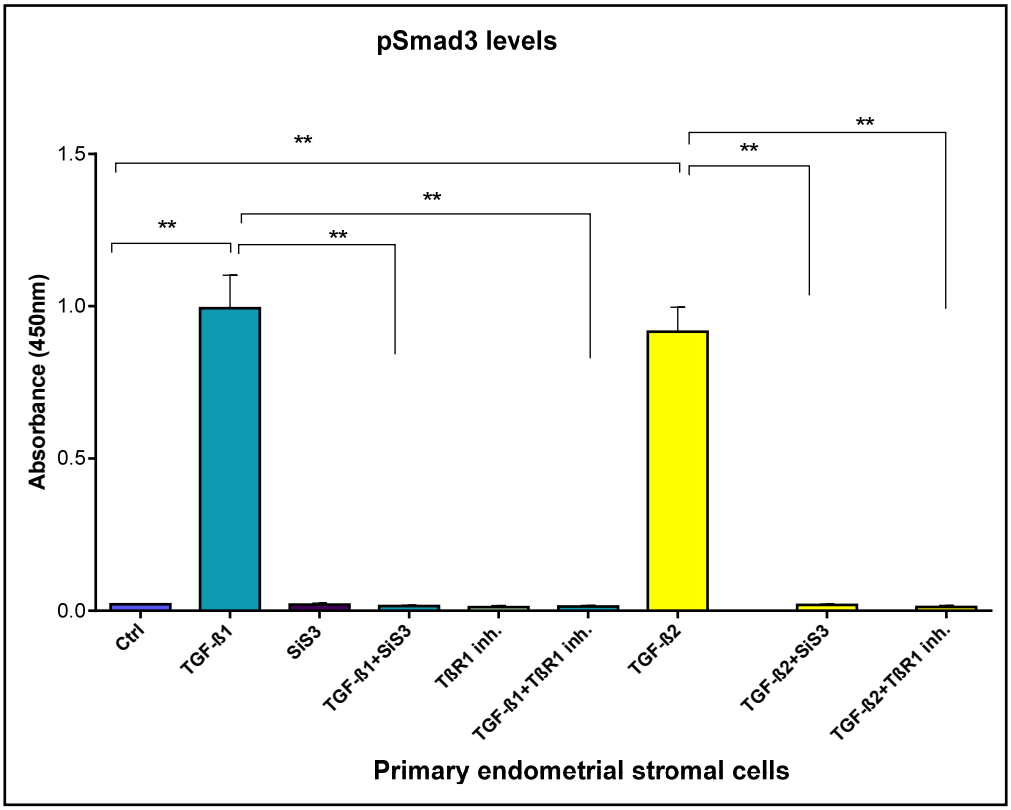


Figure 25. TGF-β1 or TGF-β2, respectively, stimulated phosphorylation of Smad3 in primary endometrial stromal cells. Smad3 phosphorylation levels were very similar to the endometrial stromal cells, T-HESC (Fig. 23B). LY364947 and SiS3 inhibitors completely blocked TGF-β-induced Smad3 phosphorylation in primary endometrial cells (**=P<0.01, n=6).

In summary, endometrial cells showed higher phosphorylation levels of Smad3 compared to endometriotic cells upon treatment with TGF-β1 or TGF-β2. Smad3 phosphorylation in endometrial epithelial cells, HES, was 2-fold higher compared to endometriotic epithelial cells, 12ZVK. Similarly, Smad3 phosphorylation in endometrial stromal cells, T-HESC, was 2-fold higher compared to endometriotic stromal cells 22B. Primary endometrial stromal cells showed similar Smad3 phosphorylation compared to endometrial stromal cells, T-HESC. LY364947 and SiS3 inhibitors completely blocked TGF-β-induced Smad3 phosphorylation in all cell lines studied.

3.5 Influence of TGF-β1 or TGF-β2 on Apoptosis of Endometrial and Endometriotic Cells in vitro

Apoptosis is characterized by activation of an endogenous cell suicide programme which involves chromatin condensation, nuclear fragmentation, cell shrinkage, membrane blebbing and dissolution of cells into apoptotic bodies which are rapidly phagocytosed by neighbouring cells or resident macrophages (Ruaidhri et al., 1998). In our experiments, we wanted to investigate whether or not TGF-βs have an apoptotic effect on human endometrial and endometriotic cells and if yes, which apoptotic pathways are involved.

Endometrial, endometriotic cell lines and primary endometrial stromal cells were treated with or without TGF-β1 or TGF-β2 (10ng/ml), respectively, for 24 hours and then apoptosis experiments were carried out by use of ELISAs. Staurosporine (0.1μM) was included in the assay as a positive control. Staurosporine is a potent inhibitor of protein kinase C, most other kinases, and topoisomerase II by blocking transfer of phosphodiester bonds from DNA to the active site of tyrosine hence inducing apoptosis (Seynaeve et al., 1994).

The apoptotic parameters investigated included quantification of phosphatidylserine on the outer membrane surface, of the inner mitochondrial membrane potential, and of Cysteinyl-asparate-specific proteases 3/7 (Caspase 3/7) proteins. The three parameters are important because they can be used to monitor necrosis and apoptosis. In apoptotic cells, phosphatidylserine which is usually located on the inner side of the cell membrane is translocated in apoptotic cells onto the outer surface of the cell membrane due to loss of cell symmetry (Sims and Wiedmer, 2001). The translocated phosphatidylserine can be quantified by ELISA. The inner mitochondrial membrane integrity is intact but once the cell undergoes apoptosis, the integrity of the inner mitochondrial potential is lost causing release of some ions among them cytochrome C which initiates caspase cascades leading to apoptosis (Kroemer et al., 2007).

The results showed an increase of phosphatidylserine, a decrease of the inner mitochondrial membrane potential and an increase of Caspase 3/7 levels upon treatment of cells with TGF-β1 or TGF-β2 (10ng/ml), respectively (Figs. 26-30).

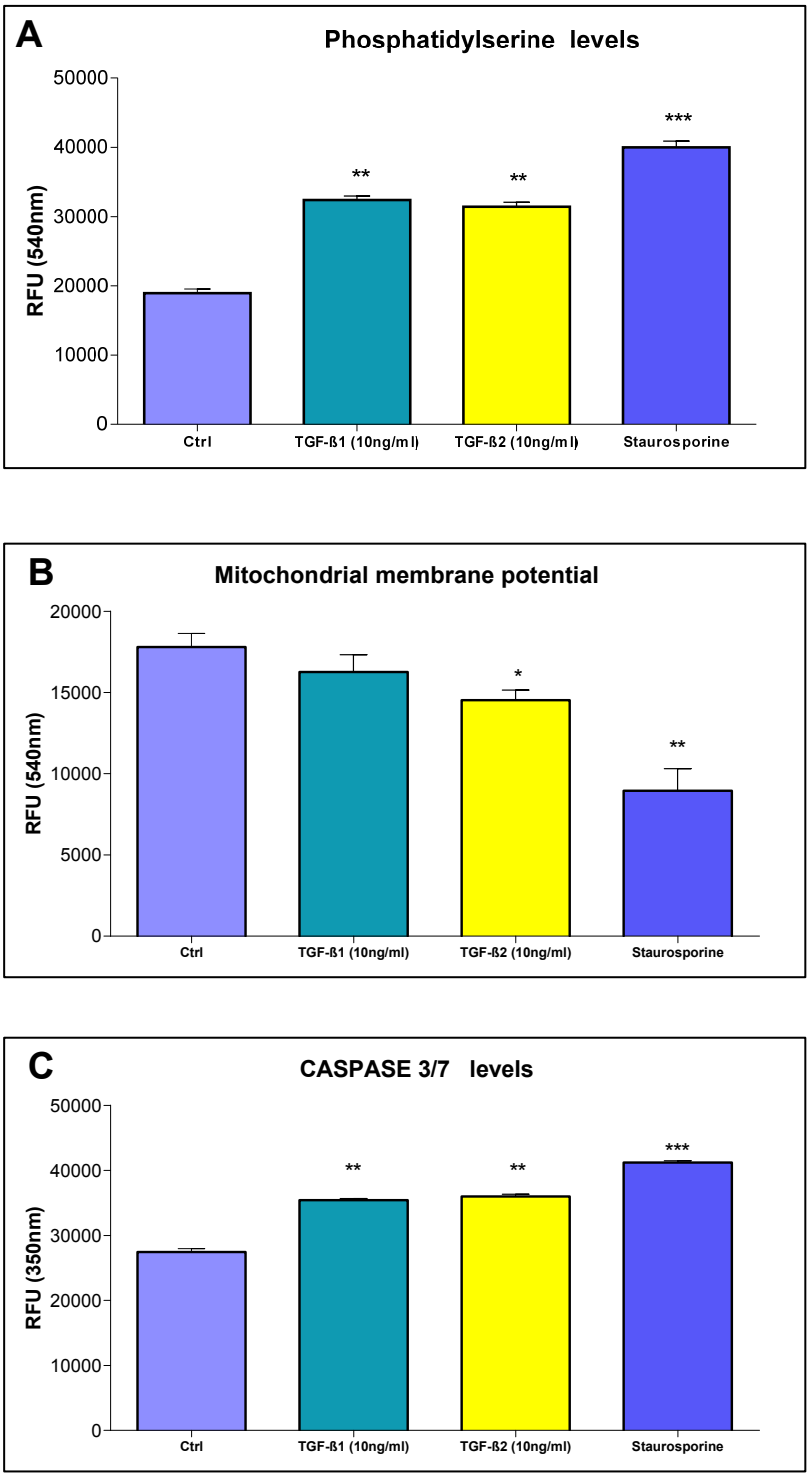


Figure 26. Treatment with TGF-β1 or TGF-β2, respectively, induced apoptosis in endometrial epithelial cells, HES. There was an increase in the amount of translocated phosphatidylserine **(A)**, a decrease of the mitochondrial membrane potential **(B)**, and an increase of the amount of Caspase 3/7 **(C)**. Staurosporine strongly induced apoptosis in HES cells (*=P<0.05, **=P<0.01, ***=P<0.001, n=6). RFU; relative fluorescence units.

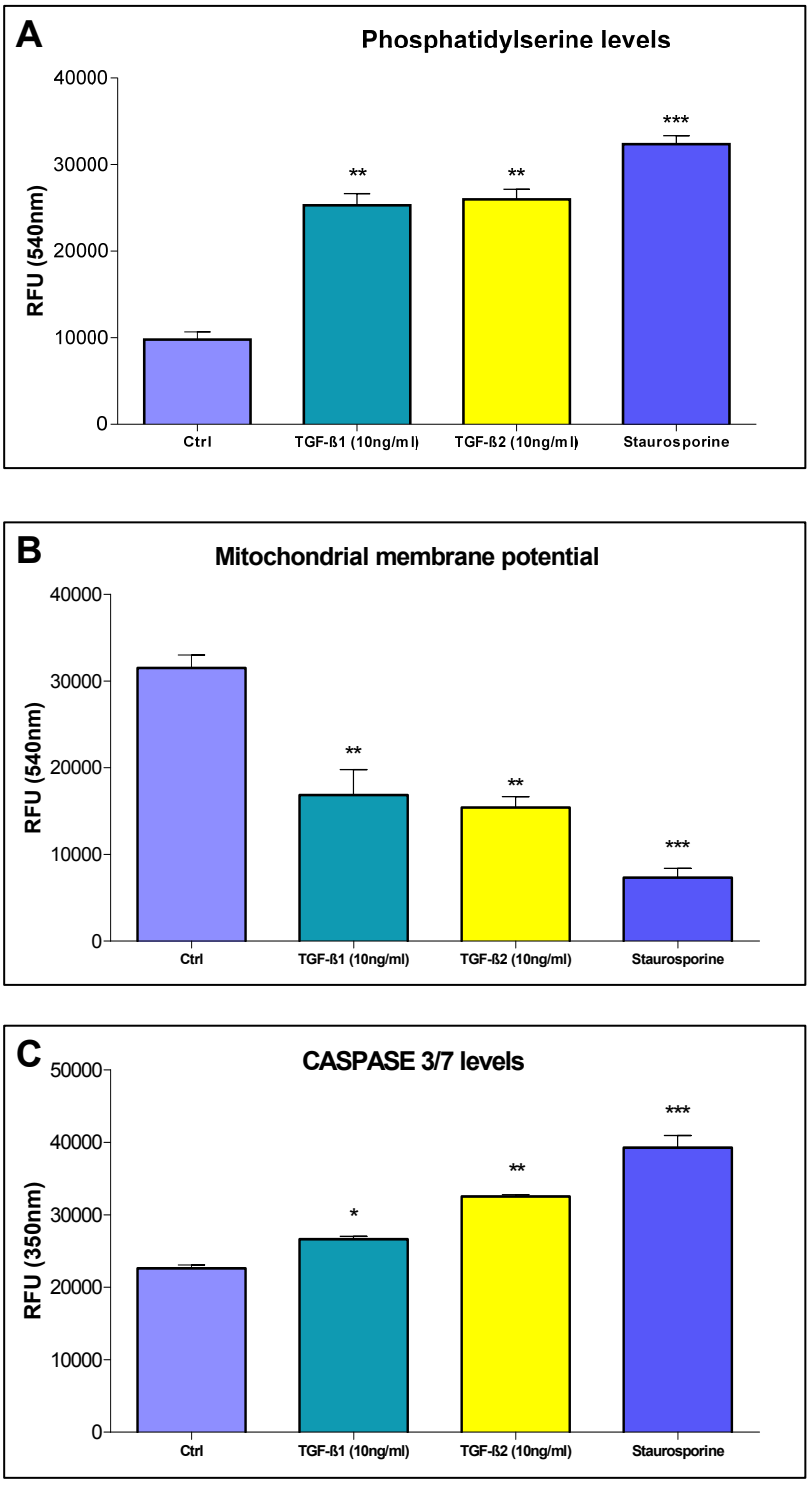


Figure 27. Treatment with TGF-β1 or TGF-β2, respectively, induced apoptosis in endometrial stromal cells, T-HESC. There was an increase in the amount of translocated phosphatidylserine **(A)**, a decrease of the mitochondrial membrane potential **(B)**, and an increase of the amount of Caspase 3/7 **(C)**. Staurosporine strongly induced apoptosis in T-HESC cells (*=P<0.05, **=P<0.01, ***=P<0.001, n=6). RFU; relative fluorescence units.

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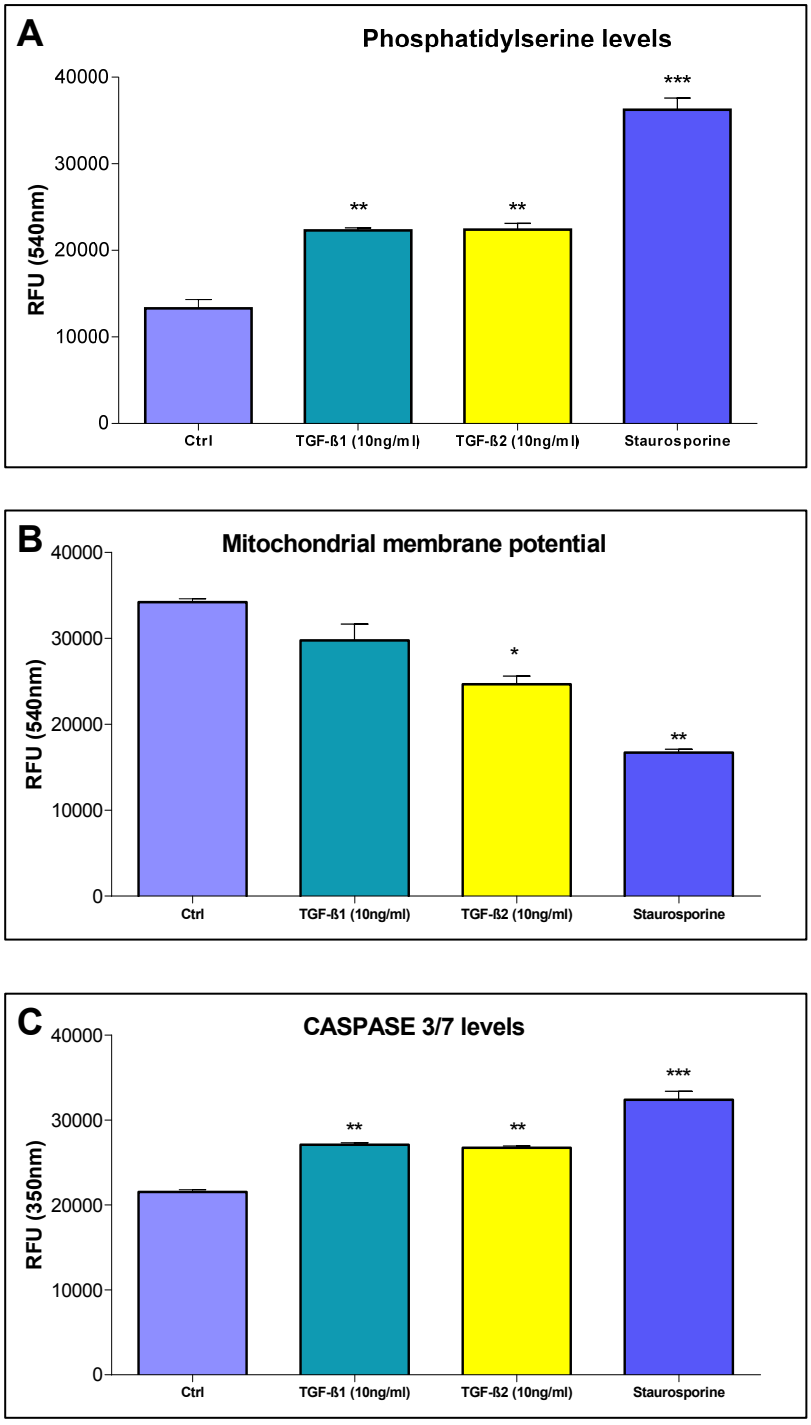


Figure 28. Treatment with TGF-β1 or TGF-β2, respectively, induced apoptosis in endometriotic epithelial cells, 12ZVK. There was an increase in the amount of translocated phosphatidylserine **(A)**, a decrease of the mitochondrial membrane potential **(B)**, and an increase of the amount of Caspase 3/7 **(C)**. Staurosporine strongly induced apoptosis in 12ZVK cells (**=P<0.01, ***=P<0.001, n=6). RFU; relative fluorescence units.

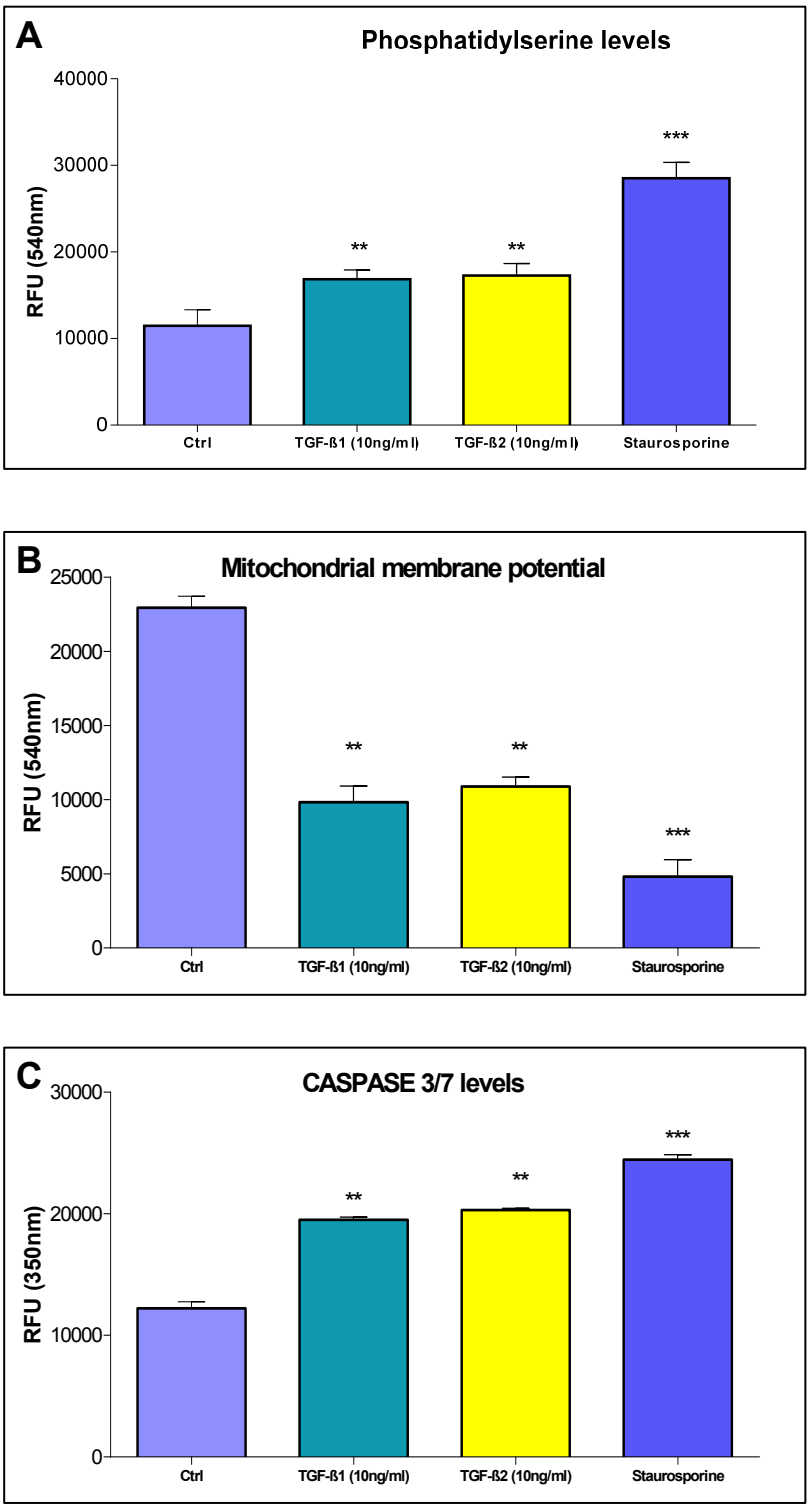


Figure 29. Treatment with TGF-β1 or TGF-β2, respectively, induced apoptosis in endometriotic stromal cells, 22B. There was an increase in the amount of translocated phosphatidylserine **(A)**, a decrease of the mitochondrial membrane potential **(B)**, and an increase of the amount of Caspase 3/7 **(C)**. Staurosporine strongly induced apoptosis in 22B cells (**=P<0.01, ***=P<0.001, n=6). RFU; relative fluorescence units.

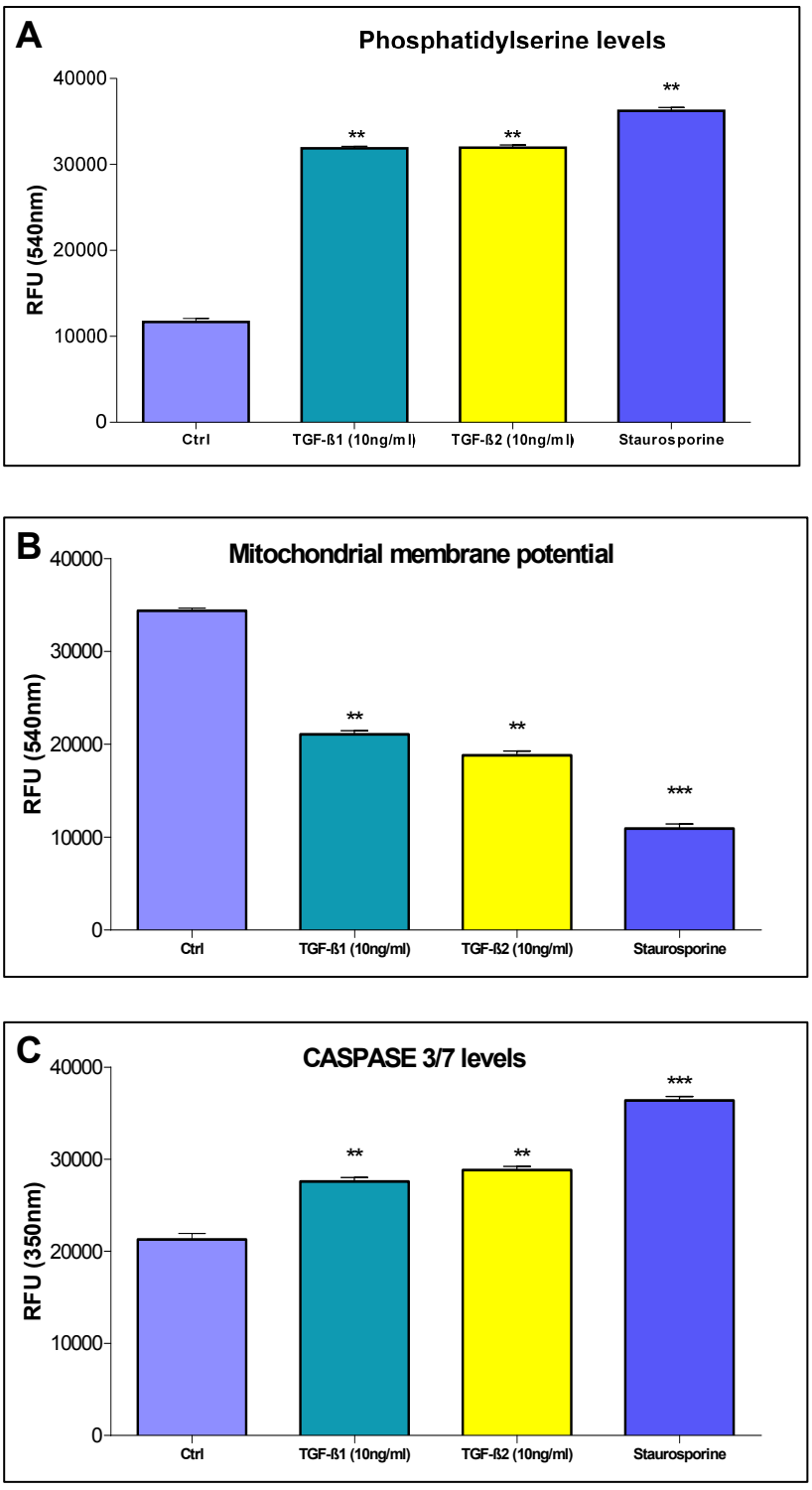


Figure 30. Treatment with TGF-β1 or TGF-β2, respectively, induced apoptosis in primary endometrial stromal cells. There was an increase in the amount of translocated phosphatidylserine **(A)**, a decrease of the mitochondrial membrane potential **(B)**, and an increase of the amount of Caspase 3/7 **(C)**. Staurosporine strongly induced apoptosis in primary endometrial cells (**=P<0.01, ***=P<0.001, n=6). RFU; relative fluorescence units.

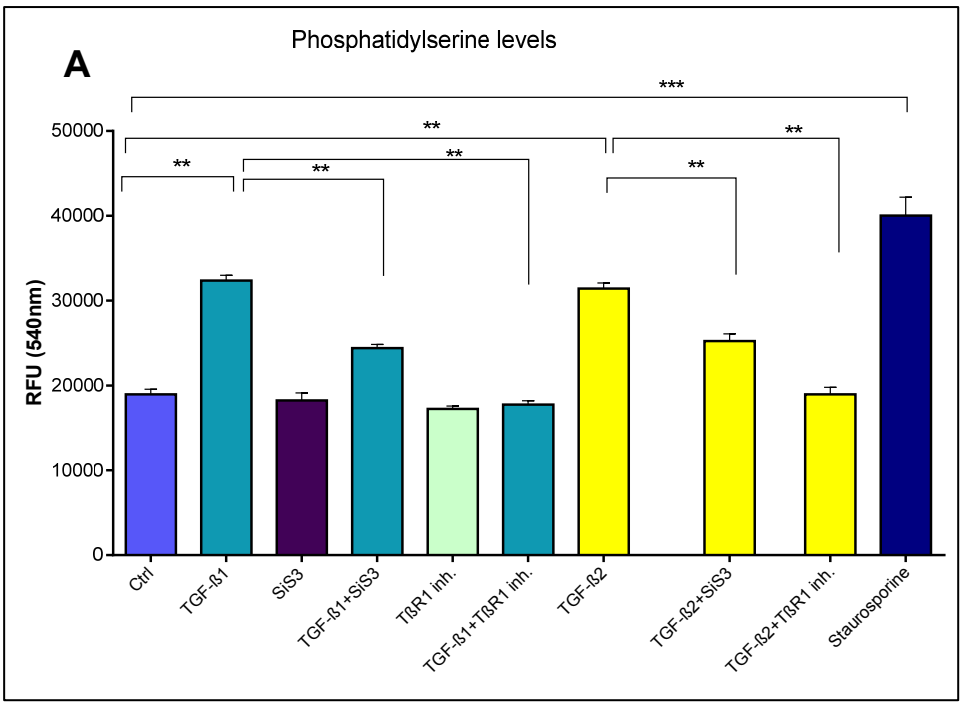
In summary, either TGF-β1 or TGF-β2 induced apoptosis in all cell lines studied. There was an increase in the amount of phosphatidylserine translocated to the outer surface of the cells, an increase of Caspase 3/7 levels and a decrease of the inner mitochondrial membrane potential. There were no significant differences between endometrial and endometriotic cell lines. Furthermore, the apoptotic effects of TGF-β1 or TGF-β2 on primary endometrial stromal cells and endometrial stromal cells were similar.

Analysis of the Smad Pathway in Apoptosis in Endometrial and Endometriotic Cells

The inhibitors LY364947 and SiS3 were used to investigate the role of the Smad-dependent pathway in apoptosis. The inhibitors block phosphorylation of Smad2/3 by selectively inhibiting TβR1 and Smad3 phosphorylation, respectively.

LY364947 (5 μM) and SiS3 (2 μM) were added two hours before adding the TGF-βs (10ng/ml) or staurosporine (0.1 μM), respectively, for 24 hours and then apoptosis experiments were carried out by ELISAs. The apoptotic parameters tested included quantification of phosphatidylserine, the mitochondrial membrane potential and Caspase 3/7 levels.

The results showed that treatment of cells with TGF-β1 or TGF-β2, respectively, increased phosphatidylserine on the outer surface of the cell membrane, decreased the inner mitochondrial membrane potential and increased Caspase 3/7 in all cells. LY364947 blocked TGF-β-mediated apoptosis in all cell lines completely, whereas SiS3 blocked TGF-β-mediated apoptosis in all cell lines only partially (Fig. 31).


A
Phosphatidylserine levels
RFU (540nm)
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10000
0
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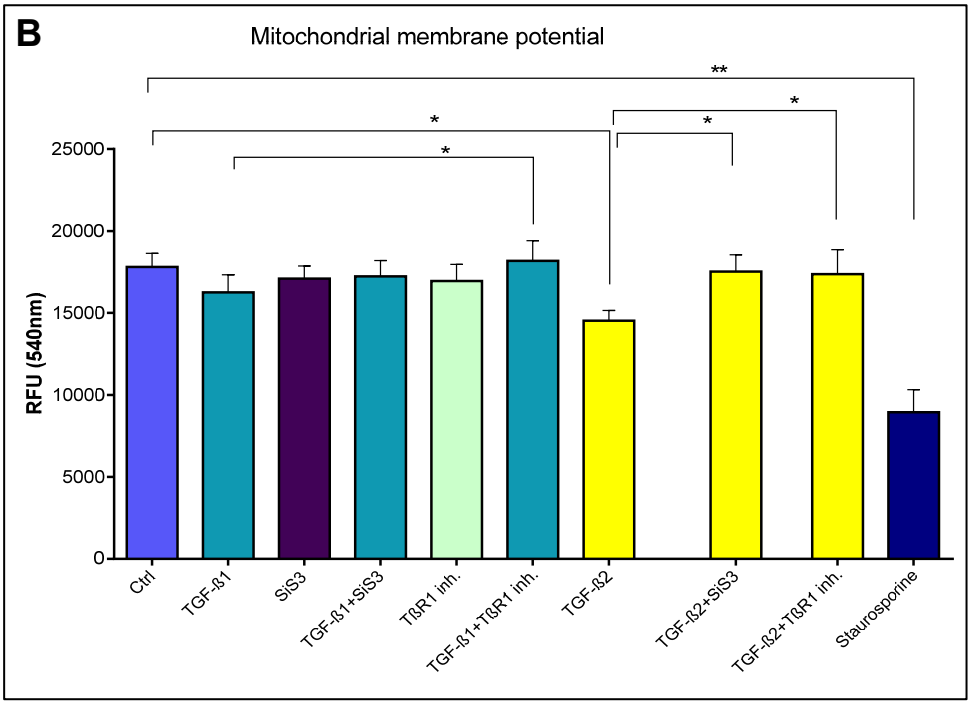
TGF-ß1
SiS3
TGF-ß1+SiS3
TßR1 inh.
TGF-ß1+TßR1 inh.
TGF-ß2
TGF-ß2+SiS3
TGF-ß2+TßR1 inh.
Staurosporine


B
Mitochondrial membrane potential
RFU (540nm)
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TGF-ß1
SiS3
TGF-ß1+SiS3
TßR1 inh.
TGF-ß1+TßR1 inh.
TGF-ß2
TGF-ß2+SiS3
TGF-ß2+TßR1 inh.
Staurosporine

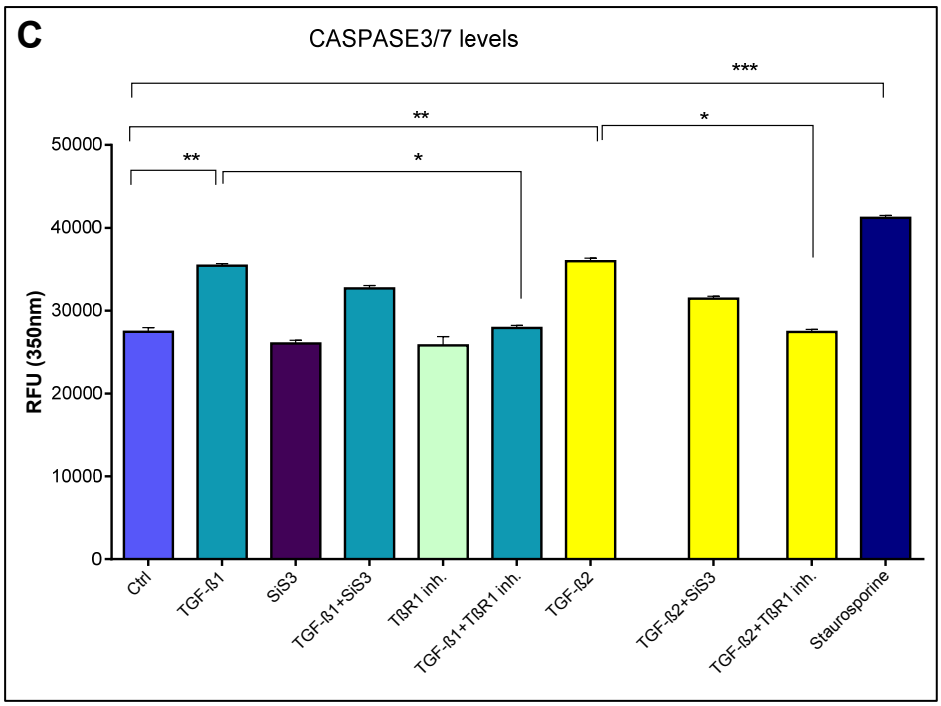


Figure 31. Treatment of endometrial epithelial HES cells with TGF-β1 or TGF-β2, respectively, induced apoptosis. There was an increase in the amount of translocated phosphatidylserine **(A)** (**=P<0.01, ***=P<0.001, n=6), a decrease of the mitochondrial membrane potential **(B)** (*=P<0.05, **=P<0.01, n=6), and an increase of the amount of Caspase 3/7 **(C)** (*=P<0.05, **=P<0.01, ***=P<0.001, n=6). LY364947 blocked TGF-β-mediated apoptosis in HES cells completely, whereas SiS3 blocked TGF-β-mediated apoptosis in cells only partially (A-C). Similar results were observed in the other cell lines (data not shown). RFU; relative fluorescence units.

3.6 Influence of TGF-β1 or TGF-β2 on Plasminogen Activator Inhibitor-1 Secretion by Endometrial and Endometriotic Cells in vitro

Plasminogen Activator Inhibitor-1 (PAI-1) belongs to the serine protease inhibitor family (SERPIN) and is a modifier of pathways that impact proliferative/migratory events (Czekay et al., 2003), PAI-1 is one of the gold standards for studying the effects of TGF-βs on gene expression. Moreover, PAI-1 disrupts integrin-containing adhesions and actin stress fibers which might result in apoptosis or reduced cell numbers (Czekay et al., 2003).

3.6.1 Analysis of TGF-β-induced PAI-1 Secretion by Endometrial and Endometriotic Cells

To investigate the effects of TGF-βs on PAI-1 secretion, endometrial and endometriotic cells were treated with 10ng/ml of TGF-β1 or TGF-β2, respectively. After 48 hours, supernatants were collected to quantitate PAI-1.

PAI-1 secretion was increased significantly by TGF-β1 or TGF-β2, approximately 40-fold in endometrial epithelial cells (HES), 5-fold in endometriotic epithelial cells (12ZVK), 3-fold in stromal cells (T-HESC, primary endometrial stromal cells, and 22B), compared to the controls (Figs. 32-34).

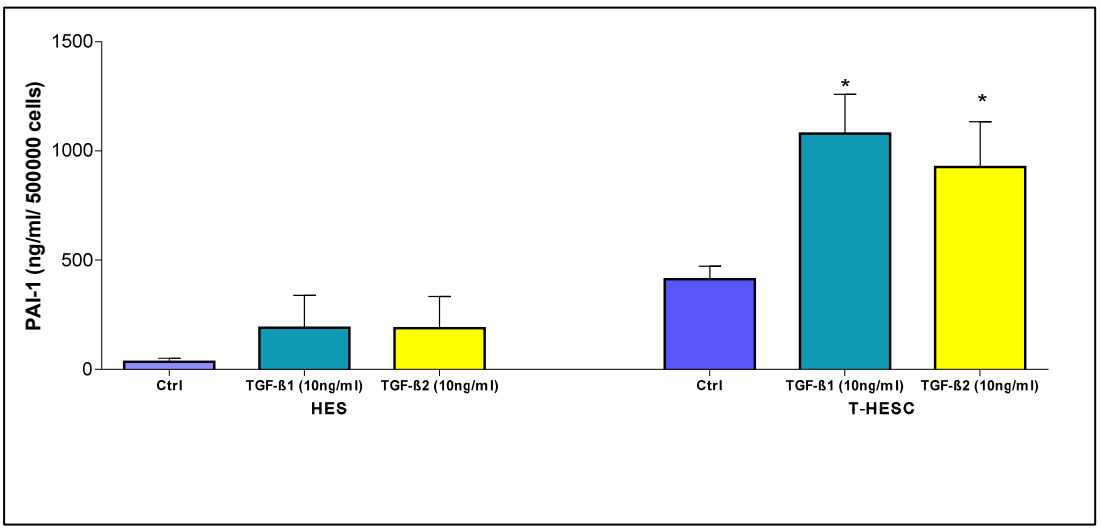


Figure 32. Quantification of PAI-1 secretion by endometrial cells with or without treatment with TGF-βs. HES cells secreted only negligible amounts of PAI-1, but secretion was slightly increased upon treatment with TGF-βs. Endometrial stromal cells (T-HESC) secreted 10-fold more PAI-1 compared to endometrial epithelial cells (HES) stimulated by TGF-β1 or TGF-β2 (10ng/ml), respectively (*=P<0.05, n=6).

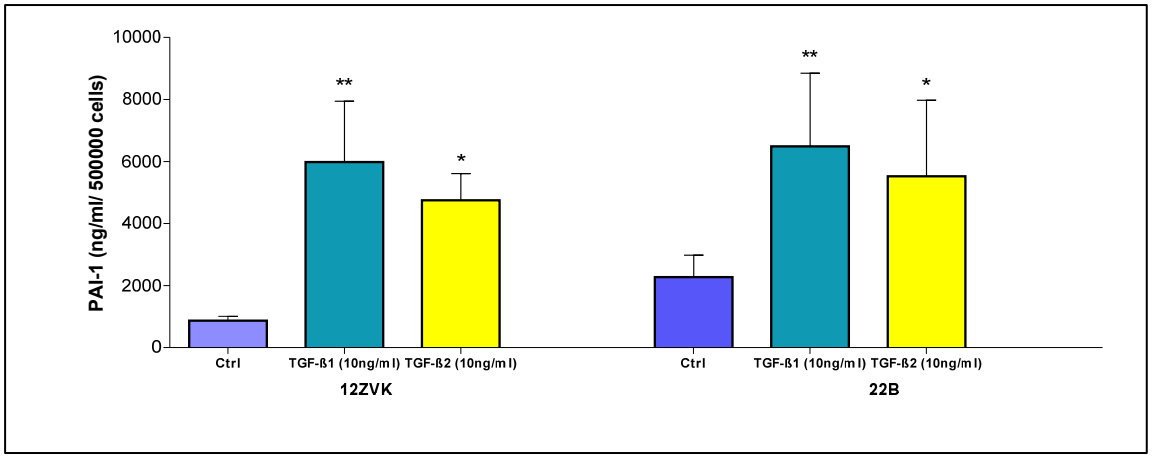


Figure 33. Quantification of PAI-1 secretion by endometriotic cells with or without treatment with TGF-βs. Both endometriotic epithelial cells (12ZVK) and endometriotic stromal cells (22B) secreted more PAI-1 upon TGF-β1 or TGF-β2 (10ng/ml) treatment, respectively (*=P<0.05, **=P<0.01, n=6).

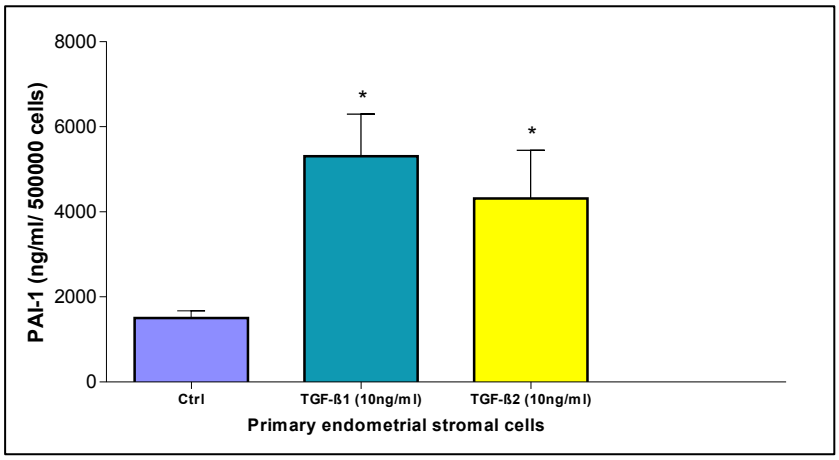


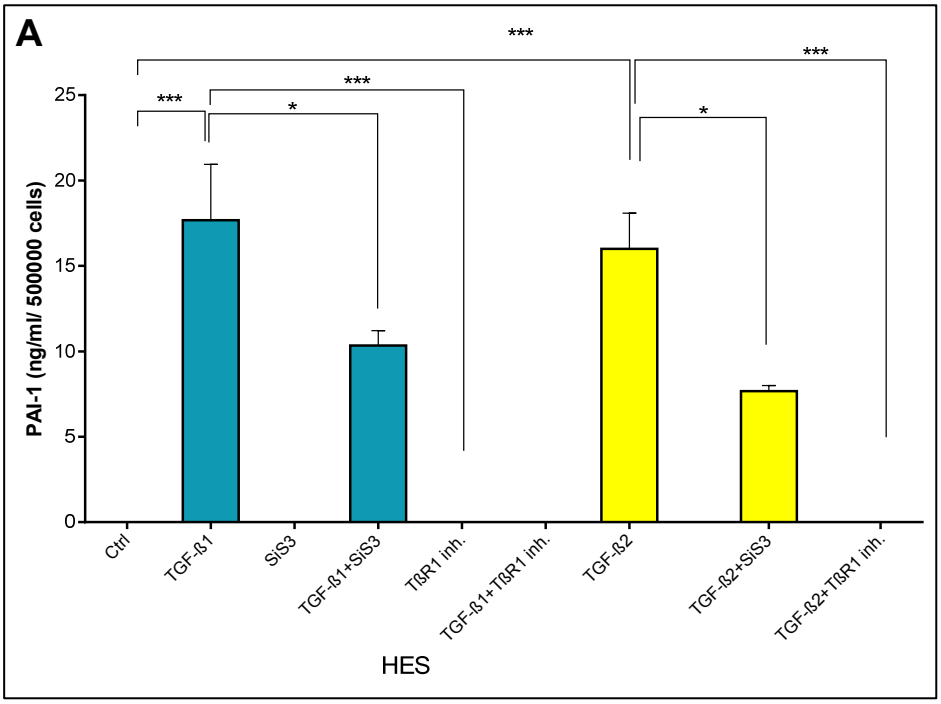
Figure 34. Quantification of PAI-1 secretion by primary endometrial stromal cells with or without TGF-βs. The primary endometrial stromal cells secreted more PAI-1 upon TGF-β1 or TGF-β2 (10ng/ml) treatment, respectively (*=P<0.05, n=6).

In summary, endometriotic epithelial cells 12ZVK secreted approximately 200-fold more PAI-1 compared to endometrial epithelial cells HES. Endometriotic stromal cells 22B secreted 4-fold more PAI-1 compared to endometrial stromal cells T-HESC. Furthermore PAI-1 secretion by primary endometrial stromal cells was nearly similar to secretion levels of endometrial stromal cells T-HESC.

3.6.2 Analysis of the Smad Pathway in PAI-1 Secretion in Endometrial and Endometriotic Cells

The inhibitors LY364947 and SiS3 were used to investigate the role of the Smad-dependent pathway in PAI-1 secretion. The inhibitors block phosphorylation of Smad2/3 by selectively inhibiting TβR1 and Smad3, respectively. LY364947 (5 µM) and SiS3 (2 µM) were added two hours before adding the TGF-βs (10ng/ml). Cells were cultured for 48 hours after stimulation with the TGF-βs. Then supernatants were collected for quantification of PAI-1 secretion by PAI-1 ELISA.

The results showed that TGF-β1 and TGF-β2 both increased PAI-1 secretion in all cell lines studied. LY364947 blocked TGF-β-mediated PAI-1 secretion in cells completely to control levels, whereas SiS3 blocked TGF-β-mediated PAI-1 secretion in all cells only partially (Fig. 35).



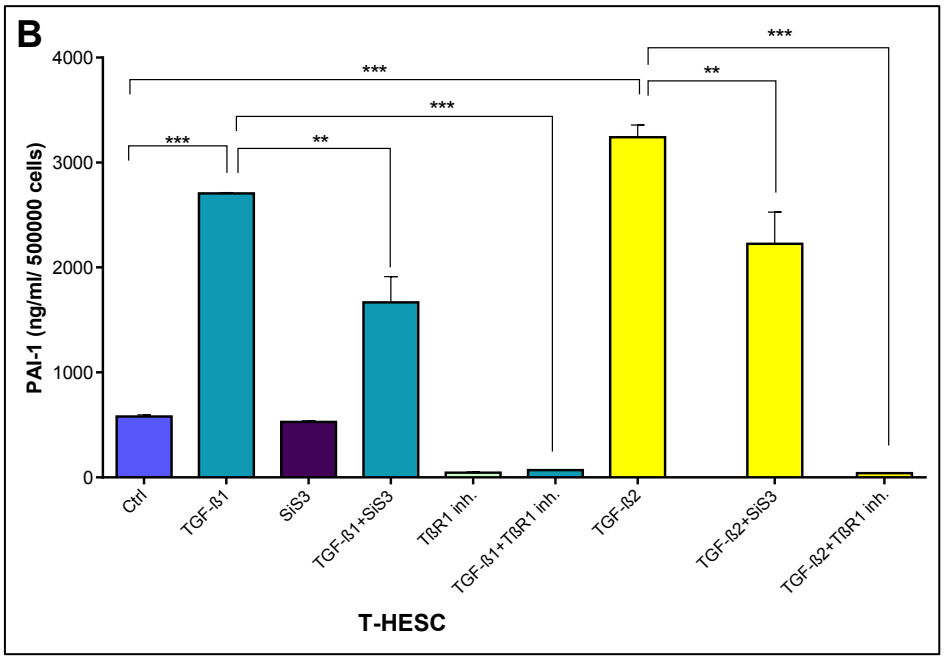


Figure 35. Treatment of cells with TGF-β1 or TGF-β2 (10ng/ml), respectively, induced PAI-1 secretion in endometrial epithelial cells, HES **(A)**, and endometrial stromal cells, T-HESC **(B)**. LY364947 blocked TGF-β-induced PAI-1 secretion of all cells completely to control levels while SiS3 blocked TGF-β-induced PAI-1 secretion of cells in a range of 40-70%. Similar results were observed with the other cell lines (data not shown).

3.6.3 Analysis of the BMP Pathway in PAI-1 Secretion

The BMP inhibitor LDN-193189 was used to study the role of the BMP pathway in PAI-1 secretion. The BMP inhibitor selectively inhibits the BMP type 1 receptors ALK2, ALK3 and ALK 6 and thus blocks BMP-mediated phosphorylation of Smad1/5/8.

The BMP inhibitor (5μM) was added two hours before adding the TGF-βs (10ng/ml). Cells were cultured for 48 hours after stimulation with the TGF-βs. Then supernatants were collected for quantification of PAI-1 secretion.

The results showed that TGF-β1 or TGF-β2, respectively, increased PAI-1 secretion in all cell lines studied. The BMP inhibitor demonstrated a complete decrease (100%) of TGF-β1 or TGF-β2 induced-PAI-1 secretion in all cell lines studied (Figs. 36-38).

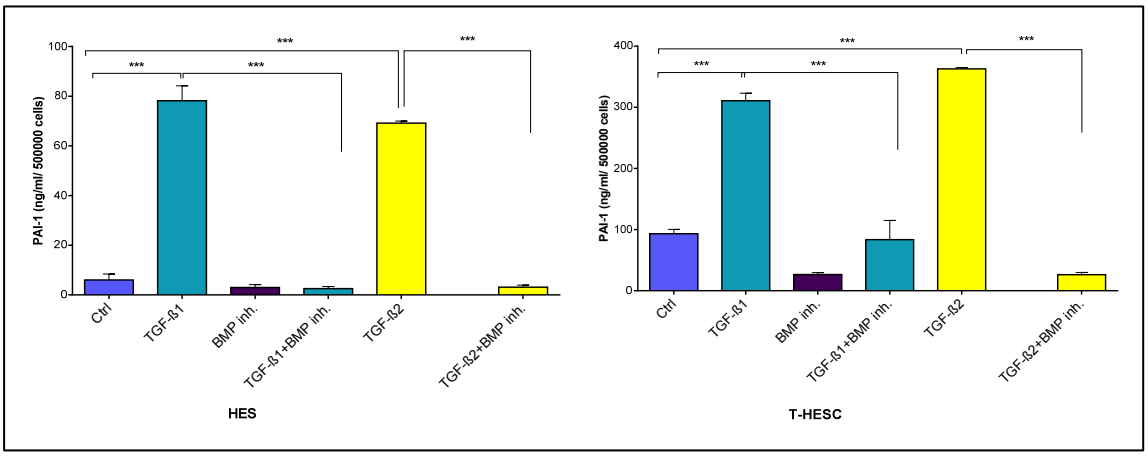


Figure 36. Treatment of endometrial cells HES or T-HESC with TGF-β1 or TGF-β2 (10ng/ml), respectively, induced PAI-1 secretion. T-HESC secreted more PAI-1 compared to endometrial epithelial cells HES. The BMP inhibitor blocked TGF-β-induced PAI-1 secretion in both cell lines to control levels (***=P<0.001, n=6).

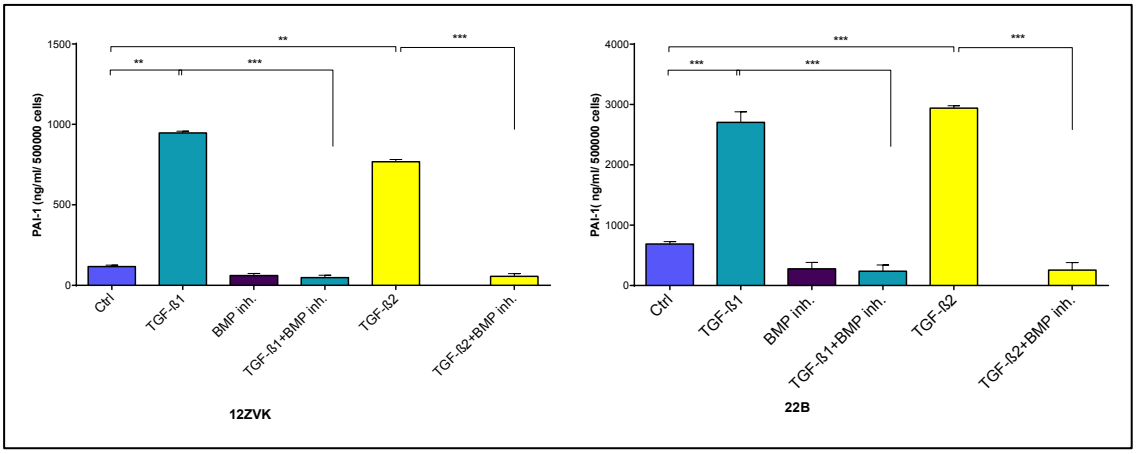


Figure 37. Treatment of endometriotic cells 12ZVK or 22B with TGF-β1 or TGF-β2 (10ng/ml), respectively, induced PAI-1 secretion. 22B secreted more PAI-1 compared to endometriotic epithelial cells 12ZVK. The BMP inhibitor blocked TGF-β-induced PAI-1 secretion in both cell lines to control levels (**=P<0.01, ***=P<0.001, n=6).

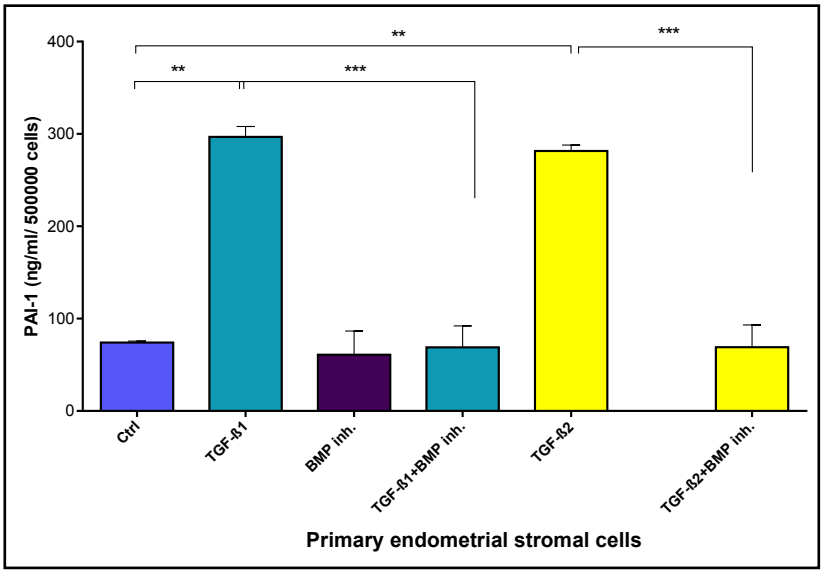


Figure 38. Treatment of primary endometrial stromal cells with TGF-β1 or TGF-β2, (10ng/ml) respectively, induced PAI-1 secretion. The BMP inhibitor blocked TGF-β-induced PAI-1 secretion of primary endometrial cells to control levels (**=P<0.01, ***=P<0.001, n=6).

3.6.4 Analysis of BMP Receptors in PAI-1 Secretion

Activin Receptor-Like Kinase (ALK), namely ALK-2, ALK-3 and ALK-6, inhibitors were used separately to study the specific BMP receptor involved in the complete decrease of TGF-β1 or TGF-β2 induced-PAI-1 secretion in cells as shown by the BMP inhibitor LDN-193189 in section 3.6.3.

The ALK-2 (10μM), ALK-3 (4μM), ALK-6 (6μM) inhibitors and IgG1 (2μM) were added two hours before adding the TGF-βs (10ng/ml). Cells were cultured for 48 hours after stimulation with the TGF-βs. Then supernatants were collected for quantification of PAI-1 secretion.

The results showed that TGF-β1 or TGF-β2 increased PAI-1 secretion of all cell lines studied. The ALK-2 inhibitor demonstrated a complete decrease (100%) of TGF-β1 or TGF-β2 induced-PAI-1 secretion in all cell lines, whereas the ALK-3 and ALK-6 inhibitors demonstrated only a partial effect of 40% and 25%, respectively. The IgG1 (control) had no affect on TGF-β1 or TGF-β2 induced-PAI-1 secretion (Figs. 39-41).

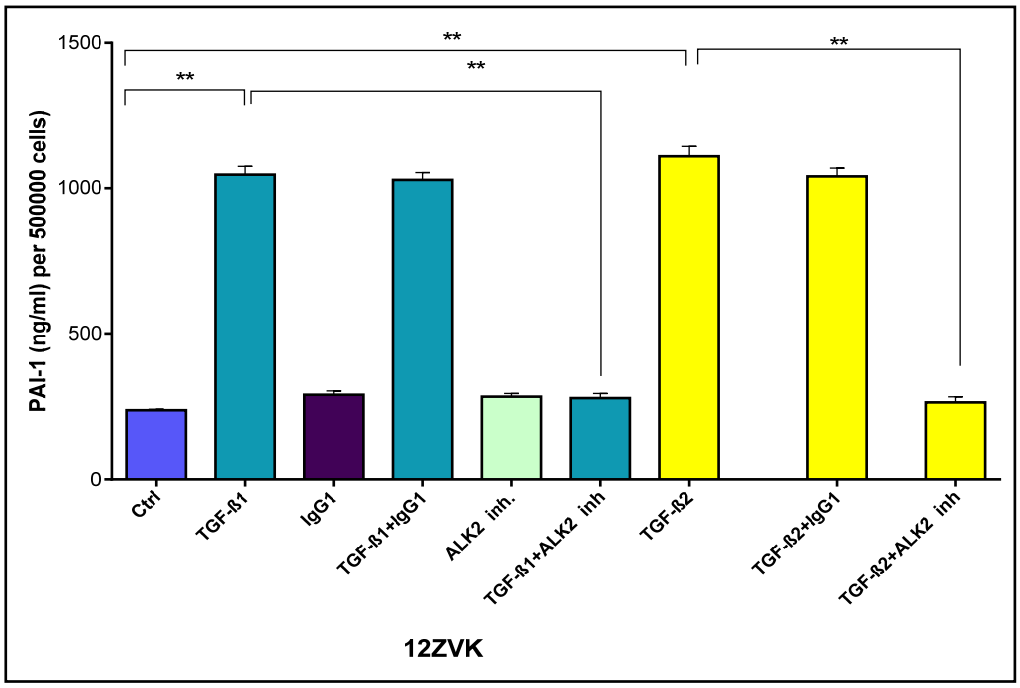


Figure 39. Treatment of endometriotic cells (12ZVK) with TGF-β1 or TGF-β2 (10ng/ml), respectively, induced PAI-1 secretion. The ALK-2 inhibitor blocked TGF-β-induced PAI-1 secretion completely to control levels, whereas IgG1 had no effect on TGF-β-induced PAI-1 secretion (**=P<0.01, n=6). Similar results were observed in the other cell lines (data not shown).

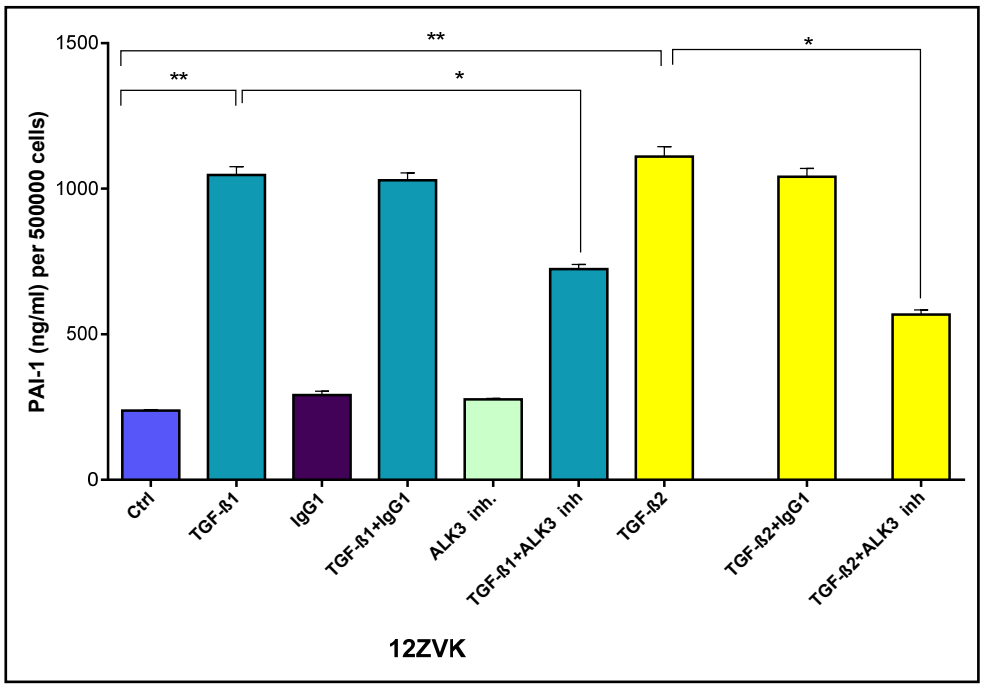


Figure 40. Treatment of endometriotic cells (12ZVK) with TGF-β1 or TGF-β2 (10ng/ml), respectively, induced PAI-1 secretion. The ALK-3 inhibitor blocked TGF-β-induced PAI-1 secretion by 40%, whereas IgG1 had no effect on TGF-β-induced PAI-1 secretion (*=P<0.05, **=P<0.01, n=6). Similar results were observed in the other cell lines (data not shown).

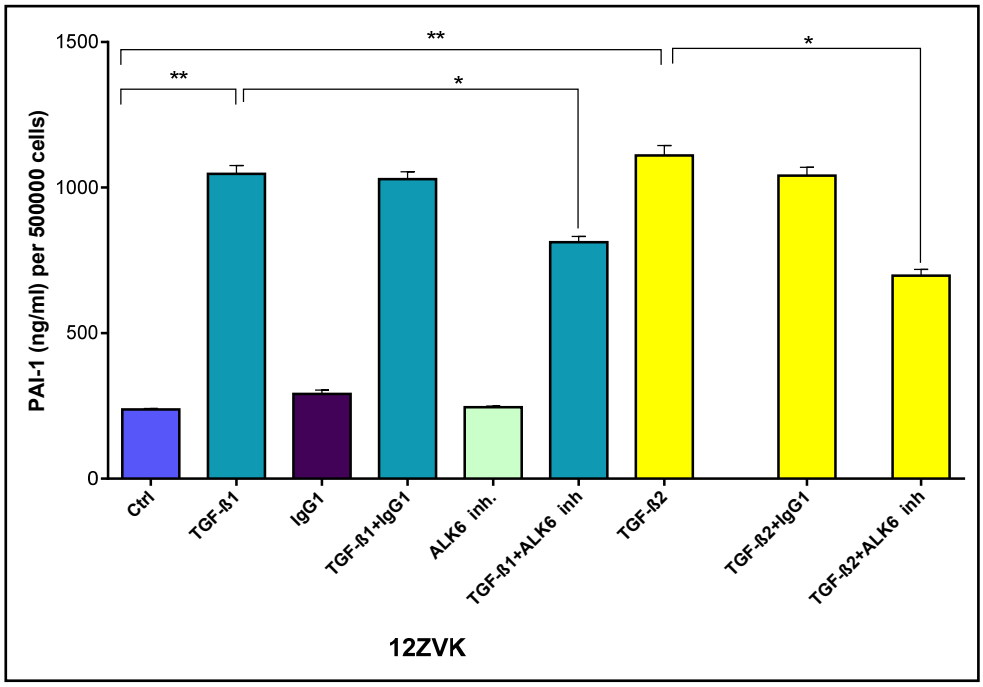


Figure 41. Treatment of endometriotic cells (12ZVK) with TGF-β1 or TGF-β2 (10ng/ml), respectively, induced PAI-1 secretion. The ALK-6 inhibitor blocked TGF-β-induced PAI-1 secretion by 25%, whereas IgG1 had no effect on TGF-β-induced PAI-1 secretion (**=P<0.05, **=P<0.01, n=6). Similar results were observed in the other cell lines (data not shown).

3.7 Influence of TGF-β1 or TGF-β2 on Inhibin B Secretion by Endometrial and Endometriotic Cells in vitro

Inhibin B belongs to the TGF-β superfamily regulates reproduction. Recent studies have shown that inhibins might be involved in tumour suppression (Gail et al., 2001). Inhibin B secretion is one possible marker for the functionality of the BMP pathway. Thus, by treating endometrial or endometriotic cells with TGF-β1 or TGF-β2, it might be possible to elucidate whether TGF-βs might also use the BMP pathway-dependent Smad1/5/8.

To investigate the effects of TGF-βs on inhibin B secretion, endometrial and endometriotic cells were treated with TGF-β1 or TGF-β2 (10 ng/ml), respectively, for 48 hours and supernatants were collected for the inhibin B ELISA.

The results showed that TGF-β1 or TGF-β2 reduced inhibin B secretion of HES cells and primary endometrial stromal cells while inhibin B secretion was not affected by TGF-β1 or TGF-β2 in the other cell lines studied (Figs. 42-44).

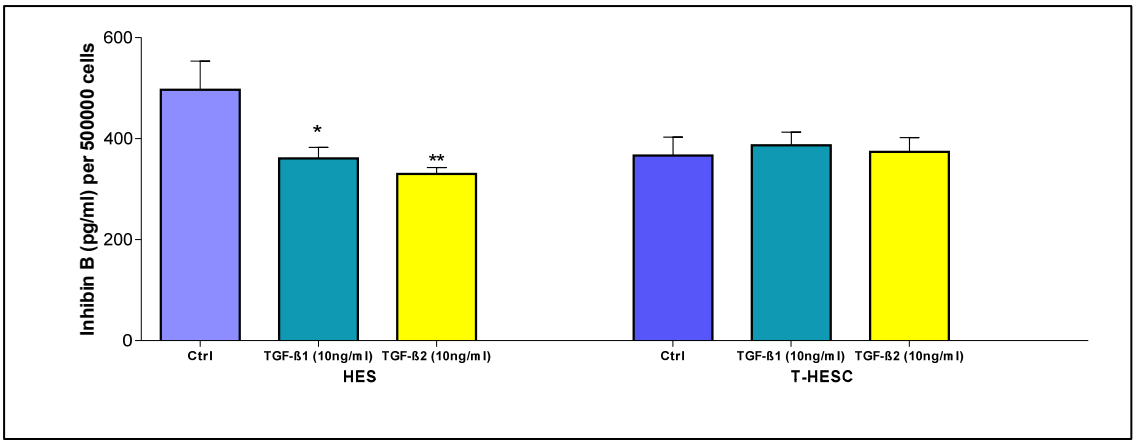


Figure 42. Treatment of endometrial epithelial cells (HES) with TGF-β1 or TGF-β2 (10ng/ml), respectively, reduced inhibin B secretion compared to control. There were no effects on inhibin B levels in T-HESC cells (*=P<0.05, **=P<0.01 n=6).

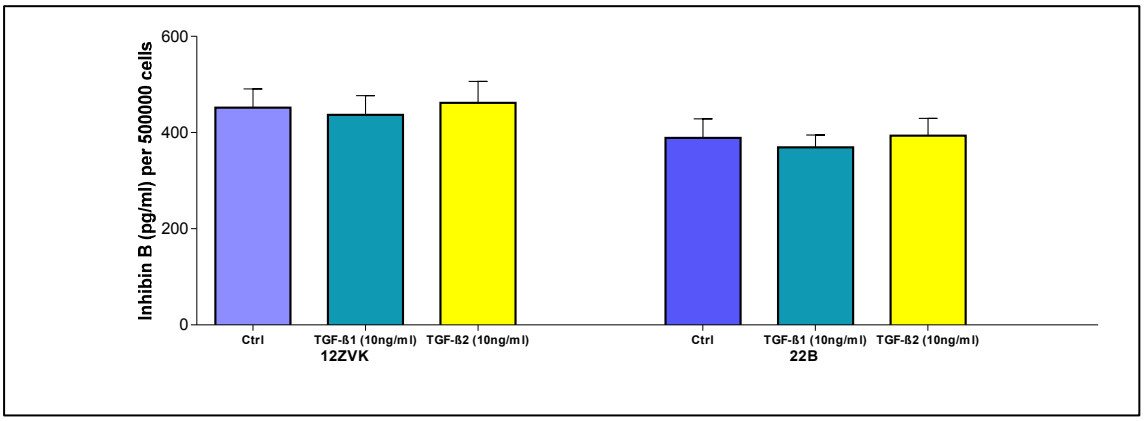


Figure 43. Treatment of endometriotic cells (12ZVK and 22B) with TGF-β1 or TGF-β2 (10ng/ml), respectively, did not show any effect on inhibin B secretion.

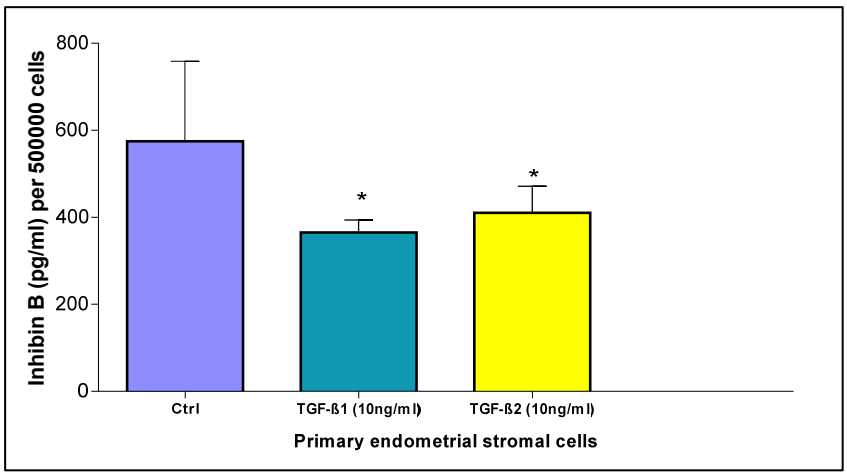


Figure 44. Treatment of primary endometrial stromal cells with TGF-β1 or TGF-β2 (10ng/ml), respectively reduced inhibin B secretion compared to the control (*=P<0.05, n=6).

3.8 Influence of TGF-β1 or TGF-β2 on TβRIII Expression of Endometrial and Endometriotic Cells in vitro

High affinity binding of TGF-β2 to Transforming growth factor beta receptor II (TβRII) is best via TβRIII (Lopez et al., 1993). However, TGF-β1 as well as TGF-β3 also bind to TβRIII.

In our experiments, 24-well plates were coated with TGF-β1 or TGF-β2 (10ng/ml), respectively, and incubated for 48 hours. The cells were fixed with ice-cold 4% paraformaldehyde (PFA). A high affinity TβRIII antibody (R&D) was used as a primary antibody. An anti-goat peroxidase (DAKO) was used as a secondary antibody. TMB substrate (Calbiochem, Germany) was added followed by addition of 0.18N H_2SO_4 to stop the reaction. Absorbance was read at 450nm.

Treatment of cells with TGF-β1 or TGF-β2 increased expression of TβRIII on stromal cells whereas epithelial cells showed little or no increase (Figs. 45-47).

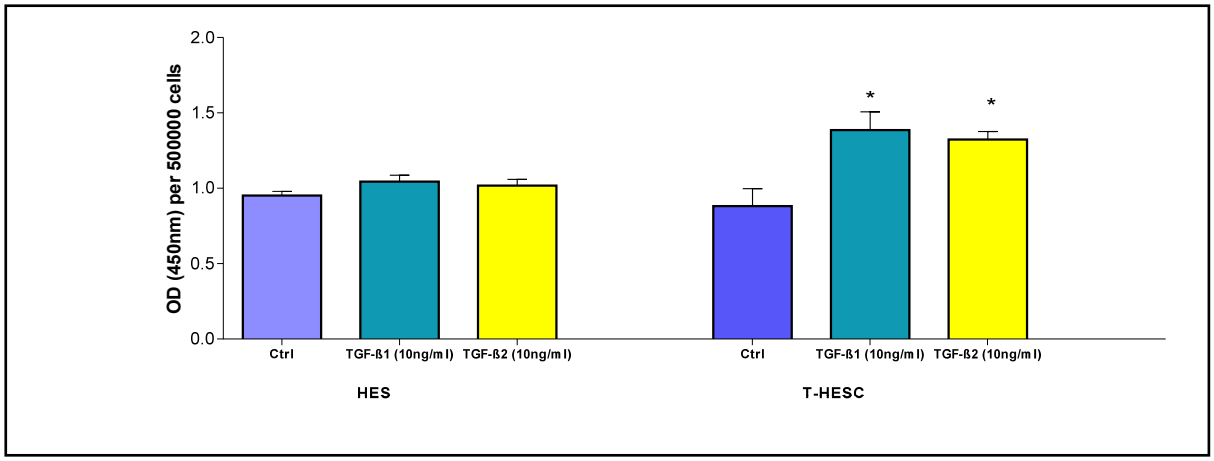


Figure 45. Treatment of endometrial cells with TGF-β1 or TGF-β2 (10ng/ml), respectively, increased expression of TβRIII only on T-HESC cells with no effect on endometrial epithelial cells HES (*=P<0.05, n=6).

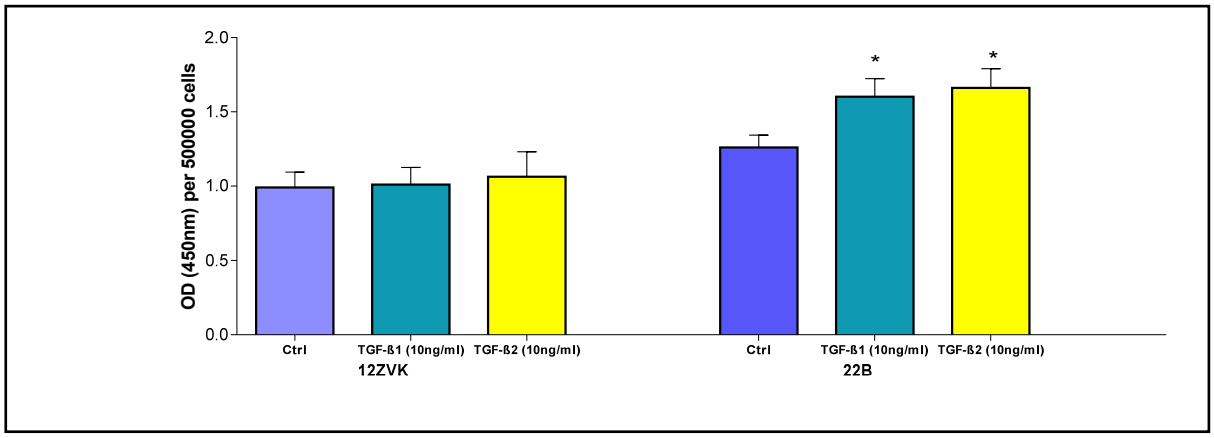


Figure 46. Treatment of endometriotic cells with TGF-β1 or TGF-β2 (10ng/ml), respectively, increased expression of TβRIII only on 22B cells with no effect on endometriotic epithelial cells 12ZVK (*=P<0.05, n=6).

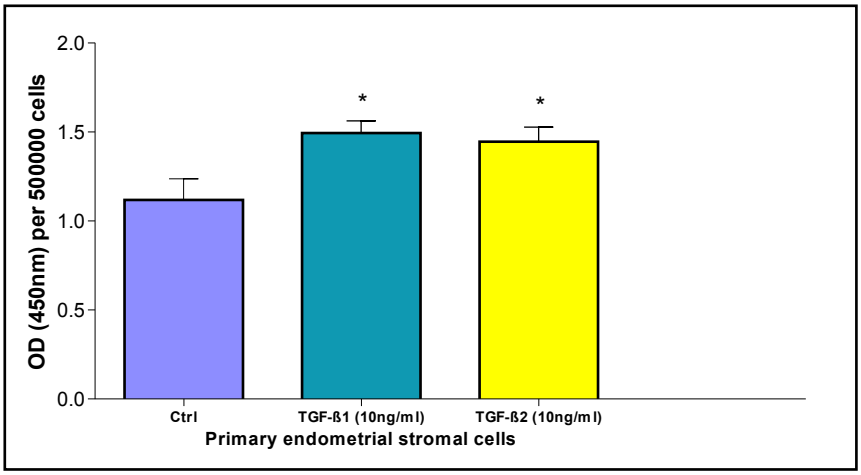


Figure 47. Treatment of primary endometrial stromal cells with TGF-β1 or TGF-β2 (10ng/ml) increased expression of TβRIII on the cells (*=P<0.05, n=6). The TβRIII expression was similar to the endometrial stromal cell line T-HESC (Fig. 45).

3.9 Influence of TGF-β1 or TGF-β2 on Secretion of TGF-β2 by Endometrial and Endometriotic Cells in vitro

To investigate the influence of TGF-β1 or TGF-β2 on secretion of TGF-β2 by endometrial and endometriotic cells, we quantitated TGF-β2 secretion by cells.

The endometrial and endometriotic cells were treated with TGF-β1 or TGF-β2 (10ng/ml), respectively. Then cell supernatants were collected after 48 hours upon TGF-β1 or TGF-β2 treatment to quantitate TGF-β1 or TGF-β2.

Treatment of cells with TGF-β1 or TGF-β2 increased secretion of TGF-β2 in TGF-β1-treated cells. No effect on TGF-β2 levels was observed in controls and TGF-β2-treated cells (Figs. 48-50).

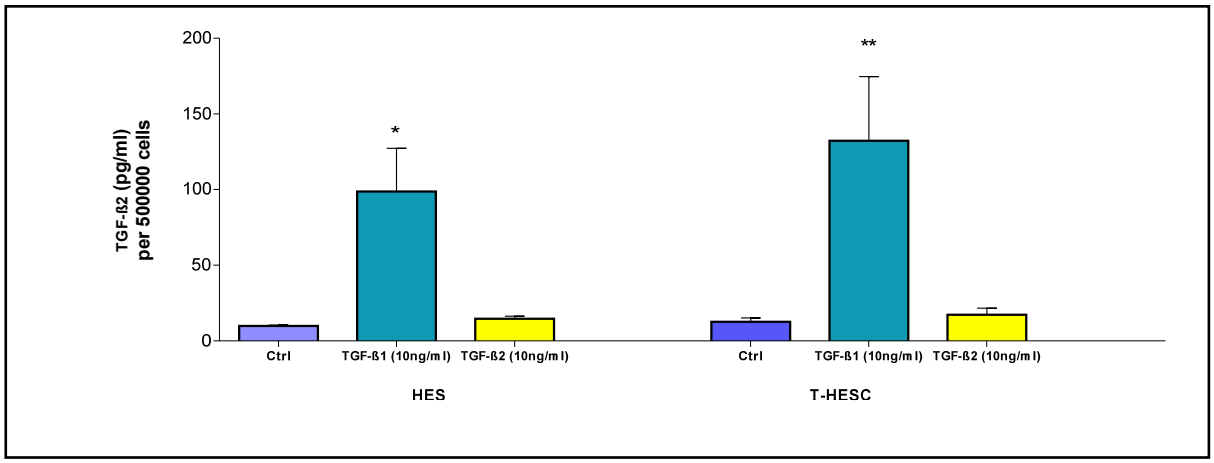


Figure 48. Treatment of endometrial cells (HES and T-HESC) with TGF-β1 or TGF-β2 (10ng/ml), respectively, increased secretion of TGF-β2 in TGF-β1-treated cells. Endometrial stromal cells, T-HESC, secreted more TGF-β2 compared to endometrial epithelial cells, HES. No effect on TGF-β2 levels was observed in controls and TGF-β2-treated endometrial cells (*=P<0.05, **=P<0.01, n=6).

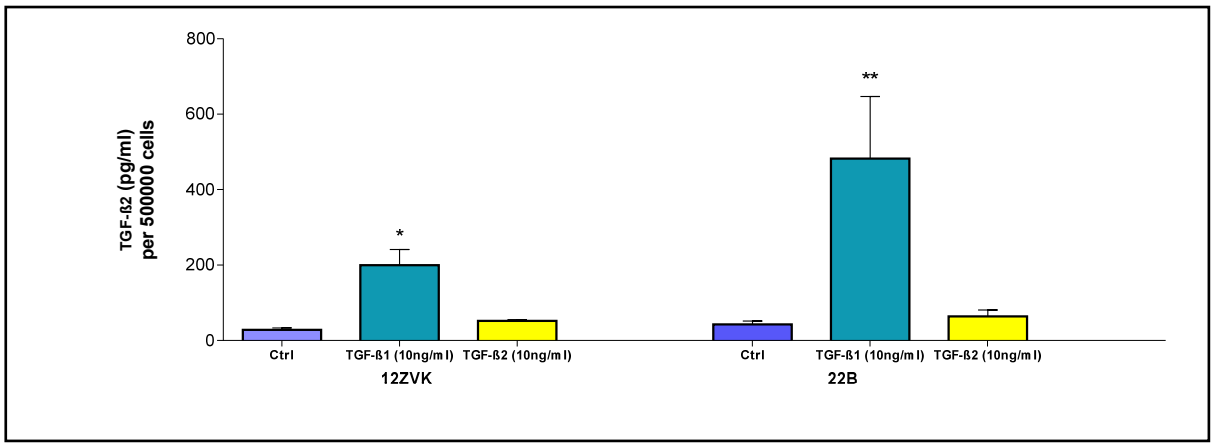


Figure 49. Treatment of endometriotic cells (12ZVK and 22B) with TGF-β1 or TGF-β2 (10ng/ml), respectively, increased secretion of TGF-β2 in TGF-β1-treated cells. Endometriotic stromal cells, 22B, secreted more TGF-β2 compared to endometriotic epithelial cells, 12ZVK. No effect on TGF-β2 levels was observed in controls and TGF-β2-treated cells in endometriotic cells (*=P<0.05, **=P<0.01, n=6).

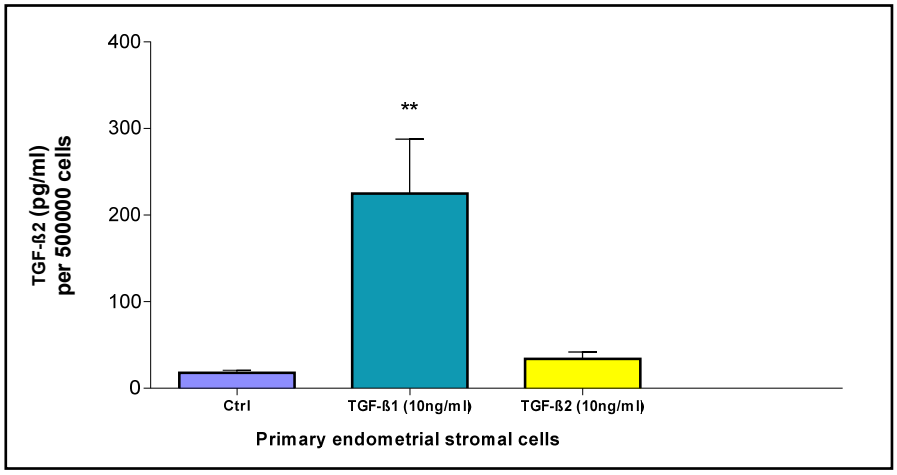


Figure 50. Treatment of primary endometrial stromal cells with TGF-β1 or TGF-β2 (10ng/ml), respectively, increased secretion of TGF-β2 in TGF-β1-treated endometrial stromal cells. No effect on TGF-β2 levels was observed in controls and TGF-β2-treated cells (**=P<0.01, n=6).

In summary, in all cell lines studied, there was increased TGF-β2 secretion in TGF-β1-treated cells. No effect was observed in control cells and cells treated with TGF-β2. Endometriotic epithelial cells, 12ZVK, secreted 2-fold more TGF-β2 compared to endometrial epithelial cells, HES, after stimulation with TGF-β1. Endometriotic stromal cells, 22B, secreted 3-fold more TGF-β2 compared to endometrial stromal cells T-HESC. TGF-β1-treated T-HESC as well as TGF-β-treated primary endometrial stromal cells secreted almost the same amount of TGF-β2.

3.10 Influence of TGF-β1 or TGF-β2 on Secretion of TGF-β1 by Endometrial and Endometriotic Cells in vitro

To investigate the influence of TGF-β1 or TGF-β2 on secretion of TGF-β1 by endometrial and endometriotic cells, we quantitated TGF-β1 secretion by cells.

The endometrial and endometriotic cells were treated with TGF-β1 or TGF-β2 (10ng/ml), respectively. Then cell supernatants were collected after 48 hours upon TGF-β1 or TGF-β2 treatment to quantitate TGF-β1.

Treatment of cells with TGF-β1 or TGF-β2 increased secretion of TGF-β1 in TGF-β1-treated cells. No effect on TGF-β1 levels was observed in controls and TGF-β2-treated cells (Figs. 51-53).

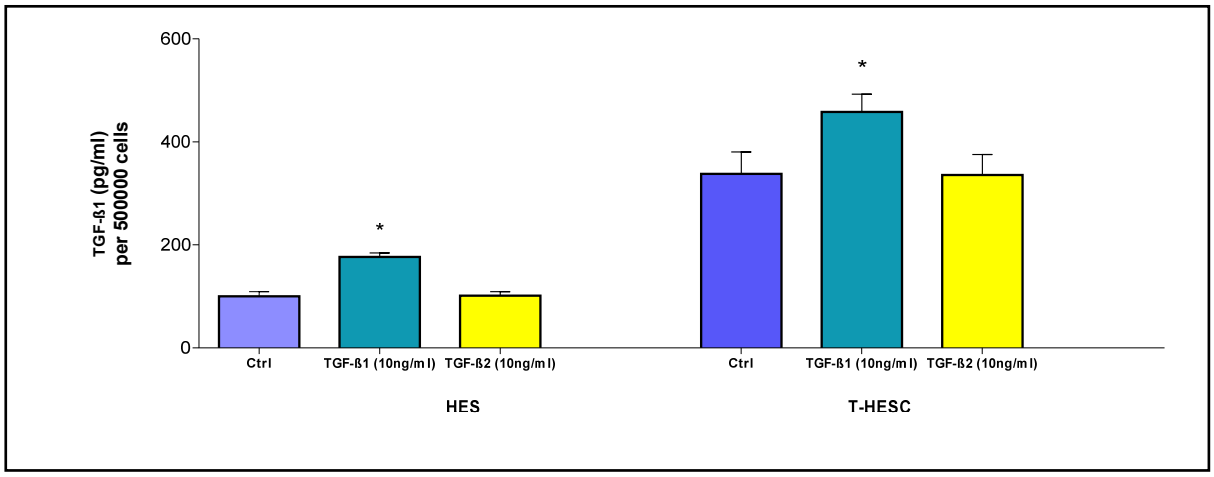


Figure 51. Treatment of endometrial cells (HES and T-HESC) with TGF-β1 or TGF-β2 (10ng/ml), respectively, increased secretion of TGF-β1 in TGF-β1-treated cells. Endometrial stromal cells, T-HESC, secreted 2-fold more TGF-β1 compared to endometrial epithelial cells HES. No effect on TGF-β1 levels was observed in controls and TGF-β2-treated cells in endometrial cells (*=P<0.05, n=6).

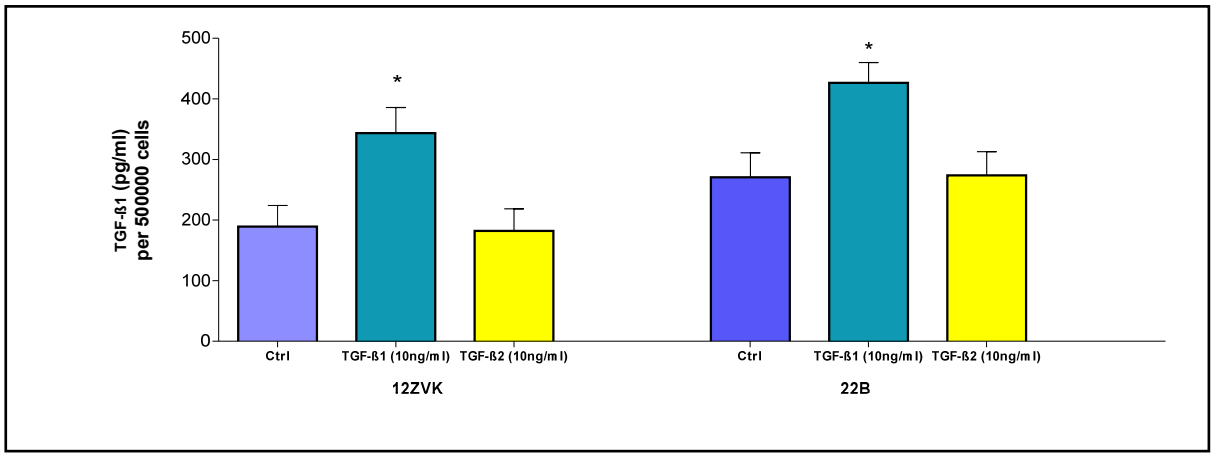


Figure 52. Treatment of endometriotic cells (12ZVK and 22B) with TGF-β1 or TGF-β2 (10ng/ml), respectively, increased secretion of TGF-β1 in TGF-β1-treated cells. Endometriotic stromal cells, 22B, secreted 1.5-fold more TGF-β1 compared to endometriotic epithelial cells 12ZVK. No effect on TGF-β1 levels was observed in controls and TGF-β2-treated endometriotic cells (*=P<0.05, n=6).

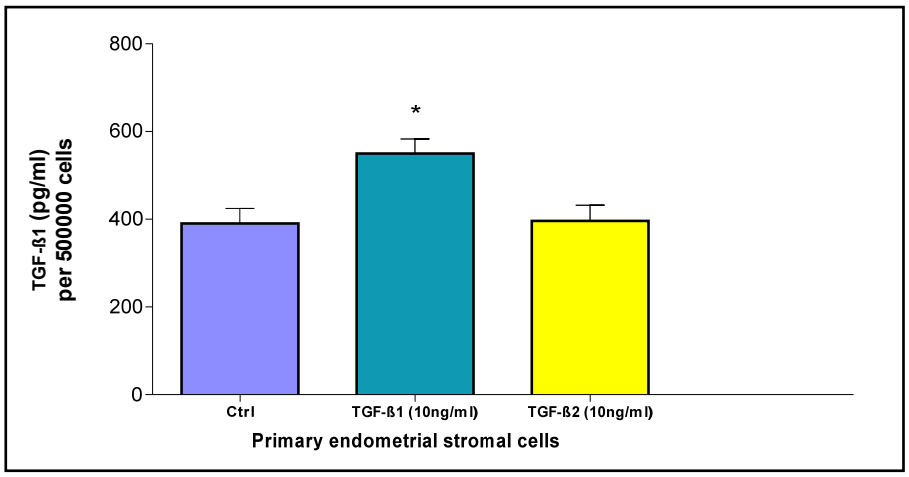
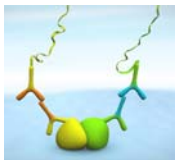


Figure 53. Treatment of primary endometrial stromal cells with TGF-β1 or TGF-β2 (10ng/ml), respectively, increased secretion of TGF-β1 in TGF-β1-treated endometrial stromal cells. No effect on TGF-β1 levels was observed in controls and TGF-β2-treated cells (*=P<0.01, n=6).

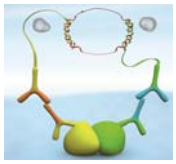
3.11 Influence of TGF-β1 or TGF-β2 on Interaction of TβRI, TβRII and TβRIII Receptors on Endometrial and Endometriotic Cells in vitro (Signalosome analysis)

To quantify and analyze the interaction of the TβRI, TβRII and TβRIII receptors on primary endometrial cells and other cell lines, we used the Proximity Ligation Assay (PLA). PLA enables detection, visualization and quantification of protein interactions in tissue and cell samples. The PLA principle is shown in Fig. 54

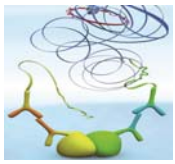
Step 1: A pair of primary antibodies raised in different species bind to the target antigens of interest



Step 2: A pair of species-specific secondary antibodies called, PLA probes (PLUS and MINUS), bind to their respective primary antibody



Step 3: Two oligonucleotides are joined by a ligase to form a circular molecule only when the two PLA probes are at a close proximity



Step 4: A polymerase amplifies the circle forming several hundred-fold replication of DNA circle

Step 5: Labeled complementary oligonucleotide probes bind the synthesized DNA strand allowing the product to be visualized with a fluorescence microscope

Figure 54. Principle of Duolink In Situ PLA assay (adapted from http//www.Olink.com)

The resulting fluorescence in each single-molecule amplification product is visible as a distinct spot under a fluorescence microscope (Soderberg et al., 2006; Jurvious et al., 2007).

In our experiments, we applied *in situ* PLA (Duolink) with a combination of antibodies that allows the detection of TβRII/TβRIII and TβRI/TβRII interaction only when these are in close proximity upon treatment of cells with TGF-β1 or TGF-β2. The details of the antibodies used are indicated in Table 1.

Table 1. Antibody specifications and dilutions used for the PLA Duolink

Antibody	Species	Dilution	Company
TGFβ RI	Goat	1:200	Santa Cruz, USA
TGFβ RII	Rabbit	1:350	Abcam, UK
TGFβ RIII	Goat	1:200	R&D Systems, USA

For the PLA assay, 20,000 cells on 8-well chamber slides were treated with or without TGF-β1 or TGF-β2 for 24 hours and afterwards fixed and permeabilized. A pair of primary antibodies against TβRII and TβRIII or TβRI and TβRII was used to detect the complexes (Table 1). Controls were performed by omissions of the primary antibodies. Phase contrast images showed the exact localization of the signals. Images were obtained with the inverse microscope FSX 100 (Olympus) using the Olympus FSX-BSW software. Images were processed with Adobe Photoshop. The quantity of PLA signals was counted and the average number of spots per cell was presented graphically.

The results showed interaction of TβRII and TβRIII or TβRI and TβRII in all cell lines as shown by the red fluorescent spots. The quantity of red fluorescent spots varied in the cell lines depending on the intensity of TGF-βs/TβRs interaction. Control cells and cells without primary antibodies showed no TβR interactions (Figs. 55-62).

3.11.1 Interaction of TβRII and TβRIII Receptors on HES Cells

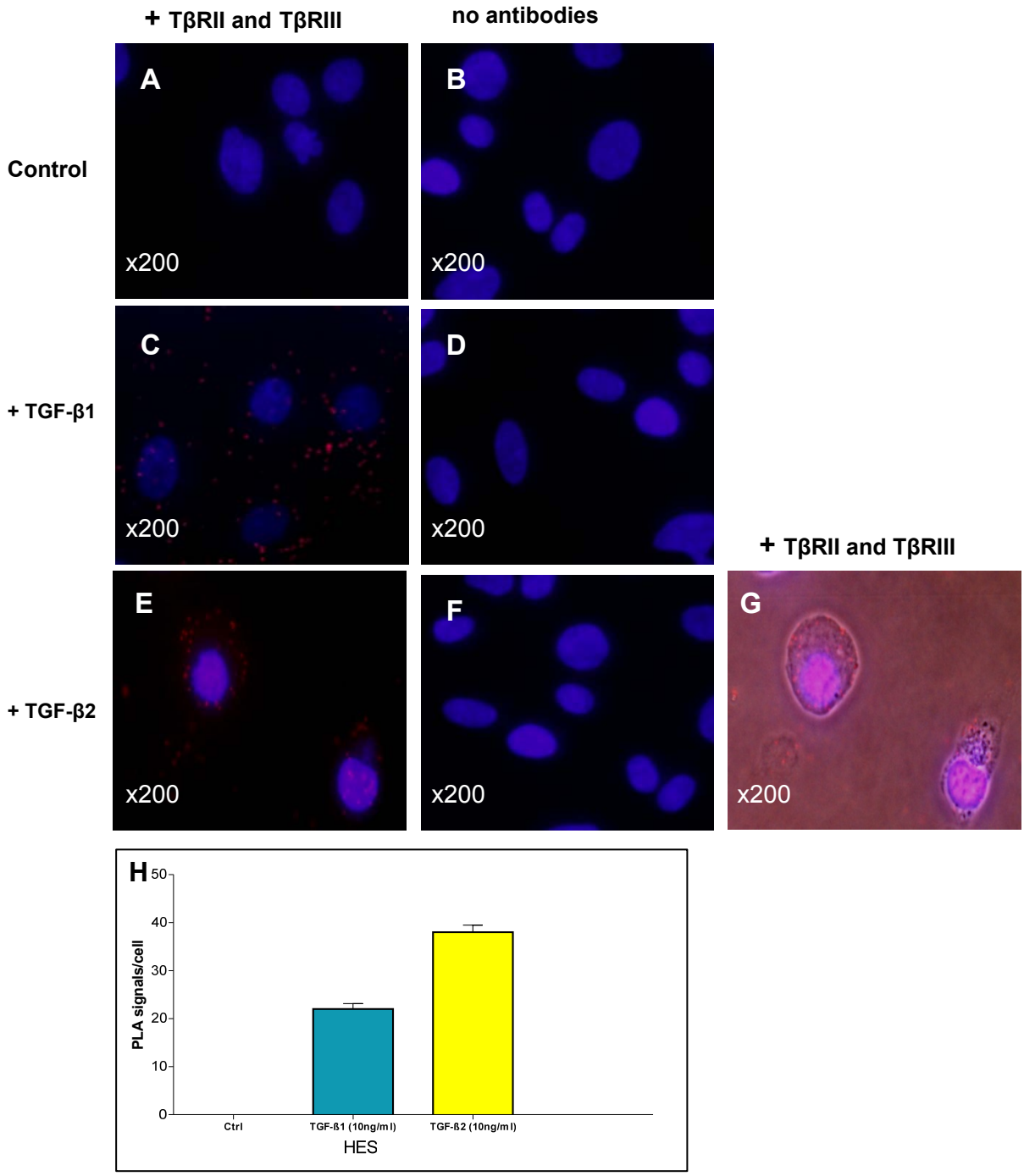


Figure 55. Interaction of TβRII/III on HES cells after TGF-β1 or TGF-β2 (10ng/ml) stimulation. Cells were treated with TGF-β1 (C, D) or TGF-β2 (E-G) for 30 minutes or left untreated (A, B). Antibodies against TβRII and TβRIII were used to detect the TβRII/TβRIII receptor complexes (A, C, E and G). The phase contrast image (G) showed the exact location of the receptor interaction on the cells. Omission of the primary antibodies was used as controls (B, D, F). Signals were counted and the average number of spots per cell is presented in the graph (H).

Treatment of HES cells with TGF-β1 or TGF-β2 (10ng/ml) induced interaction of TβRII and TβRIII receptors. The interaction was stronger in TGF-β2- compared to TGF-β1-treated cells and the receptor complex interactions were localized mainly in the proximity of the membranes. Untreated cells and cells without primary antibodies showed no receptor interactions.

3.11.2 Interaction of TβRI and TβRII Receptors on HES Cells

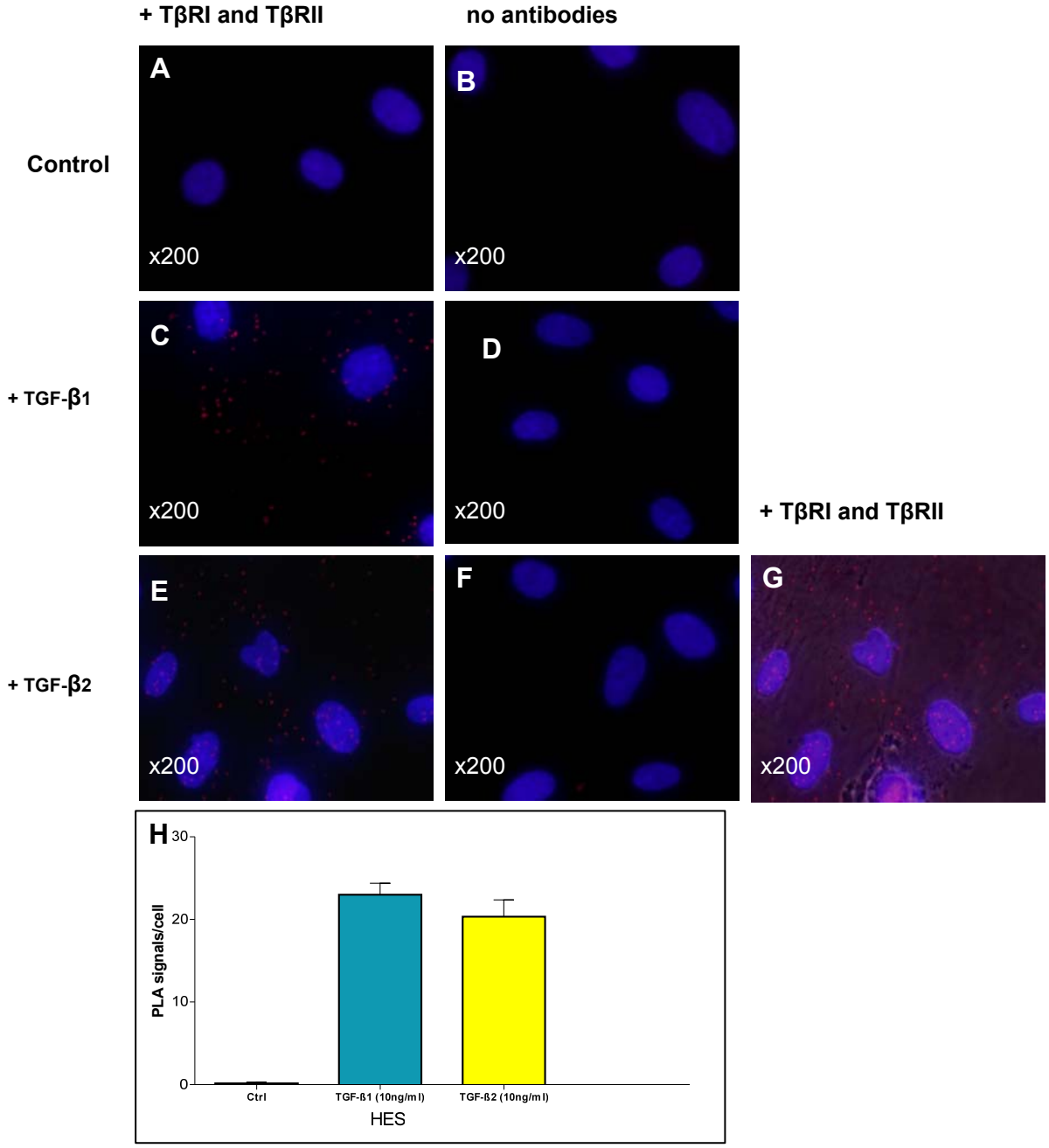


Figure 56. Interaction of TβRI/II on HES cells after TGF-β1 or TGF-β2 (10ng/ml) stimulation. Cells were treated with TGF-β1 (C, D) or TGF-β2 (E-G) for 30 minutes or left untreated (A, B). Antibodies against TβRI and TβRII were used to detect the TβRI/TβRII receptor complexes (A, C, E and G). The phase contrast image (G) showed the exact location of the receptor interaction on the cells. Omission of the primary antibodies was used as controls (B, D, F). Signals were counted and the average number of spots per cell is presented in the graph (H).

Treatment of HES cells with TGF-β1 or TGF-β2 (10ng/ml) induced interaction of TβRI and TβRII receptors. The interaction was moderate in both TGF-β1- and TGF-β2-treated cells and the receptor complex interactions were localized mainly found in the proximity of the membranes. Untreated cells and cells without primary antibodies showed no receptor interaction. All experiments were performed only once.

3.11.3 Interaction of TβRII and TβRIII Receptors on T-HESC Cells
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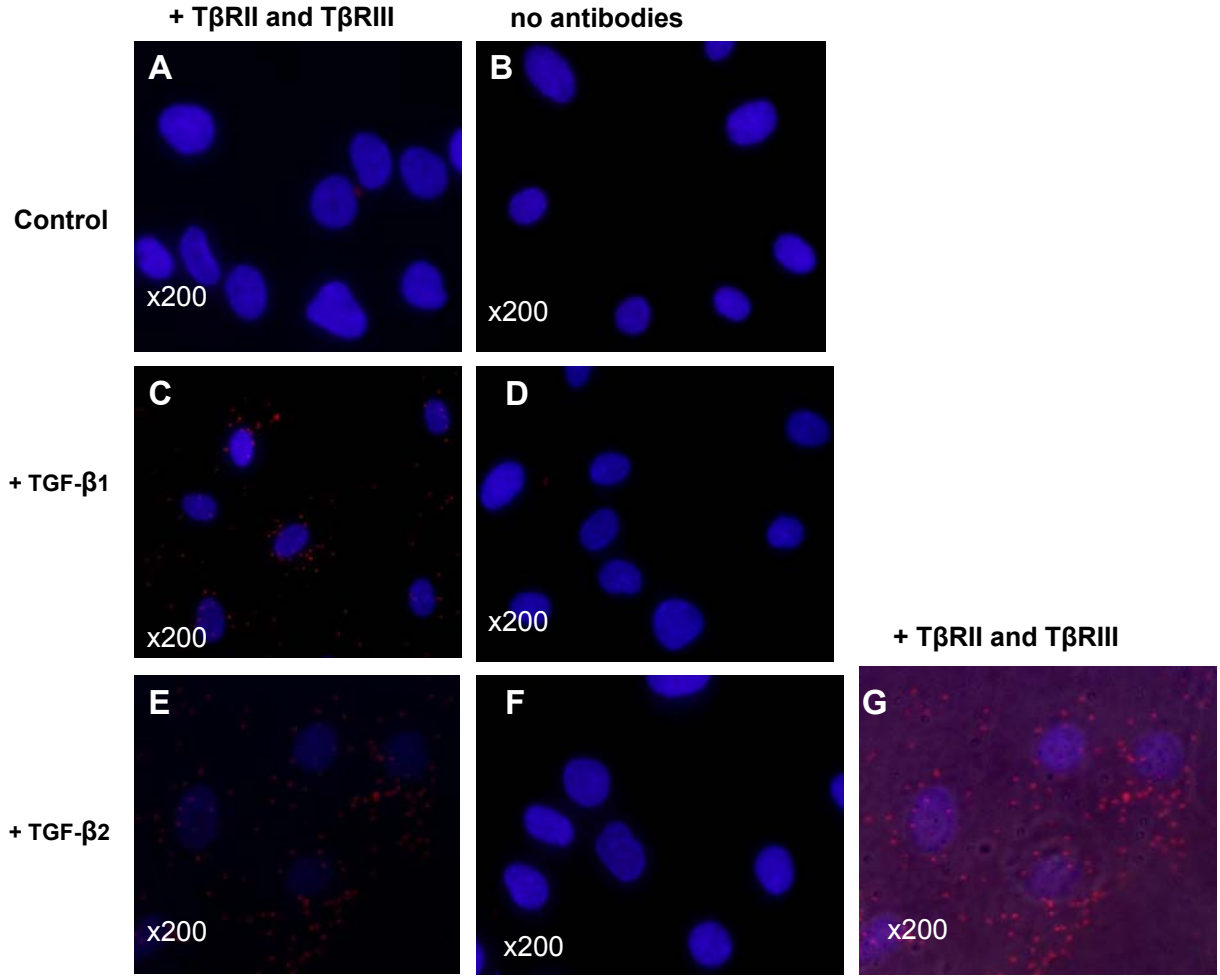


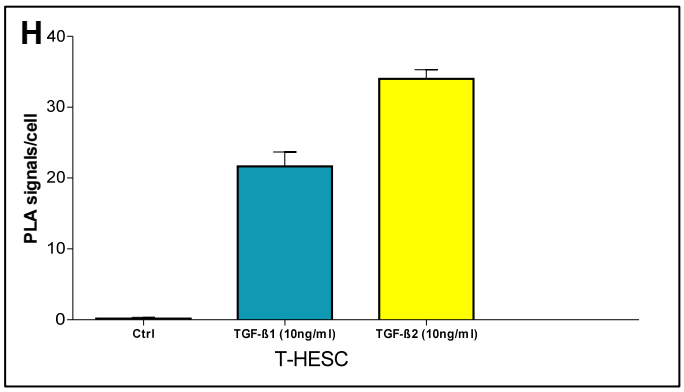


Figure 57. Interaction of TβRII/III on T-HESC cells after TGF-β1 or TGF-β2 (10ng/ml) stimulation. Cells were treated with TGF-β1 (C, D) or TGF-β2 (E-G) for 30 minutes or left untreated (A, B). Antibodies against TβRII and TβRIII were used to detect the TβRII/TβRIII receptor complexes (A, C, E and G). The phase contrast image (G) showed the exact location of the receptor interaction. Omission of the primary antibodies was used as controls (B, D, F). Signals were counted and the average number of spots per cell is presented in the graph (H). Similar results were observed with primary endometrial stromal cells (data not shown).

Treatment of T-HESC cells with TGF-β1 or TGF-β2 (10ng/ml) induced interaction of TβRII and TβRIII receptors. The interaction was stronger in TGF-β2- compared to TGF-β1-treated cells and the receptor complex interactions were localized mainly in the proximity of the membranes. Untreated cells and cells without primary antibodies showed no receptor interaction.

3.11.4 Interaction of TβRI and TβRII Receptors on T-HESC Cells

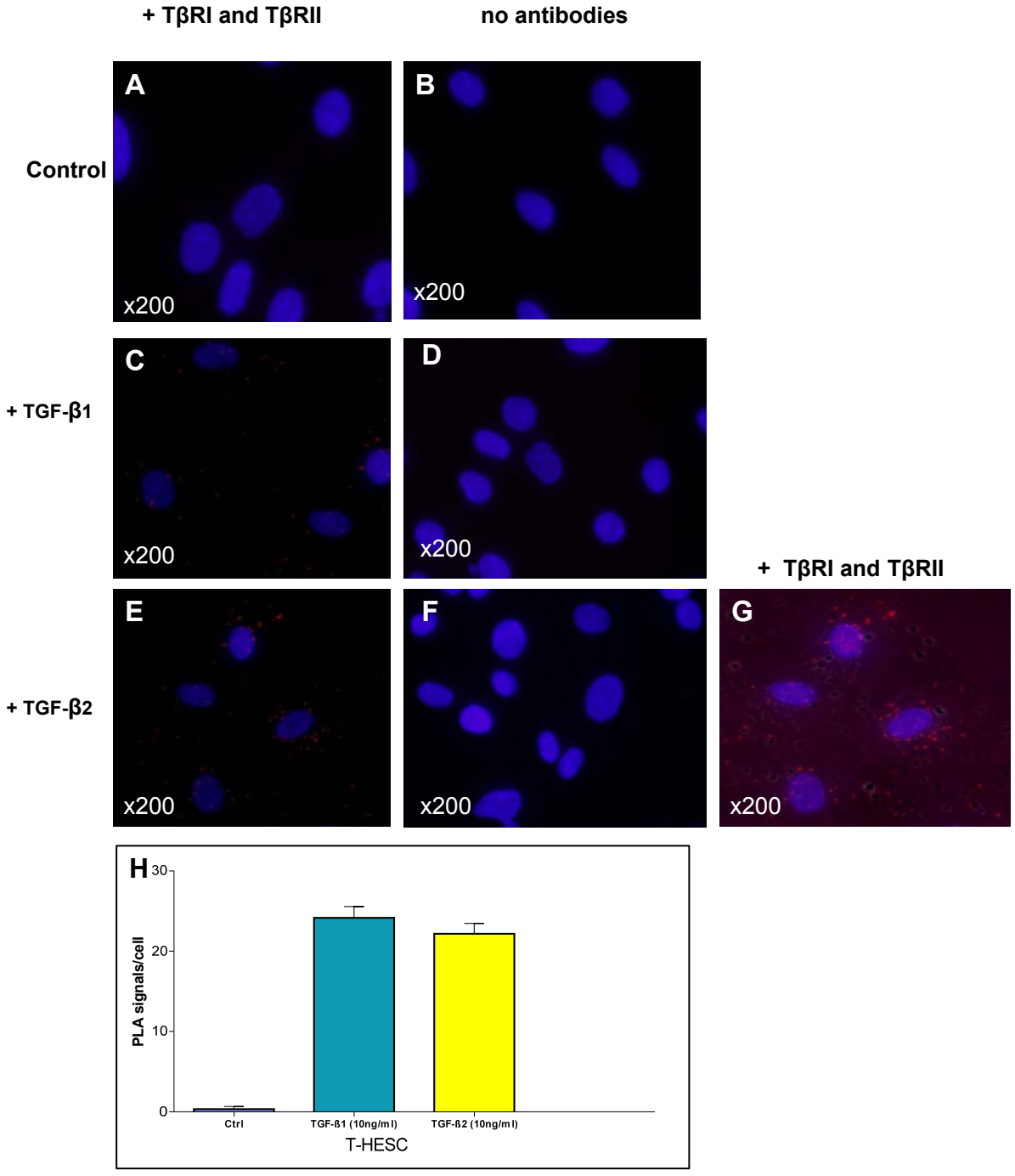


Figure 58. Interaction of TβRI/II on T-HESC cells after TGF-β1 or TGF-β2 (10ng/ml) stimulation. Cells were treated with TGF-β1 (C, D) or TGF-β2 (E-G) for 30 minutes or left untreated (A, B). Antibodies against TβRI and TβRII were used to detect the TβRI/TβRII receptor complexes (A, C, E and G). The phase contrast image (G) showed the exact location of the receptor interaction. Omission of the primary antibodies was used as controls (B, D, F). Signals were counted and the average number of spots per cell is presented in the graph (H). Similar results were observed with primary endometrial stromal cells (data not shown).

Treatment of T-HESC cells with TGF-β1 or TGF-β2 (10ng/ml) induced interaction of TβRI and TβRII receptors. The interaction was moderate in both TGF-β1- and TGF-β2-treated cells and the receptor complex interactions were mainly revealed in the proximity of the membranes. Untreated cells and cells without primary antibodies showed no receptor interaction. All experiments were performed only once.

3.11.5 Interaction of TβRII and TβRIII Receptors on 12ZVK Cells

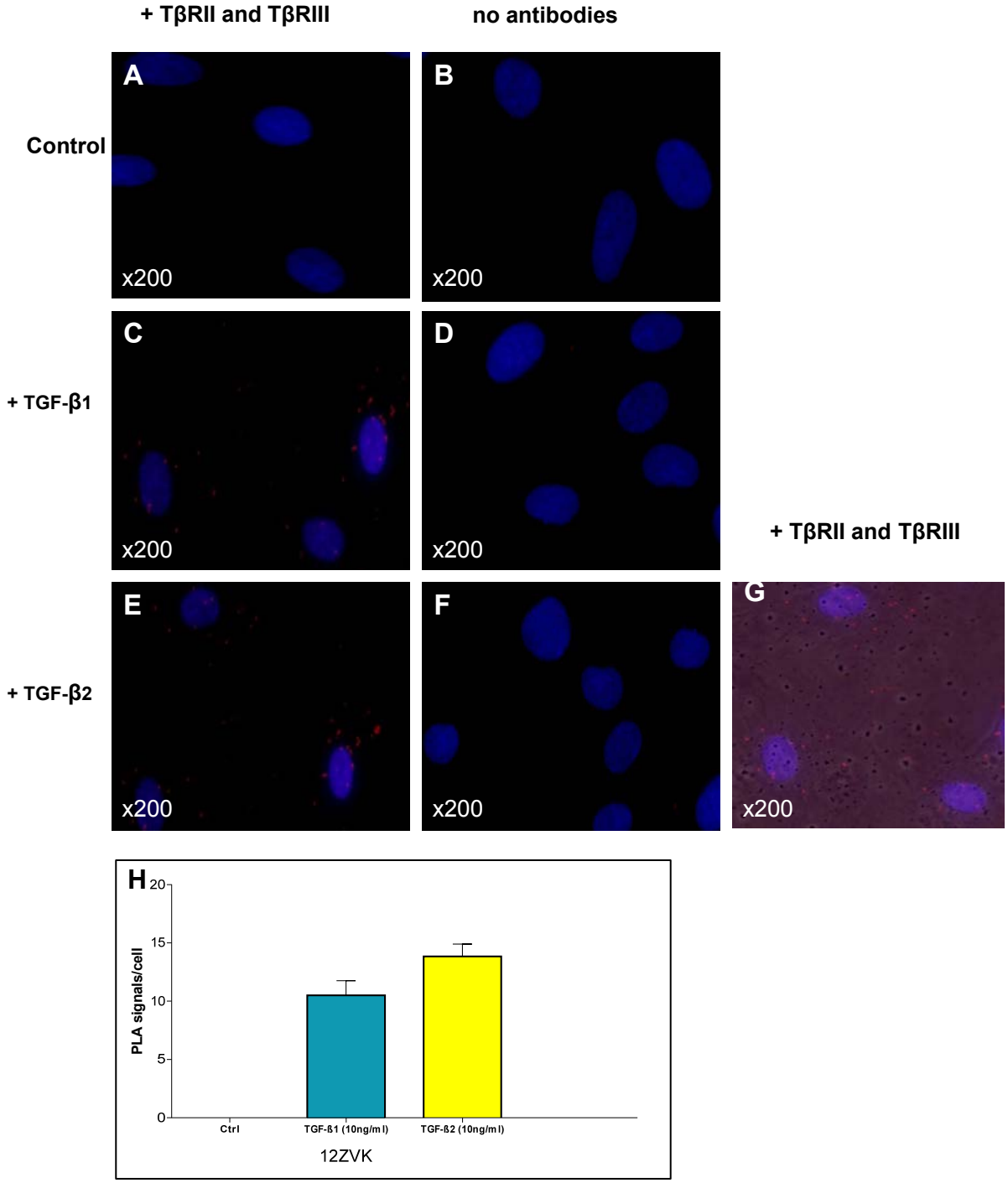
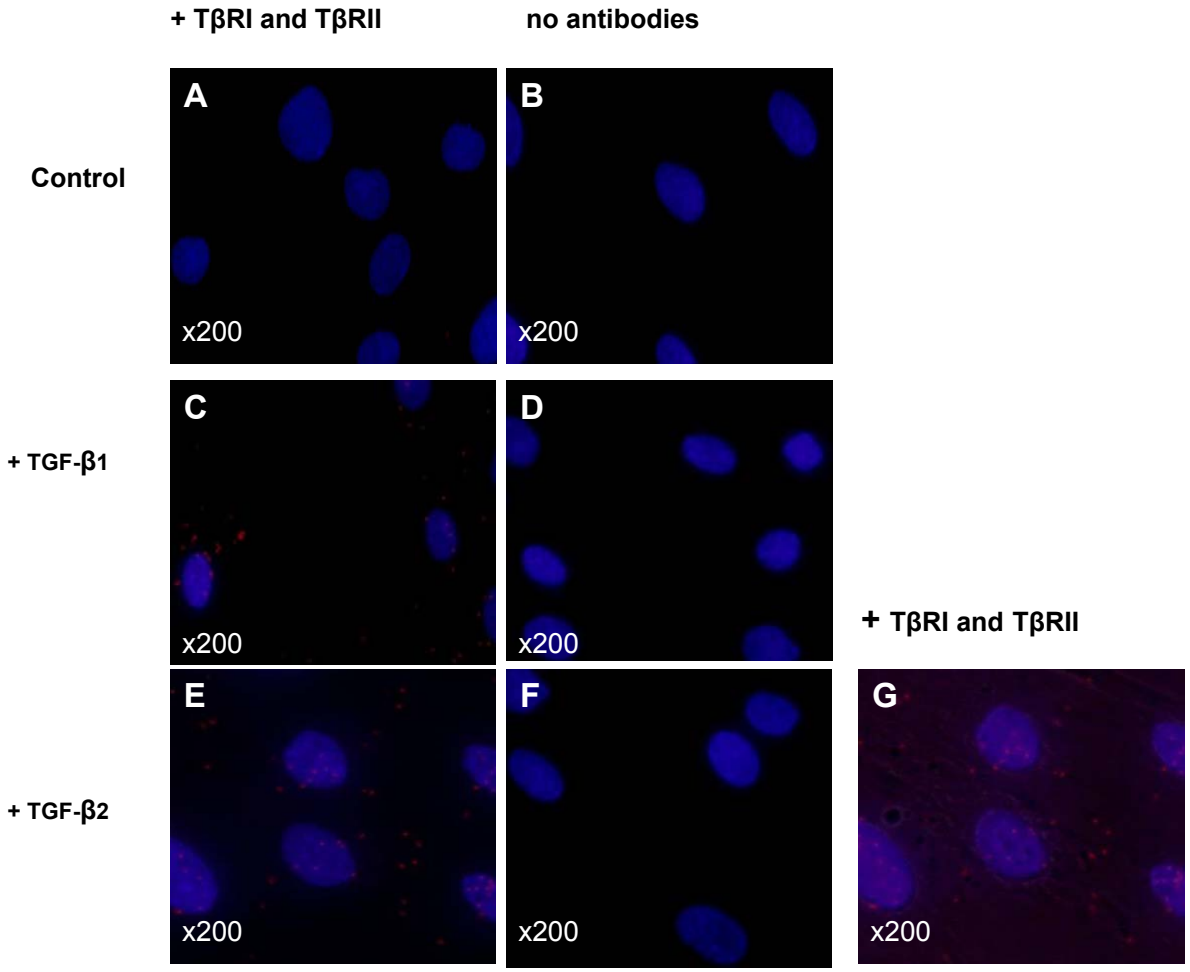


Figure 59. Interaction of TβRII/III on 12ZVK cells after TGF-β1 or TGF-β2 (10ng/ml) stimulation. Cells were treated with TGF-β1 (C, D) or TGF-β2 (E-G) for 30 minutes or left untreated (A, B). Antibodies against TβRII and TβRIII were used to detect the TβRII/TβRIII receptor complexes (A, C, E and G). The phase contrast image (G) showed the exact location of the receptor interaction. Omission of the primary antibodies was used as controls (B, D, F). Signals were counted and the average number of spots per cell is presented in the graph (H).

In summary, treatment of 12ZVK cells with TGF-β1 or TGF-β2 (10ng/ml) induced interaction of TβRII and TβRIII receptors. The interaction was stronger in TGF-β2- compared to TGF-β1-treated cells and the receptor complex interactions were mainly located in the proximity of the membranes. Untreated cells and cells without primary antibodies showed no receptor interaction.

3.11.6 Interaction of TβRI and TβRII Receptors on 12ZVK Cells



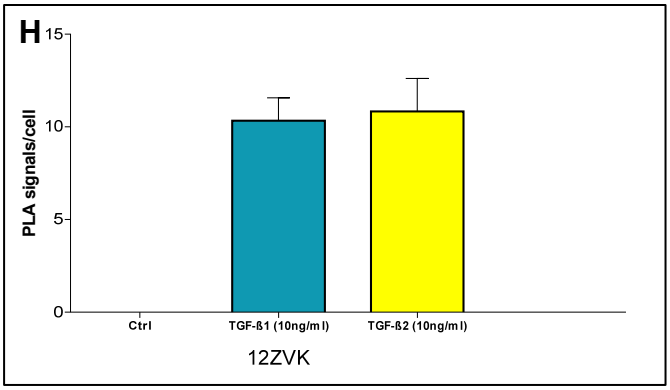


Figure 60. Interaction of TβRI/II on 12ZVK cells after TGF-β1 or TGF-β2 (10ng/ml) stimulation. Cells were treated with TGF-β1 (C, D) or TGF-β2 (E, F) for 30 minutes or left untreated (A, B). Antibodies against TβRI and TβRII were used to detect the TβRI/TβRII receptor complexes (A, C ,E and G). Omission of the primary antibodies was used as controls (B, D, F). The phase contrast image (G) showed the exact location of the receptor interaction. Signals were counted and the average number of spots per cell is presented in the graph (H).

In summary, treatment of 12ZVK cells with TGF-β1 or TGF-β2 (10ng/ml) induced interaction of TβRI and TβRII receptors. The interaction was moderate in both TGF-β1- and TGF-β2-treated cells. The receptor complex interactions were mainly in the proximity of the membranes. Untreated cells and cells without primary antibodies showed no receptor interaction.

3.11.7 Interaction of TβRII and TβRIII Receptors on 22B Cells

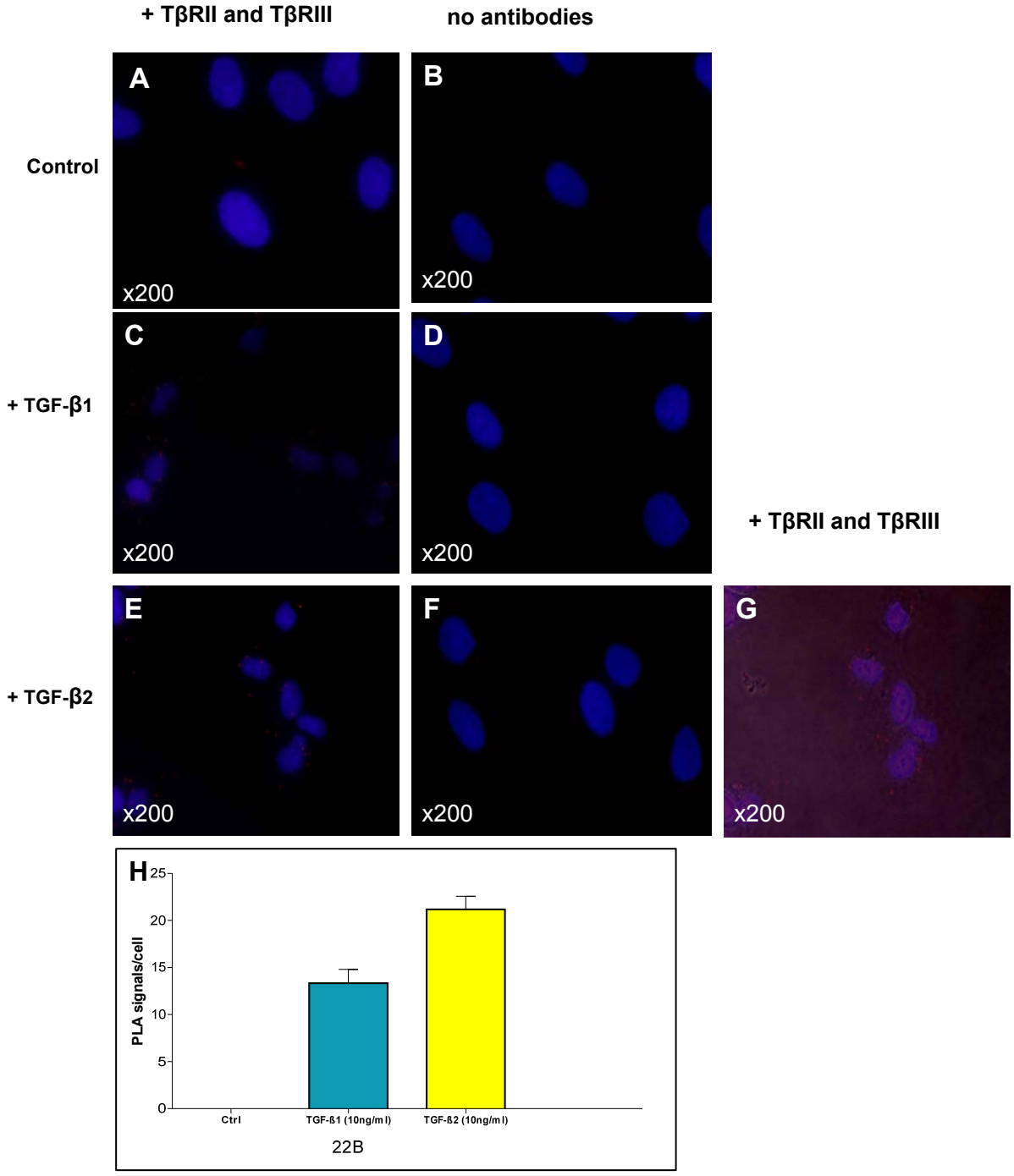


Figure 61. Interaction of TβRII/III on 22B cells after TGF-β1 or TGF-β2 (10ng/ml) stimulation. Cells were treated with TGF-β1(C, D) or TGF-β2 (E-G) for 30 minutes or left untreated (A, B). Antibodies against TβRII and TβRIII were used to detect the TβRII/TβRIII receptor complexes (A, C, E and G). The phase contrast image (G) showed the exact location of the receptor interaction. Omission of the primary antibodies was used as controls (B, D, F). Signals were counted and the average number of spots per cell is presented in the graph (H).

In summary, treatment of 22B cells with TGF-β1 or TGF-β2 (10ng/ml) induced interaction of TβRII and TβRIII receptors. The interaction was stronger in TGF-β2- compared to TGF-β1-treated cells and the receptor complex interactions were mainly found in the proximity of the membranes. Untreated cells and cells without primary antibodies showed no receptor interaction.

3.11.8 Interaction of TβRI and TβRII Receptors on 22B Cells

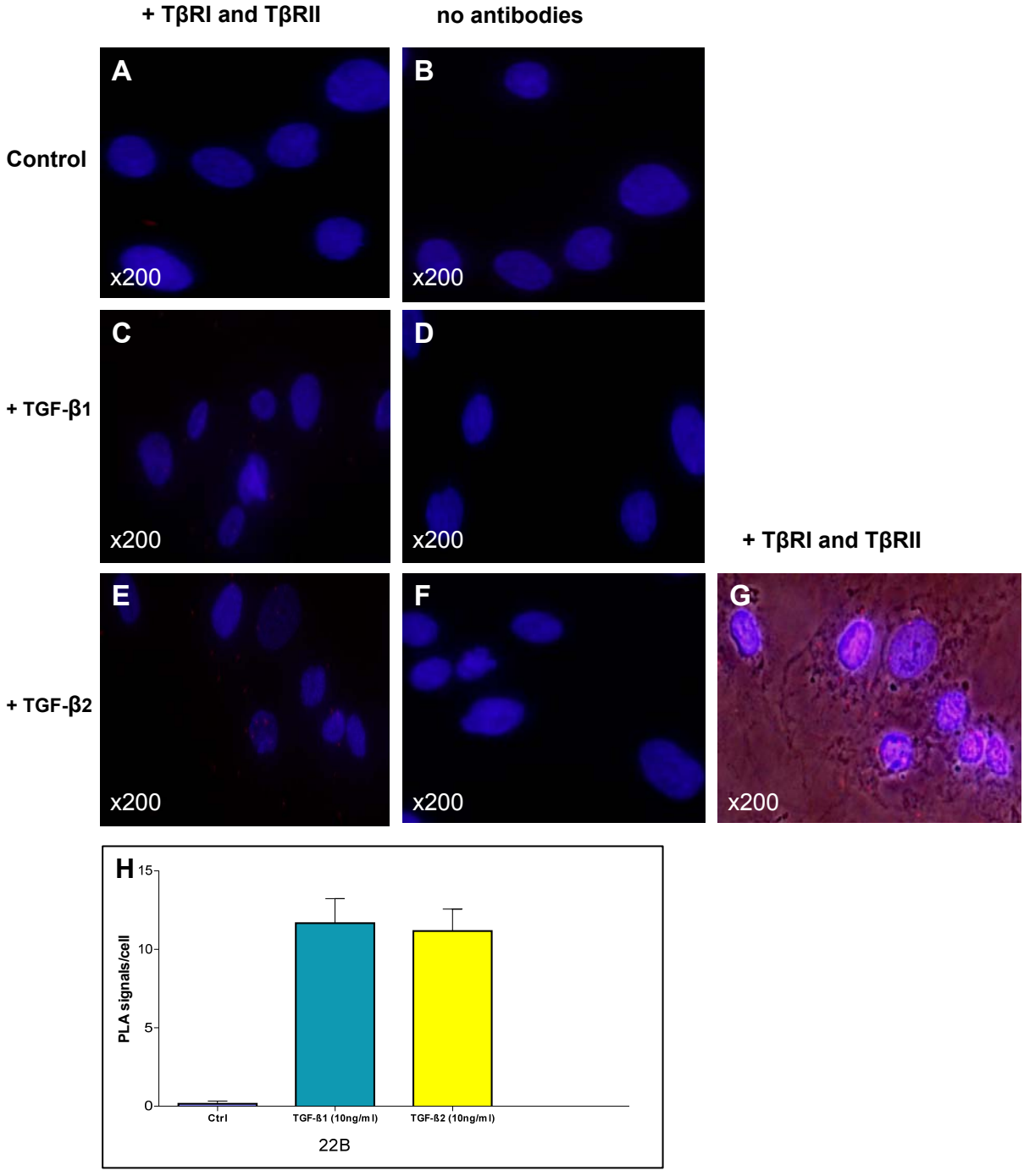


Figure 62. Interaction of TβRI/II on 22B cells after TGF-β1 or TGF-β2 (10ng/ml) stimulation. Cells were treated with TGF-β1 (C, D) or TGF-β2 (E-G) for 30 minutes or left untreated (A, B). Antibodies against TβRI and TβRII were used to detect the TβRI/TβRII receptor complexes (A, C, E and G). The phase contrast image (G) showed the exact location of the receptor interaction. Omission of the primary antibodies was used as controls (B, D, F). Signals were counted and the average number of spots per cell is presented in the graph (H).

Treatment of 22B cells with TGF-β1 or TGF-β2 (10ng/ml) induced interaction of TβRI and TβRII receptors. The interaction was moderate in both TGF-β1- and TGF-β2-treated cells and the receptor complex interactions were mainly located in the proximity of the membranes. Untreated cells and cells without primary antibodies showed no receptor interaction.

4 Discussion

TGF-βs are one of the most essential growth factors involved in cell differentiation, proliferation, motility and apoptosis in various cell types. Given the great importance of TGF-βs in the process of menstruation and endometriosis (Pizzo et al., 2002; Gaide Chevronnay et al., 2008), TGF-βs and their receptors (TβRs) are suspected to be involved in establishment and maintenance of endometriosis (Omwandho et al., 2010).

In this study, we investigated the influence of TGF-βs on cell numbers, regulation of some proteins essential in endometriosis and TGF-β receptor interactions in endometrial, endometriotic cells and primary endometrial stromal cells. Furthermore, we studied the signaling pathways of TGF-βs and possible cross-talks with other pathways, especially the Smad-dependent and Smad-independent pathways were investigated in this study. Also the possible cross-talk between TGF-βs and BMPs and TGF-β/TβR in endometrial and endometriotic cells were investigated for the first time.

4.1 Role of TGF-βs in Endometrial and Endometriotic Cells and Primary Endometrial Stromal Cells

4.1.1 Influence of TGF-βs on Cell Numbers

Cell numbers in tissues are usually determined by the rate of proliferation and apoptosis (Sommer and Rao, 2002). Up to date, it remains unclear how the TGF-β pathways results in induction of apoptosis. Because the effects of TGF-βs on endometrial and endometriotic cells numbers are controversial, we investigated the effects of TGF-βs on endometrial and endometriotic cell numbers after treatment with TGF-β1 and TGF-β2. TGF-βs decreased the cell numbers in all cell lines studied. Our results agree with those of Meresman et al. (2003) and Sui. (2012) who showed that the influence of TGF-βs on cell numbers decrease when the initial cell number is high. Of note, in this study, the reduction of cell numbers was higher in endometrial cells compared to endometriotic cells, suggesting that possibly the endometriotic cells are less responsive to stimulation by TGF-βs as similarly shown for cancer cells and will be discussed later.

Furthermore, we showed that the effect of TGF-β1 or TGF-β2 on cell numbers was completely blocked with a TβR1 inhibitor, while a Smad3 inhibitor blocked TGF-βs

effects on cell numbers only partially. Our results further showed that the Smad-dependent pathway is utilized in the TGF-β-dependent reduction of cell numbers in endometrial and endometriotic cells as also shown by Sui. (2012).
Apart from cell proliferation, other factors like apoptosis or cell attachment might influence the cell numbers as will be explained in detail in the following sections.

4.1.2 Effects of TGF-βs on Apoptosis

According to Garcia-Velasco et al. (1999), the escape of endometrial fragments from apoptosis as they enter/transit the peritoneal cavity is very important for their survival. Gebel et al. (1998) showed greatly reduced apoptosis in sloughed endometrial cells from women who develop endometriosis, implying that high numbers of surviving cells can enter the peritoneal cavity. Similarly, apoptosis was found to decrease as the severity of endometriosis increased (Dmowski et al., 2001). Also, Bcl-2 protein expression was found to be increased in proliferative eutopic endometrium in women with endometriosis and FasL was highly expressed by endometriotic tissues (Meresman et al., 2000; Garcia-Velasco et al., 2002).
Up to date, the connection between the TGF-β pathway and the apoptotic pathways still remains unclear. Since TGF-βs are increased in the serum and peritoneal fluid of women with endometriosis and their levels are enhanced markedly with the severity of the disease (Pizzo et al., 2002; Garcia-Velasco et al., 2002), we suspect that TGF-βs possibly induce apoptosis which might play an important role in the pathogenesis of endometriosis by increasing survival rate of endometriotic cells because less cells die upon TGF-β treatment. In our experiments, we wanted to investigate whether or not TGF-βs exert an apoptotic effect on human endometrial and endometriotic cells and if yes, which apoptotic pathways are involved. We quantified phosphatidylserine on the outer membrane surface, the inner mitochondrial membrane potential, and activation of Caspase 3/7 proteins. The three parameters are important because they can be used to discriminate between necrosis and apoptosis.
The results showed that TGF-β1 or TGF-β2 increased phosphatidylserine, decreased the inner mitochondrial membrane potential and increased Caspase 3/7 levels in endometrial and endometriotic cells. However, in our results, TGF-β1- or TGF-β2-induced apoptosis did not show any significant differences among the four cell lines and the primary endometrial stromal cells. This implies that concerning the aspect of

apoptosis, endometrial cells, endometriotic cells, as well as primary endometrial stromal cells, response to the stimulation by TGF-βs in a similar manner.

Furthermore, we showed that the effect of TGF-β1 or TGF-β2 on apoptosis was completely blocked by a TβR1 inhibitor, while a Smad3 inhibitor blocked TGF-β-induced apoptosis in all cells only partially. Our results indicate that the Smad-dependent pathway is mainly utilized in TGF-β-induced apoptosis in endometrial and endometriotic cells. Thus, we showed that both the mitochondrial apoptotic pathway (intrinsic) and the death receptor apoptotic pathway (extrinsic) are involved in TGF-β-induced apoptosis in endometrial and endometriotic cells. Our results further agrees with Sánchez-Capelo (2005) who showed that TGF-βs cooperate with the death receptor apoptotic pathway (Fas), the mitochondrial apoptotic pathway (Bcl-2) and a number of intracellular apoptotic modulators in mediating apoptosis.

Since, the effects of TGF-βs on apoptosis are similar in both endometrial and endometriotic cell numbers, we suppose that other factors like the amount of Smad3 phosphorylation or PAI-1 levels might possibly affect cell numbers or proliferation as will be discussed in the following sections.

4.1.3 The Role of TGF-βs in Phosphorylation of Smad3

Despite many studies on Smad proteins, there is so far no direct evidence implicating Smad3 as an intermediate protein in TGF-β signaling pathway in endometriotic cells. Luo et al. (2003) showed that TGF-β1 induced phosphorylation of Smad3 in endometrial epithelial cell line (HES) and endometrial stromal cells (ESC) in a dose-dependent manner. He quantified Smad3 phosphorylation in the HES cell line and endometrial stromal cells using Western blot, IHC and semiquantitative Polymerase Chain Reaction. The effect of TGF-β1 on pSmad3 induction was in part abrogated upon treatment of HES and ESC cells with TGF-β type II receptor antisense (Luo et al., 2003). Given the fact that the six developmental stages leading to endometriosis are similar to tumourigenesis (Omwandho et al., 2010), also Smad proteins have been found to be frequently mutated in cancer cells (Liu et al., 1997). Moreover, Smad3 is frequently downregulated in cancer (Jones et al., 2008). Thus, we hypothesized that the effects of TGF-βs on Smad3 phosphorylation in endometriosis might be similar to those observed in tumour cells. We further hypothesized that the effects of TGF-βs on Smad3 phosphorylation might have an indirect impact on cell numbers in endometrial or endometriotic cells. In our experiments, we quantified

Smad3 phosphorylation with a phospho-Smad3 ELISA upon TGF-β1 or TGF-β2 stimulation.

Our results showed that TGF-βs stimulated phosphorylation of Smad3 in endometrial, endometriotic cell lines and primary endometrial stromal cells. Our results agree with those of Luo et al. (2003) who also observed phosphorylation of Smad3 in HES and endometrial stromal cells upon stimulation with TGF-β1. Interestingly, we also demonstrated that Smad3 phosphorylation was significantly higher in endometrial cells compared to endometriotic cells upon stimulation with TGF-βs, suggesting that possibly the endometriotic cells are somehow less responsive to stimulation by TGF-βs. Furthermore, we showed that the effects of TGF-β1 or TGF-β2 on pSmad3 induction were abrogated completely following treatment of cells with Smad3 or TGF-β type I receptor inhibitors. Thus, our results provide strong evidence that Smad3 is involved in TGF-β-dependent responses and we suppose that its activity is downregulated in endometriosis. These observations might explain the reduced responsiveness in reduction of cell numbers of endometriotic cells upon stimulation with TGF-βs. However, expression levels of Smad3 need to be determined in all cell lines studied.

Liu et al. (1997) showed that Smad3 phosphorylation might be involved in TGF-β-dependent growth inhibition and activation of genes encoding PAI-1 protein in lung epithelial cells. Also, constitutive phosphorylation of Smad3 was associated with increased transcription of PAI-1 genes in activated hepatic stellate cells (Inagaki et al., 2001). Czekay et al. (2011) observed that PAI-1 lowered attachment of cells to ECM through inhibition of uPAR-vitronectin interaction. Based on these observations, we hypothesised that PAI-1 might have an effect on endometrial and endometriotic cell numbers since it is directly correlated to Smad3 phosphorylation. In our experiments, we quantified secretion of PAI-1 by endometrial and endometriotic cells as described in the next chapter.

4.1.4 The Role of TGF-βs in PAI-1 Secretion

PAI-1 is the major inhibitor of tissue/urokinase plasminogen activator (tPA/ uPA) and is important in extracellular matrix turnover (Vial and Longo, 2008). Expression of PAI-1 and uPA was found to be higher in endometriotic and endometrial tissue of women with endometriosis compared to tissue of women without endometriosis (Bruce et al., 1998; 2004). Also, PAI-1 levels were higher in ectopic tissues compared

to eutopic tissues of women with endometriosis (Bruce et al., 2004) indicating that PAI-1 is important in the pathogenesis of endometriosis.

In our experiments, we wanted to investigate whether or not PAI-1 is involved in endometriosis and which pathways are concerned. We compared secretion of PAI-1 in endometrial and endometriotic cells upon stimulation with TGF-β1 or TGF-β2 in vitro. We demonstrated that endometriotic cells secreted more PAI-1 than endometrial cells. Since PAI-1 was found to favour detachment of cells from various substrates and also to increase their motility (Akkawi et al., 2006; Czekay et al., 2011), then increased secretion of PAI-1 in endometriotic cells compared to endometrial cells observed in our experiments suggests that endometriotic cells are more easily detached and thus more cells are possibly retrograded to ectopic sites hence enhancing establishment of endometriosis.

Furthermore, we showed that the effects of TGF-β1 or TGF-β2 on PAI-1 secretion were completely blocked by a TβR1 inhibitor, while a Smad3 inhibitor blocked PAI-1 only partially. Our results further affirm that the Smad-dependent pathway is mainly utilized in TGF-β-dependent PA1-1 secretion by endometrial and endometriotic cells. Since TGF-β-dependent PA1-1 secretion was observed to be mainly Smad-dependent, we hypothesized that by the use of PAI-1 secretion, we might study possible cross-talks between the TGF-βs and BMP pathways. Both pathways utilize Smad proteins as their signal transducers from the cell surface to the nucleus. The cross-talk between the two pathways is described in the following section.

4.1.5 Cross-talk of the TGF-β and BMP Pathways.

The BMP ligands can bind to any of the three type II receptors (BMPRII, ActRIIa and ActRIIb) and the three type 1 receptors (ALK-2, ALK-3 and ALK-6). Upon binding, the constitutively active type II receptor phosphorylates type 1 receptor. The activated type 1 receptor phosphorylates the BMP-responsive Smad proteins namely Smad1, Smad5 and Smad8. The activated Smads bind Smad4 either in the cytoplasm or in the nucleus to facilitate signaling (Yu et al., 2008).

Perturbations of both BMP and TGF-β signaling have been reported to cause distinct and also overlapping phenotypic bone diseases (Jansens et al., 2000). Furthermore, alterations of both TGF-β and BMP pathways have been associated with vascular diseases like pulmonary hypertension (Boeck and Dijke, 2011). These two observations clearly indicate that the two pathways might be cross-talking at some

point. Thus, it might be crucial to determine whether or not a cross-talk exists between the two pathways in endometrial and endometriotic cells. This will enhance further understanding of the roles of the two pathways in the pathogenesis of endometriosis.

In our experiments, we used a general BMP inhibitor LDN-193189 selectively inhibiting the BMP type 1 receptors ALK-2, ALK-3 and ALK-6 and thus blocking BMP-mediated phosphorylation of Smad1/5/8. In addition, we used distinct inhibitors for ALK-2, ALK-3 and ALK-6 separately to determine the possible cross-talk of the TGF-β and BMP pathways upon TGF-β1 or TGF-β2 stimulation.

Our results showed that LDN-193189 as well as the ALK-2 inhibitor completely inhibited the TGF-β-induced secretion of PAI-1 in endometrial and endometriotic cells. Interestingly, both ALK-3- and ALK-6-inhibitors reduced the TGF-β-induced secretion of PAI-1 only partially. Our results suggest a cross-talk between the TGF-β and BMP pathways and also that the Smads are the most important intracellular transducers of TGF-β and BMP signals from the receptors to the nucleus.

Furthermore, we also showed for the first time that an ALK-2 inhibitor completely blocked the TGF-β-induced secretion of PAI-1 in endometrial and endometriotic cells. Our results are in agreement with those of Chen et al. (2003) and Bharathy et al. (2008) who showed that TGF-βs can also strongly but only transiently phosphorylate Smad1, Smad5 and Smad8 in endothelial, epithelial, fibroblasts and cancer-derived cells. Our results are further supported by Barnet et al. (2002) who showed that TGF-βs signals via ALK-2 and ALK-5 in chick atrial cells and Olivey et al. (2006) who implicated ALK-2 in the TGF-βs stimulated epithelial-mesenchymal transformation in mammary glands of the mouse.

To further understand other cross-talks between the TGF-β and BMP pathways, we measured secretion of inhibin B which is a marker for the functionality of the BMP pathway as described in the following section.

4.1.6 The Effect of the TGF-βs on Inhibin B Secretion

Inhibin B is a member of the TGF-β superfamily and regulates reproduction. Inhibin B secretion is a marker for the functionality of the BMP pathway. Petraglia et al. (1998) showed that inhibin B is found in peritoneal fluid and serum of women with or without endometriosis. They showed that there was no significant difference in inhibin B levels in women with and without endometriosis although inhibin B levels were higher

in the peritoneal fluid compared to serum in women with or without endometriosis. Cultured endometrial stromal and epithelial cells also expressed inhibin B with no significant differences between women with or without endometriosis (Petraglia et al., 1998). Since inhibin B was expressed by both endometrial and endometriotic cell cultures, we hypothesized that by treating endometrial or endometriotic cells with TGF-β1 or TGF-β2, it might be possible to elucidate whether TGF-β cross-talks with the BMP pathway in these cells with respect to inhibin B secretion.

In our experiments, we stimulated endometrial or endometriotic cells with TGF-β1 or TGF-β2 and measured inhibin B secretion. Our results showed that TGF-β1 or TGF-β2 slightly reduced inhibin B secretion by HES cells and primary endometrial stromal cells with no effects observed in the other cell lines. There were no significant differences between endometrial and endometriotic cells. Thus, the result shows that inhibin B is not a common protein as PAI-1 in both BMP and TGF-β pathways.

4.1.7 Effects of TGF-βs on Interaction of TβRI, TβRII and TβRIII Receptors

TGF-βs are secreted into the extracellular matrix as a latent protein complex and only after activation they become biologically active (Rebecca, 2000). Once activated, the TGF-βs bind to their high affinity cell surface receptors (TβRI, TβRII and TβRIII). However, TβRI and TβRII were found to be inactivated in various human tumors (Levy and Hill, 2006).

Given the fact that the stages for endometriosis resemble closely metastasis thus, we suspect that the relevance of TGF-βs in metastasis might be similar in endometriosis.

In our experiments, we characterized the TGF-β signalosome in endometrial and endometriotic cells. Upon stimulation with TGF-β1 or TGF-β2, TβRII and TβRIII or TβRI and TβRII interaction were quantified by in situ Proximity Ligation Assay. Expression of TβRIII by cell surface ELISA was also further investigated.

Our results showed that stimulation of endometrial and endometriotic cells with TGF-β1 or TGF-β2 increased TβRIII expression on the cell surfaces. Of note, stromal cells showed higher expression of TβRIII compared to epithelial cells in both endometrial and endometriotic cells. We observed no significant differences in TβRIII receptor expression between endometrial and endometriotic cells. Our results are in contrast to the observations by Dong et al. (2007), Hempel et al. (2007), Turley et al. (2007) and Gordon et al. (2008) who showed reduction of TβRIII receptor expression in pancreatic, breast, prostrate and ovarian cancers. The increased TβRIII receptor

expression upon TGFβ stimulation in both endometrial and endometriotic cells in this study might be attributed to the fact that unlike cancer cells, endometriotic cells still form functional glands.

Stimulation of endometrial and endometriotic cells with TGF-β1 or TGF-β2 increased TβRII and TβRIII interaction. Interestingly, the interaction was stronger in TGF-β2-treated cells compared to TGF-β1-treated cells. This shows that TGF-β2 has a higher sensitivity to TβRIII compared to TGF-β1. Also, endometrial cells showed stronger interaction compared to endometriotic cells. Furthermore, we observed a moderate TβRI/TβRII interaction in TGF-β1-treated as well as in TGF-β2-treated endometrial cells which was slightly stronger compared to endometriotic cells. Overall our results showed that the TGF-βs exert a stronger influence in endometrial cells compared to endometriotic cells with respect to the interaction of the high-affinity TGF-β receptors upon TGF-β treatment. This might explain the stronger reduction in cell numbers of endometrial cells due to stronger receptor interactions on these cells. Thus we predict that the weak interaction of TβRI, TβRII and TβRIII receptors expression in endometriotic cells might be associated with the pathogenesis of endometriosis. Our results agrees with those of Meng et al. (2011) who observed a decreased expression of TβRII and TβRIII in oral squamous cell carcinoma and oral carcinoma-associated fibroblasts upon TGF-β1 treatment. Also TβRIII expression decreased during breast cancer and lung cancer progression (Dong et al., 2007; Malkoski et al., 2012). In addition, Chen et al. (1997) and Kim et al. (1999) observed a decreased expression of TβRI, TβRII and TβRIII in some human cancers. In summary, the different expression levels of TβRs in endometrial cells compared to endometriotic cells suggest an involvement in the pathogenesis of endometriosis.

4.1.8 Characteristic Differences between Endometrial and Endometriotic Cells and Tissues

Several epithelial cell markers have been used in tumor biology by pathologists to characterize metastatic cells; most investigators use a panel of epithelial markers which are like fingerprints for certain tissues. In this study we characterized endometrial and endometriotic tissues using antibodies for Cytokeratin 18 (CK18) and Mucin-1 (MUC1) proteins which are expressed on epithelial cells and polarized epithelial cells, respectively. We characterized endometrial biopsies obtained from patients with or without endometriosis and we assessed endometrial glands from the

endometrium and the ovary. Our results showed that both proteins are expressed in the endometrial and endometriotic glands of both the endometrium and the ovary. The identical expression profile of MUC1 and CK 18 in both tissues examined suggests a common origin. Thus, our data are consistent with Sampson's hypothesis, who postulated that all endometriotic lesions result from dissemination of endometrial cells, however, more markers need to be tested to fully characterise endometrial and endometriotic tissues.

4.1.9 TGF-β Signaling in Endometrial Cells, Endometriotic Cells and Primary Endometrial Stromal Cells

The Smad-dependent pathway is the main pathway of TGF-β signaling and is activated by phosphorylation of TβRI which in turn phosphorylates Smad2/3 (Massagué and Wotton, 2000).

In our experiments, we demonstrated a reduction in cell numbers, increased PAI-1 secretion, increased Smad3 phosphorylation and increased apoptosis upon stimulation of endometrial, endometriotic cells and primary endometrial stromal cells with TGF-βs. In addition, we studied both Smad-dependent and Smad-independent pathways by using specific inhibitors that target downstream cascades of TGF-β signaling in endometrial, endometriotic cells and primary endometrial stromal cells.

4.1.10 The Smad-dependent Pathway in TGF-β Signaling in Endometrial Cells, Endometriotic Cells and Primary Endometrial Stromal Cells

TGF-βs can also activate Smad1, Smad5 and Smad8 (BMP-responsive Smads) in endothelial cells, epithelial cells, fibroblasts and epithelium-derived cancer cells (Bharathy et al., 2008; Liu et al., 2009).

In our study, we investigated the Smad-dependent pathway by using LY364947 and SiS3 inhibitors that selectively block the kinase activity of TβR1 and Smad3 phosphorylation, respectively. Our results showed that LY364947 can completely inhibit reduction in cell numbers, block increased PAI-1 secretion, inhibit increased Smad3 phosphorylation and block increased apoptosis upon stimulation of endometrial, endometriotic cells and primary endometrial stromal cells with TGF-βs. The Smad3 inhibitor SiS3 completely blocked the TGF-β-induced Smad3 phosphorylation but only partially blocked reduction in cell numbers, increased PAI-1 secretion, and increased apoptosis, implying that Smad2 is also involved in TGF-β

signaling. These data further supports that Smads are the most important mediators of TGF-β signaling from the receptors to the nucleus as published by Chen et al. (2003).

4.1.11 The Smad-independent Pathways in TGF-β Signaling in Endometrial and Endometriotic Cell Lines and Primary Endometrial Stromal Cells

A number of Smad-independent pathways which modulate the signal transduction by TGF-βs have been described (Yue and Mulder, 2000; Massagué and Chen, 2000; Moustakas and Heldin, 2005; Zhang et al., 2009). For example, Engel et al. (1999) showed that TGF-β-activated JNK phosphorylated Smad3 inducing its nuclear translocation. TGF-β1 mediated activation of TGF-β2 expression in human hepatic, murine fibroblasts and keratinocytes cells (Bascom et al., 1989; Shimada et al., 2011). Also, TGF-β2 is significantly upregulated in breast cancer cells (Dave et al., 2011). The mechanisms used by TGF-β1 to activate TGF-β2 have not been fully understood. In our experiments, we investigated the influence of TGF-β1 or TGF-β2 on secretion of TGF-β1 or TGF-β2 by endometrial and endometriotic cells.

Our results showed increased TGF-β2 secretion in TGF-β1-treated cells but no effects upon treatment with TGF-β2. Of note, endometriotic epithelial cells as well as endometriotic stromal cells secreted more TGF-β2 compared to endometrial epithelial and stromal cells, respectively. Thus, the increased expression of TGF-β2 in endometriotic cells compared to endometrial cells suggests the possible TGF-β involvement in pathogenesis of endometriosis. Our results concur with those of Dave et al. (2011) who observed higher expression of TGF-β2 in advanced stages of breast cancer. Also our results agree with the observations by Shimada et al. (2011) who showed that TGF-β1 mediated activation of TGF-β2 in human hepatic cells. Of note, TGF-β1 treated stromal T-HESC and primary endometrial stromal cells secreted almost the same amount of TGF-β2.

Furthermore we analyzed the mechanisms used by TGF-β1 in mediating activation of TGF-β2 by investigating several Smad-dependent and Smad-independent pathways using specific inhibitors. Our results showed that only the JNK inhibitor blocked TGF-β1-induced secretion of TGF-β2 partially by about 50% (data not shown). Our results possibly suggest that TGF-β1 might stimulate secretion of TGF-β2 by the JNK pathway. Our results concur with those of Li and Wicks. (2001) who observed that JNK interacts with the retinoblastoma protein in stimulating TGF-β2.

Also, we observed increased TGF-β1 secretion in TGF-β1 treated cells indicating autostimulation. However, no effect on TGF-β1 secretion was observed in both controls and TGF-β2 treated cells. These results possibly indicate that TGF-β1 autostimulated itself through the JNK pathway. Our results are in agreement with those of Pardouk and Derynck, (2004) and Ventura et al. (2004) who showed that JNK regulates expression of TGF-β1 in fibroblasts. Further support for our results is shown by Zhang et al. (2006) who found that TGF-β1 autoinduced itself through JNK/p38 signaling in proximal tubular epithelial cells.

4.2 Conclusions

In this study, we investigated the influence of TGF-βs on endometrial, endometriotic cell lines and primary endometrial stromal cells in vitro. The findings showed that TGF-βs dramatically increased secretion of PAI-1, Smad3 phosphorylation, apoptosis and TBRIII receptor expression on endometrial, endometriotic cell lines and primary endometrial stromal cells. We also demonstrated reduction in cell numbers in all cells studied. The TGF-β-induced strong increase in PAI-1 secretion in endometriotic cells compared to endometrial cells suggest that PAI-1 might cause also an increased breakdown of endometrial tissue during menstruation, possibly resulting in increased invasiveness and implantation of endometrial tissues at ectopic sites after retrograde menstruation. Our findings that reduced Smad3 phosphorylation and reduced TBRI, TBRII and TBRIII interactions in endometriotic cells compared to endometrial cells indicate that endometriotic cells might become somehow less responsive to TGF-β signals. Furthermore, this might possibly also explain for example why there was a lower reduction in cell numbers in endometriotic cells compared to endometrial cells upon treatment with TGF-βs. Also, the results obtained with MUC1 and CK 18 expression in nearly all endometrial glands as well as ovarian cysts indicate that endometrial cells are disseminated like tumor cells thus further corroborating the importance of the results obtained with PAI-1 and the TGF-βs.

Our study also affirmed that the Smad pathway is the main pathway of TGF-β signaling in endometrial, endometriotic cell lines and primary endometrial stromal cells. Our results also showed for the first time that ALK-2 acts as a link between BMP and TGF-β signaling in endometrial, endometriotic cell lines and primary endometrial stromal cells. Furthermore, we reported for the first time that the JNK

pathway is utilized by TGF-β1 to stimulate TGF-β2 and TGF-β1 secretion in endometrial, endometriotic cell lines and endometrial stromal cells.

We showed for the first time that TGF-β-induced apoptosis of endometrial and endometriotic cells is Smad-dependent and both intrinsic and extrinsic pathways are involved. In addition, we showed for the first time the TGF-βs and TBRs interaction (Signalosome analysis) in endometrial, endometriotic cells and primary endometrial stromal cells. It is tempting to speculate that the observed downregulation of receptors on endometriotic cells might suggest an escape mechanism by which endometriotic cells tries to evade the growth regulation by TGF-βs.

Interestingly, we were able to demonstrate ALK-2 as a possible point of cross-talk between the BMP and TGF-β pathways besides ALK-3 and ALK-6. However, further studies are required to clarify this connection between the two pathways. Finally, for the first time, we showed that the endometrial stromal cell line T-HESC demonstrated similar characteristics compared to primary endometrial stromal cells. Further investigations are needed to analyse the characteristics of other primary cells compared to cell lines as these will show a clear picture of what might happen in vivo.

5 Summary

Endometriosis is characterized by the presence of endometrial-like cells outside the uterus mostly in the ovary and peritoneum. TGF-βs are expressed significantly higher in the serum and peritoneal fluid of patients with endometriosis. TGF-βs have been also observed in endometriotic sites. Thus, TGF-βs might be involved in the pathogenesis of endometriosis. The aim of this study was to investigate the signaling pathways of the TGF-βs and possible cross-talks with other pathways. Also, we investigated the interaction of the TGF-βs to their receptors.

In this study, we used four different cell lines including endometrial epithelial and stromal cell lines, endometriotic epithelial and stromal cell lines and primary endometrial stromal cells. Also, endometrial and ovarian tissues were used. Our results showed that in all four cell lines and primary cells studied: (1) TGF-β1 or TGF-β2 decreased cell numbers in all cells and the reduction was higher in endometrial cells compared to endometriotic cells, (2) TGF-β1 or TGF-β2 induced apoptosis in all cells with no significant differences between endometrial or endometriotic cells, (3) TGF-β1 or TGF-β2 induced Smad3 phosphorylation in all cells studied with higher phosphorylation levels observed in endometrial cells compared to endometriotic cells, (4) a TβRI inhibitor completely blocked the TGF-β-induced reduction in cell numbers, apoptosis, PAI-1 secretion and Smad3 phosphorylation. A Smad3 inhibitor only partly blocked it. (5) TGF-β1 or TGF-β2 increased TBRII and TBRIII or TBRI and TBRII interaction with a stronger interaction observed in endometrial cells compared to endometriotic cells. (6) A BMP as well as an ALK-2 inhibitor completely blocked the TGF-β-induced PAI-1 secretion. In contrast, ALK-3 and ALK-6 inhibitors only partly blocked it. (7) A JNK inhibitor blocked increased secretion of TGF-β2 and TGF-β1 in TGF-β1-treated cells. (8) Both endometrial glands and ovarian endometriotic foci express CK 18 and MUC1 proteins.

From these results, we suppose that the reduced responsiveness upon TGF-β treatment observed in endometriotic cells compared to endometrial cells in regard to reduction in cell numbers, Smad3 phosphorylation and TBR receptor interaction indicates that endometriotic cells are more resistant to TGF-β signals. This suggests that endometriotic cells might acquire tumor-like characteristics which might contribute to their survival, evasion of the immune system and subsequent

implantation during the pathogenesis of endometriosis. In addition, we provided evidence that endometriotic cells have possibly the same origin from endometrial cells and thus are disseminated like tumor cells. Furthermore, we demonstrated for the first time that endometrial and endometriotic cells undergo apoptosis upon TGF-β treatment and both intrinsic and extrinsic pathways are involved. In addition, we demonstrated the participation of the JNK and BMP pathways in TGF-β signaling in endometrial, endometriotic and primary endometrial stromal cells. These findings might provide new insights into the roles of TGF-βs in the pathophysiology of endometriosis. However, more studies are needed on BMP and JNK pathways in TGF-β signaling in endometrial, endometriotic and primary cells to elucidate the connection between BMP or JNK with TGF-βs since our study only gave a first glimpse into involvement in TGF-β signaling in endometrial, endometriotic and primary endometrial stromal cells.

6. Zusammenfassung

Endometriose ist charakterisiert durch die Anwesenheit von endometrium-ähnlichen Zellen ausserhalb des Uterus meistens im Ovar und im Peritoneum. TGF-βs sind signifikant stärker exprimiert im Serum und Peritonealflüssigkeit von Patienten mit Endometriose. TGF-βs wurden auch beobachtet an endometriotischen Stellen und könnten deshalb in der Pathogenese der Endometriose involviert sein. Das Ziel der Studie war die Untersuchung der Signalwege der TGF-βs und mögliche Wechselwirkungen mit anderen Signalwegen. Ebenso analysierten wir die Interaktion der TGF-βs mit ihren Rezeptoren.

In dieser Studie nutzten wir vier verschiedene Zelllinien eingeschlossen endometriale epitheliale und stromale Zelllinien, endometriotische epitheliale and stromale Zelllinien und primäre endometriale stromale Zellen. Ebenso wurden endometriale und ovarielle Gewebe verwendet. Unsere Resultate zeigten in allen untersuchten vier Zelllinien und den primären Zellen: (1) TGF-β1 oder TGF-β2 verminderten die Zellzahlen in allen Zelllinien und die Reduktion war höher in den endometrialen Zelllinien verglichen mit den endometriotischen Zelllinien, (2) TGF-β1 oder TGF-β2 induzierten die Apoptose in allen Zelllinien mit keinem signifikanten Unterschied zwischen endometrialen oder endometriotischen Zellen, (3) TGF-β1 oder TGF-β2 induzierten die Phosphorylierung von Smad3 in allen untersuchten Zellen mit höheren Phoshorylierungsspiegeln in endometrialen Zellen verglichen zu endometriotischen Zellen, (4) ein TβRI Inhibitor blockierte komplett die TGF-β-induzierte Reduktion der Zellzahlen, der Apoptose, der PAI-1 Sekretion und der Smad3 Phosphorylierung. Ein Smad3 Inhibitor dagegen blockierte nur teilweise. (5) TGF-β1 oder TGF-β2 erhöhten die Interaktion von TBRII mit TBRIII oder von TBRI mit TBRII mit einer stärkeren Interaktion in den endometrialen Zellen verglichen mit den endometriotischen Zellen. (6) Ein BMP als auch ein ALK-2 Inhibitor inhibierte vollständig die TGF-β-induzierte PAI-1 Sekretion. Im Gegensatz dazu blockierten ein ALK-3 oder ein ALK-6 Inhibitor nur teilweise. (7) Ein JNK Inhibitor blockierte die erhöhte Sekretion von TGF-β2 und TGF-β1 in TGF-β1-behandelten Zellen. (8) Sowohl endometriale Drüsen als auch ovarielle endometriotische Foci exprimieren die Proteine CK 18 und MUC1.

Ausgehend von diesen Resultaten vermuten wir, dass die reduzierte Responsivität nach TGF-β Stimulation beobachtet in endometriotischen Zellen im Vergleich zu

endometrialen Zellen bezüglich Reduktion der Zellzahlen, der Smad3 Phosphorylierung und TBR Rezeptor Interaktion darauf hinweist, dass endometriotische Zellen resistenter gegenüber TGF-β Signalen sind. Das deutet daraufhin, dass endometriotische Zellen möglicherweise Tumor-ähnliche Eigenschaften erwerben, die beitragen zu ihrem Überleben, Umgehen des Immunsystems und der nachfolgenden Implantation während der Pathogenese der Endometriose. Zusätzlich zeigten wir, dass endometriotische Zellen möglicherweise alle von endometrialen Zellen abstammen und sich deshalb wie Tumorzellen ausbreiten. Des weiteren zeigten wir erstmalig, dass endometriale und endometriotische Zellen die Apoptose einleiten nach Stimulation mit TGF-βs und daran sowohl der intrinsische als auch der extrinsische Weg beteiligt sind. Ebenso konnten wir zeigen, dass es eine Beteiligung des JNK und BMP Signalwegs beim TGF-β Signalweg gibt in endometrialen, endometriotischen und primären endometrialen stromalen Zellen. Diese Befunde erlauben möglichwerweise neue Einblicke in die Rolle der TGF-βs in der Pathophysiologie der Endometriose. Dennoch sind mehr Studien zu den BMP und JNK Signalwegen in endometrialen, endometriotischen und primären Zellen nötig um die Zusammenhänge zwischen BMP oder JNK mit den TGF-βs aufzuklären, weil unsere Studie nur einen ersten Eindruck in die Beteiligung der TGF-β Signalwege in endometrialen, endometriotischen und primären endometrialen stromalen Zellen vermitteln konnte.

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8. Acknowledgements

Firstly, I wish to express my sincere gratitude to my supervisor, PD. Dr. Lutz Konrad for his invaluable guidance, encouragement and positive criticism without which I would have not accomplished my objectives. He helped me to develop an understanding of the subject and offered me opportunities of advancing my research career. I wish to sincerely thank my co-supervisor Prof. Charles Omwandho of the Department of Biochemistry, University of Nairobi for his constant advice, invaluable assistance and encouragement throughout my PhD studies.

Secondly, special thanks go to Prof. Hans-Rudolf Tinneberg, the director of the Department of Gynecology and Obstetrics of UKGM, who invited me to his laboratory in Giessen and provided me with research facilities that enabled me to accomplish my PhD studies. He also offered me constant advice and gave me a lot of help during the entire period of my PhD studies here in Giessen.

Thirdly, I wish to acknowledge the “Deutscher Akademischer Austauschdienst” (DAAD) for the generous financial support right from the Germany language course and throughout the PhD programme at the Justus-Liebig University, Giessen.

Fourthly, I would like to thank in a special way Mrs. Zoltan, MTA of the Department of Gynecology and Obstetrics who taught me cell culture techniques. Thanks a lot to Dr. Hersemeyer who supported me with confocal microscopy experiments and Dr Assad who I consulted during thesis write-up. Special thanks go to Mrs Hof, Mrs Lepper and Mrs Neeb for their help and support during my PhD studies in the laboratory.

Last but not least, my heartfelt thanks go to my lovely wife Carolynne Kemunto Ayienda who amidst despair on my side gave me an impetus to trudge on. I have known the true meaning and value of patience through her. Her personal interest in my daily experiments helped me a lot. Special thanks goes to my lovely son Steve-Michael Onyonka, I dedicate to you this thesis to work hard for greater heights. To all members of Mecha family, I say thank you very much for standing by me throughout my studies.

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STAUFENBERGRING 15
D-35396 GIESSEN

Tel: 0641-5599888 Fax: -5599890
redaktion@doktorverlag.de
www.doktorverlag.de

ISBN: 978-3-8359-6174-6