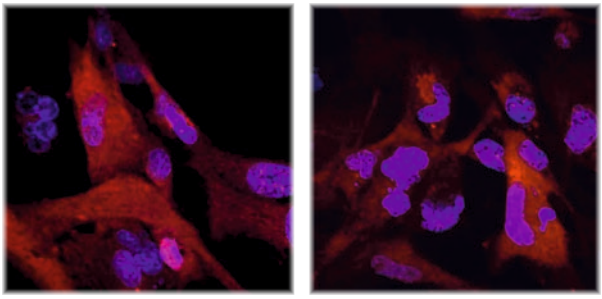
Induction of Smad-dependent and -independent Pathways by TGF-βs in Human Endometrial and Endometriotic Cells

CONG SUI



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

édition scientifique
VVB LAUFERSWEILER VERLAG

1. Auflage 2012

1st Edition 2012

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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Justus-Liebig-Universität Gießen

vorgelegt von **Cong Sui**

aus Wuhan, China

Gießen, 2011

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: 25.04.2012

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Abbreviations

Approx.	approximately
BMP	bone morphogenetic protein
BSA	bovine serum albumin
CAM	cellular adhesion molecule
CDK	cyclin-dependent kinase
Ctrl	control
DIE	deep infiltrated endometriosis
DMEM	Dulbecco's modified eagle's medium
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
ECM	extracellular matrix
EDTA	ethylene diamine tetraacetic acid
EGF	epidermal growth factor
EGTA	ethylene glycol tetraacetic acid
ELISA	enzyme-linked immunosorbent assay
ERK	extracellular signal-regulated kinase
FCS	fetal calf serum/ fetal bovine serum
FN	fibronectin
g	gravitational force
GDF	growth differentiation factor
GnRH	gonadotropin releasing hormone
GRB2	growth factor receptor-bound protein-2
h	hours
HRP	horseradish peroxidase
IL-10	Interleukin 10
IFN-γ	Interferon-gamma
JNK	c-Jun N-terminal kinase
LAP	latency associated peptide
LTBP	latent TGF-β binding protein
MEK	mitogen-activated protein kinase kinase
min.	minutes

ml	mililiter
mM	millimolar
MMP	matrix metalloproteinase
MPA	medroxyprogesterone 17-acetate
MRI	magnetic resonance imaging
mRNA	messenger ribonucleic acid
MSK1	mitogen and stress activated protein kinase-1
ng	nanogram
n.s.	not significant
OD	optical density
p38 MAPK	p38 mitogen-activated protein kinase
PAK2	p21 activated kinase-2
PAI-1	plasminogen activator inhibitor-1
pen/strep	penicillin/streptomycin
PBS	phosphate buffered saline
pg	pictogram
PMSF	phenylmethylsulfonyl fluoride
Raf	proto-oncogene serine/threonine-protein kinase
RAS	rat sarcoma
rh	recombinant human
RT	room temperature
SBE	Smad binding element
SCID	severe combined immune deficiency
SDS	sodium dodecyl sulfate
ShcA	src homology 2 domain-containing adaptor protein
SLC	small latency complex
SOS	son of sevenless
TFE3	transcription factor E3
TGF-β	transforming growth factor-beta
TIMP	tissue inhibitor of metalloproteinase
TMB	3,3’,5,5’-Tetramethylbenzidine
tPA	tissue plasminogen activator
TβR	receptor of transforming growth factor-beta
Tris	tris (hydroxymethyl)-aminomethane

TVUS	transvirginal ultrasound
uPA	urokinase-type plasminogen activator
uPAR	urokinase-type plasminogen activator receptor
UV light	ultraviolet light
µg	microgram
µl	microliter
µM	micromolar

1 Introduction

1.1 Endometriosis

1.1.1 General Characteristics of Endometriosis

Endometriosis is a chronic gynaecological disorder which occurs in 10% of the general female population during their reproductive ages (Roger et al., 2009). It is characterized by uterine endometrial tissue growing outside the uterine cavity, most commonly in the ovary and the peritoneum. In few cases, endometriotic tissue can also be found on other locations, such as intestinal tract, pericardium, pleura, lung or brain (Giudice and Kao, 2004). Pain, menstrual disorders and infertility are the main symptoms of endometriosis patients. Approximately 30-50% of the patients with endometriosis suffer from pain (Roger et al., 2009). Endometriosis is generally associated with inflammation and tissue remodelling. In severe cases, it may result in pelvic adhesions and distortion, which could lead to infertility (Giudice and Kao, 2004). Non-invasive methods for diagnosis of endometriosis before surgery, such as pelvic ultrasound, transvirginal ultrasound (TVUS) or magnetic resonance imaging (MRI) are helpful for deep infiltrated endometriosis (DIE) but often fail to diagnose superficial lesions (Champaneria et al., 2010). Altough a lot of biomarkers were tested in recent years (Mihalyi et al., 2010), none of them are currently used in the clinical routine (May et al., 2010). Because of similar symptoms like other gynaecological disorders, the diagnostic time normally delays up to 6.7 years in average (Nnoaham et al., 2011), even though some patients still get misdiagnosis. Furthermore, there might be patients with pain, but without any obvious lesions of endometriosis. Diagnosis of endometriosis can only be achieved by laparoscopy, subsequently followed by tissue histological confirmation of ectopic gland formation with endometrial and stromal cells (Kennedy et al., 2005). However, sometimes (6.6%), endometriosis occurs without glands, showing only stromal cells (Boyle and McCluggage, 2009).

According to the revised classification of the American Society of Reproductive Medicine (ASRM), four stages of endometriosis have been defined (Table 1).

Table1. Stages of Endometriosis

Stage	Name	Description
I	Minimal endometriosis	Only superficial lesions and possibly a few filmy adhesions
II	Mild endometriosis	In addition, some deep lesions are present in the rectouterine pouch
III	Moderate endometriosis	As above, plus presence of endometriomas on the ovary and more adhesions
IV	Severe endometriosis	As above, plus large endometriomas, extensive adhesions.

Endometriosis is often treated surgically, but with a recurrence rate of 5-25% after two years (Meuleman et al., 2011). The most widely used medical treatment of endometriosis involves using combined oral contraceptives, danazol, gonadotropin-releasing hormone (GnRH) analogues and progestins (Kennedy et al., 2005; Vercellini et al., 2008; Kappou et al., 2010). However, most of the drug treatments cause undesirable side effects and are not suitable for long-term use (Kennedy et al., 2005). Recently, dienogest, a highly selective progestin was shown to be effective with fewer side effects (Strowitzki et al., 2010).

1.1.2 Pathogenesis of Endometriosis

The exact cause of endometriosis still remains unknown. However, many theories have been presented to understand the etiology of endometriosis.

The concept which is most widely accepted is the retrograde menstruation theory, which was first proposed by John Albertson Sampson (Sampson, 1922; 1927). He suggested that during menstruation, some of the endometrial tissue fragments are transported to the pelvic cavity through the fallopian tube. His theory is credited because higher volumes of refluxed menstrual blood (Halme et al., 1984) and more frequent myometrial contractions (Salamanca and Beltran, 1995) were observed in women with endometriosis, compared to healthy women.

However, Sampson's theory is not able to explain all causes of endometriosis. For example, though most women have retrograde menstrual blood flow during their reproductive age, not all of them develop endometriosis (Eskenazi and Warner,

1997). There must be other factors which contribute to the survival of ectopic endometrial cells and the following pathological process. Thus, the suppression of the immune system should not be underestimated in the pathogenesis of endometriosis. Physiologically the immune system should recognize the ectopic endometrial cells and then induce an immune response against them and finally destroy them. However, in pathological cases, the immune system of patients with endometriosis seems not to perform such a process, so that the ectopic endometrial cells survive ectopically (Herington et al., 2011). Dysfunction of immunity in women with endometriosis was demonstrated with evidence of increased abnormal B and T cells (Vinatier et al., 1996) and reduced activity of natural killer cells (Osuga et al., 2011). Berbic and Fraser (2011) also showed that disturbance of regulatory T cells failed to recruit leukocytes to initiate effective immune responses against ectopic endometrial fragments in endometriosis.

The pathogenesis of endometriosis is a complex process associated with various factors. To better understand the process of endometriosis, Omwandho et al. (2010) summarized its process and categorized it into six stages, which will be described in a later paragraph (1.2.5).

1.1.3 In vivo Models of Endometriosis

According to the most widely accepted theory for the pathogenesis of endometriosis, the implantation of retrograde endometrial tissues is essential for the establishment of peritoneal endometriosis. Because of that, spontaneous menstruation, which only occurs in human and primates, is required in the pathogenesis of endometriosis. However, it is quite difficult to compare humans and primates because of species specificity. Recently, Dehoux et al. (2011) questioned the baboon model because of the low rate of spontaneous endometriosis (4.8%) and induced endometriosis (27.6%).

In recent years, nude or severe combined immune deficiency (SCID) mice, with transplanted human endometrial tissue, have been used as models for the study of endometriosis (Grümmer, 2006; Liu et al., 2010; Katayama et al., 2010; Becker et al., 2011). These models showed that human endometrial and endometriotic tissue or cells grafted into the peritoneal cavity of immunodeficient mice were able to implant and to develop into endometriotic lesions. Furthermore, in such models, the

peritoneal environment or the physical condition of the hosts can be changed, according to the aim of the studies. Tabibzadeh et al. (1999) reported that peritoneal fluid from patients with endometriosis increased the implantation of the human endometrial tissue in the mouse model. These investigations confirm that the immunodeficient mouse model with implantation of human endometrial tissue is an appropriate method for the study of endometriosis.

However, the SCID mouse model also has disadvantages. Because of the lack of the immune system, the mice often die within a short lifespan. More importantly, endometriosis is a disease in which the immune system is highly involved. Thus, the immunodeficient mouse model has also limitations in the study of endometriosis.

1.1.4 In vitro Models of Endometriosis

The primary advantage of in vitro models is that it simplifies the system, so that the investigators can focus on a small number of components, especially the investigation on intracellur proteins and pathways is more easily achieved.

For in vitro models of endometriosis, endometrial and endometriotic tissues are obtained directly from patients after surgical treatment or from in vivo models. For cell culture, the primary epithelial or stromal cells are isolated and then kept in cell culture. The invasiveness of endometriotic cells was provn in vitro with the collagen invasion assay (Gaetje et al., 1995; Starzinski-Powitz et al., 1998), which showed the possibility to study endometriosis in vitro. However, this technique has disadvantages and restrictions. For example, primary endometrial cell cultures usually contain heterogeneous cell types and die after few passages (Starzinski-Powitz et al., 1998).

The establishment of immortalized endometrial and endometriotic cell lines overcomes this problem. Starzinski-Powitz et al. (1998) established immortalized endometriotic cell lines, which could be kept in culture for 40 passages, by inducing the expression of the SV40 T antigen, a viral oncogene. In addition, cell lines used for studies of endometriosis should be similar to primary endometriotic cells, e.g. should express estrogen and progesterone receptors, which are also expressed in primary endometriotic cells (Segars, 1997).

1.2 Transforming Growth Factor-beta

1.2.1 General Characteristics of Transforming Growth Factor-beta

The transforming growth factor-beta (TGF-β) family is a group of secreted proteins which comprises three isoforms, TGF-β1, TGF-β2 and TGF-β3. This subfamily belongs to a group of growth factors called transforming growth factor β superfamily, which also includes the bone morphogenetic protein (BMP) subfamily, the inhibin/activin subfamily and the growth differentiation factor (GDF) subfamily (Peng, 2003).

The biological functions of TGF-βs are multiple. They regulate many aspects of cell functions, such as proliferation, differentiation, apoptosis, and cell motility in diverse cell types. They are also involved in many biological processes, such as morphogenesis, embryonic development, immune regulation, wound healing, inflammation and tumorigenesis (Kaminska et al., 2005; Padua and Massagué, 2009; Taylor, 2009; Santibañez et al., 2011).

All three isoforms are secreted in a latent form containing the TGF-β homodimer (Derynck et al., 1985), which binds the latency associated peptide (LAP), forming a complex called small latency complex (SLC). This complex is released and stored in the ECM after binding to latent-TGF-β binding proteins (LTBPs) (Rifkin, 2005).

The biological functions of the TGF-βs have been studied through gene knockout techniques. TGF-β1 knockout mice die at midgestation due to defective haematopoiesis and vasculogenesis (Dickson et al., 1995), and the few survivors show an altered immune response (Koglin et al., 1998), or decreased bone mass (Geiser et al., 1998), or propensity to colon cancer (Engle et al., 2002). TGF-β2 knockout mice demonstrate multiple malformation defects (Sanford et al., 1997) and impairment of central synapse function (Heupel et al., 2008). TGF-β3 knockout mice die shortly after birth due to defective palate growth (Taya et al., 1999) and delayed lung development (Shi et al., 1999).

The receptors of the TGF-βs are single pass serine/threonine kinase receptors which exist in three isoforms with distinct structural and functional properties (Chang et al., 2002). TGF-β receptor type I (TβRI) and type II (TβRII) have similar binding affinity which is high for TGF-β1 and -β3, but low for TGF-β2. The type III (TβRIII) receptor shows high affinity to all three isoforms (Gordon et al., 2008). Typical TGF-β signaling starts with binding of the ligands to the TβRII, which phosphorylates the

TβRI. Then TβRI subsequently phosphorylates the C-terminus of Smad2 and/or Smad3, leading to downstream signal transduction (Wrighton et al., 2009).

1.2.2 Smad Pathway in TGF-β Signaling

The Smad proteins are the most important intercellular proteins, which transduce the extracellular signals from the TGF-β receptors to the nucleus (Massagué, 1998). There are three different types of Smads. The receptor-regulated Smads (R-Smads), including Smad1, 2, 3, 5 and 8, interact with the common-mediator Smad (co-Smad, Smad4), which is necessary for the downstream signaling as a heteromeric partner of the R-Smads. Smad6 and Smad7 are inhibitory Smads (I-Smads), which inhibit the activity of R-Smads and co-Smad (Padgett et al., 1998). In general Smad2 and Smad3 are phosphorylated by receptors of TGF-βs or activin (Shimizu et al., 1998), while Smad1, 5 and 8 are phosphorylated by the BMP type I receptor (Aoki et al., 2001).

The basic TGF-β induced Smad-pathway is shown in Figure 1 (Massagué, 2000).

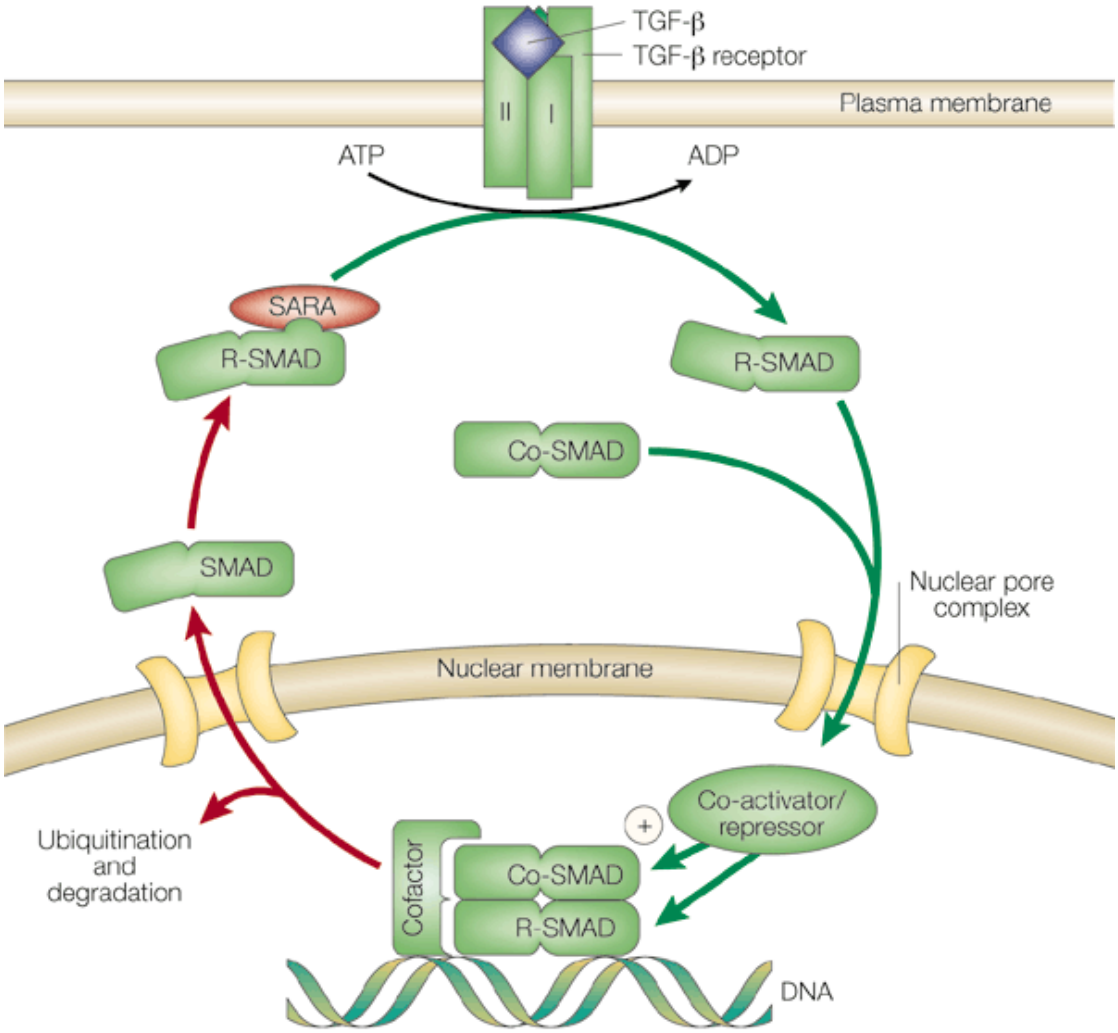

Figure 1. The basic Smad pathway (Massagué, 2000)

TβRI is activated by TβRII when TGF-βs induces the association of TβRI and TβRII. Then R-Smads are phosphorylated, and together with the co-Smad assemble transcription regulatory complexes. However, to be accessible to membrane receptors, R-Smads are tethered in the cytoplasm by Smad anchor for receptor activation (SARA). The phosphorylation of R-Smads decreases the affinity of R-Smads for SARA and increases their affinity for co-Smads. The resulting Smad complex is free to move into the nucleus and competent to associate with transcriptional co-activators or co-repressors.

1.2.3 Smad-independent Pathways in TGF-β Signaling

The identification of non-Smad signalling proteins that participate in TGF-β signal transduction pre-dates the discovery of the Smads (Yue and Mulder, 2000). Zhang (2009) summarized the Smad-independent pathways, which reinforce or modulate the signal transduction of the TGF-βs. Most of the studies on Smad-independent pathways were performed in cell models in vitro. The small GTPase Ras, the extracellular signal-regulated kinases (ERKs), p38 mitogen-activated protein kinase (p38 MAPK) and c-Jun N-terminal kinases (JNKs) are found to be implicated in TGF-β signaling (Yue and Mulder, 2000).

These Smad-independent pathways provide other possibilities of TGF-β signal transduction without the participation of the Smads. For example, TβRI can directly phosphorylate ShcA, which induces the association with growth factor receptor-bound protein-2 (GRB2) and son of sevenless (SOS), then leading to sequentially activation of Ras, Raf, MEK1/2 and ERK1/2 (Lee et al., 2007). Besides, TGF-β can activate the Ras cascades through the activation of p21 activated kinase-2 (PAK2) (Suzuki et al., 2007). These data suggests that TGF-β can directly activate the ERKs without participation of the Smad pathway.

Furthermore, these Smad-independent signal proteins have been suggested to interact with the Smad pathway (Massague et al., 2000; Moustakas and Heldin, 2005). The ERK pathway can lead to direct phosphorylation of R-Smads, thus blocking their nuclear translocation and transcriptional output (Kretzschmar et al., 1997). The p38 substrate kinase, MSK1 (mitogen and stress activated protein kinase-1) regulates the transcriptional activity of Smad3 by promoting its association with the co-activator p300 (Abécassis et al., 2004). In addition, TGF-β-activated JNK phosphorylates Smad3 and induces its nuclear translocation and transcriptional activity (Engel et al., 1999).

1.2.4 TGF-βs in the Normal Endometrium

TGF-βs are differentially and stage-specifically expressed in the endometrium during the menstrual cycle (Figure 2). According to the results from Gaide Chevronnay et al. (2008), the mRNA and protein expression of TGF-β1 was the highest, which was 2.5-fold higher than TGF-β2 and 20-fold higher than TGF-β3. Because of their high expression, TGF-βs are suggested to participate in the biological processes during

menses, such as tissue breakdown and uterine contraction (Omwandho et al., 2010).

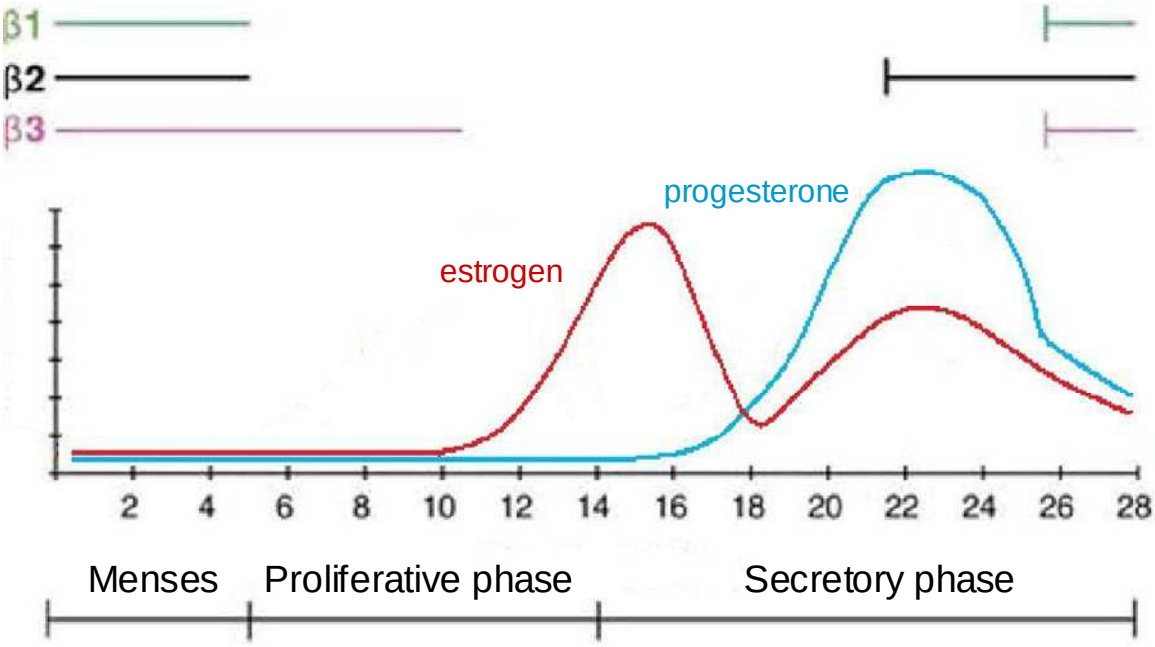

Figure 2. Levels of TGF-βs in human endometrium and hormonal changes during the menstrual cycle. Only the strongest expression of the TGF-βs is shown. All three isoforms are highly expressed during menses (Omwandho et al., 2010).

1.2.5 TGF-βs in Endometriosis

TGF-βs are suggested to be crucial in the pathogenesis of endometriosis because they are highly detected in the serum and peritoneal fluid of women with endometriosis (Pizzo et al., 2002). The process of endometriosis can be categorized into six stages (Omwandho et al., 2010), including cell shedding and reflux, cell survival, immune suppression, cell adhesion and invasion, angiogenesis, and bleeding. TGF-βs are involved directly or indirectly in most of these stages.

According to Sampson's theory, endometriosis originates from retrograde endometrial tissue. Thus, shedding and reflux of endometrial cells are the prerequisites for the establishment of endometriosis. The highest expression of TGF-βs was detected during menses (Gaide Chevronnay et al., 2008), coincident with cell shedding, thus suggesting the possible involvement of the TGF-βs in cell shedding. Furthermore, abnormal myometrial contractions with higher frequencies were described in women with endometriosis, and were suggested to be associated with the increased dissemination of endometrial fragments (Bulletti et al., 2002, 2004).

Although involvement of TGF-βs in uterine contractions still needs to be further investigated, TGF-βs were shown to induce the contraction of endometrial stromal cells (Nasu et al., 2005).

TGF-βs regulate cell survival by regulating cell proliferation and apoptosis. However, these effects are extremely dependent on the cell types and the cellular context. For example, TGF-βs trigger apoptosis in epithelial cells, but induce proliferation in fibroblasts (Rahimi and Leof, 2007). With respect to endometrial cells, TGF-βs showed different effects on regulation of cell proliferation, compared to cells from other organs. TGF-β1 stimulated proliferation of low epithelial cell numbers, but inhibited it at high cell numbers in women with and without endometriosis (Meresman et al., 2003). All three TGF-β isoforms inhibited the proliferation of endometrial stromal cells dose-dependently (Nasu et al., 2005).

After endometrial fragments enter the peritoneal cavity, the escape from the immune attack is important for their survival. TGF-β1 can strongly suppress the immune system, which was demonstrated by lethal multi-organ inflammation in gene knockout mice (Shull et al., 1992). In addition, TGF-βs suppressed proliferation and differentiation of lymphocytes including cytolytic T cells, natural killer cells and macrophages (Pardali and Moustakas, 2007). Blocking TGF-β1 function increased the levels of IFN-γ and IL-10, which are secreted by natural killer cells derived from human endometrium (Eriksson et al., 2004). Thus, high levels of TGF-βs may trigger immune escape, which might decrease the response of natural killer cells to the ectopic tissues. All in all, TGF-β might increase the survival potential of ectopic endometrial fragments by suppressing the immune system.

The process of cell-to-ECM adhesion is mainly regulated by integrins, which connect cells to the ECM (Witz et al., 2003). Koth et al. (2007) showed that integrins can activate latent TGF-β1. However, so far there is no evidence that TGF-βs could affect cell adhesion directly. The regulation might be indirect, by regulating the balance of matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs). MMPs are a goup of proteinases which degrade the ECM. TIMPs are the natural inhibitors of MMPs. The balance of MMPs and TIMPs regulates cell adhesion by controlling degradation and remodeling of the ECM (Bourboulia and Stetler-Stevenson, 2010). The regulation of MMPs and TIMPs by TGF-βs will be introduced in the following paragraph.

Conclusively, TGF-βs may be involved in most of the biological processes of the establishment and/or maintenance of endometriosis. However, the exact role remains to be studied further and clarified.

1.3 Matrix Metalloproteinases

Matrix Metalloproteinases are a subfamily of metalloproteases, which consists of 23 distinct proteases in humans (Table 2). They are zinc- and calcium-dependent enzymes, involved mainly in ECM degradation, as well as in some other biological functions (Borkakoti, 2000; Bode and Maskos, 2003; Klein and Bischoff, 2011). The newly synthesized MMPs mainly exist in the form of proenzymes without proteolytic activities. The pro-MMPs can be activated by other MMPs or serine proteinases like plasmin or oxidants. However, the mechanism of activation for most pro-MMPs remains unknown (Ra and Parks, 2007).

Gelatinase A (MMP-2) is one of the most important MMPs in ECM degradation. It is primarily secreted by fibroblasts during tissue development and regeneration (Morgunova et al., 1999). Together with gelatinase B (MMP-9), it degrades type IV collagen, gelatin (denatured collagen) and other types of collagen (V, VII and X), as well as elastin and fibronectin (Klein and Bischoff, 2011).

Tissue inhibitors of metalloproteinases are characterized as natural inhibitors of MMPs. So far, four TIMPs (-1, -2, -3 and -4) have been identified. They inhibit the activity of MMPs by forming TIMP-MMP complexes (Brew and Nagase, 2010). Thus, the balance between activated MMPs and TIMPs plays an essential role in regulating ECM degradation and remodeling.

In the human endometrium, MMPs and TIMPs are mainly secreted by stromal cells (Salamonsen and Woolley, 1996). In addition, macrophages are also able to secrete large amounts of various MMPs (Goetzl et al., 1996). The expression of most MMPs is repressed during the secretory phase and increases dramatically at or slightly before menstruation (Vassilev et al., 2005). With respect to endometriosis, the degradation of the ECM by MMPs results in the cell shedding during menstruation and thus might contribute to the invasiveness of ectopic endometrial cells (Juhasz-Böss et al., 2010).

Table 2

Overview of the identified human matrix metalloproteinases

MMP	Alternative Name	MMP	Alternative Name
1	Collagenase-1	16	Membrane type-3 MMP
2	Gelatinase A	17	Membrane type-4 MMP
3	Stromelysin-1	19	None
7	Matrilysin	20	Enamelysin
8	Collagenase-2	21	None
9	Gelatinase B	23	Femlysin
10	Stromelysin-2	24	Membrane type-5 MMP
11	Stromelysin-3	25	Membrane type-6 MMP
12	Macrophage metallo-elastase	26	Matrilysin/endometase
13	Collagenase-3	27	None
14	Membrane type-1 MMP	28	Epilysin
15	Membrane type-2 MMP		

Both MMPs and TIMPs can be regulated by TGF-βs. In the human endometrium, TGF-βs show a dual effect in regulating MMPs, which suppress the synthesis of MMP-3 and MMP-7 (Bruner et al., 1995), but increase the expression of MMP-2 and MMP-9 (Hirata et al., 2003). In human myometrial smooth muscle cells, TGF-β1 decreases the mRNA and protein synthesis of MMP-1 and MMP-3, whereas the mRNA and protein synthesis of TIMP-1 is increased (Ma and Chegini, 1999).

1.4 Plasminogen Activator Inhibitor-1

Plasminogen activator inhibitor-1 is the primary inhibitor of tissue plasminogen activator (tPA) and urokinase (uPA), the activators of plasminogen (Czekay and Loskutoff, 2004). The main function of PAI-1 is to inhibit the tPA and/or uPA induced fibrinolysis, and hence accelerates the progression to fibrosis (Binder et al., 2002). PAI-1 can be regulated by growth factors, cytokines and hormones, among which TGF-β1 is the most important one, because the PAI-1 promoter contains three Smad binding elements (SBEs), the main response elements of TGF-β signaling (Binder et al., 2002).

PAI-1 is mainly produced by endothelial cells, and released into the plasma. However, PAI-1 can be expressed and synthesized in various cell types (Binder et al., 2002). With respect to endometrial and endometriotic tissues, Bruse et al. (1998, 2004) showed that the expression of PAI-1 and uPA is much higher in the endometriotic tissue and endometrial tissue of women with endometriosis, compared to tissue of women without endometriosis. Also the PAI-1 level was higher in ectopic tissues than in eutopic tissues of the same woman (Bruse et al., 2004). Czekay et al. (2003, 2011) showed that cell migration was regulated by uPA and its high affinity receptor, uPAR. They demonstrated that PAI-1 perturbed the cell attachment to the ECM by inhibiting uPAR-vitronectin and integrin-vitronectin interactions, and thus facilitated cell migration by regulating ECM proteolysis. Furthermore, PAI-1 is able to inhibit the activity of MMPs, by inhibiting plasmin. However, this inhibition only affected the activity of MMP-1 and MMP-3 (Murphy et al., 1999), but had no effect on the activity of gelatinases (MMP-2 and MMP-9) (Ramos-DeSimone et al., 1999). Thus, in conclusion, PAI-1 might be an important protein in the pathogenesis of endometriosis.

1.5 Objectives

Though TGF-βs has been strongly suggested to be involved in the pathogenesis of endometriosis, the exact functions remain to be clarified. TGF-βs are strong regulators of cell growth, but extremely cell type- and context-dependent. Recent studies showed that the regulation of cell proliferation by TGF-β in endometrial and endometriotic cells seems to be different from cells from other organs, and it is still not clear whether TGF-βs increase or reduce the number of endometrial and endometriotic cells. Furthermore, TGF-βs are able to regulate other proteins, like MMPs and PAI-1, which contribute to the etiology of endometriosis as well. Thus, it is also important to understand how TGF-βs regulate these proteins and the pathways through which the regulation proceeds.

The immortalized endometriotic cell lines used in this study have been well characterized and proven to be sufficient in studying endometriosis. Together with endometrial cell lines, we were able to generate a reliable in vitro model, in order to study the basic aspects of endometriotic cells and their differences from normal endometrial cells, as well as the functions of TGF-βs and their signaling pathways in endometrial and endometriotic cells.

Conclusively, the aim of this study was to investigate some aspects of the biological functions of TGF-βs, such as influence on cell numbers and regulation of some proteins, which are important in endometriosis, as well as to study the signaling pathway of TGF-βs in endometrial and endometriotic cells in vitro. Also, the different characteristics of normal endometrial cells and endometriotic cells will be documented.

2 Materials and Methods

2.1 Human Immortalized Endometrial and Endometriotic Cell Lines

2.1.1 Endometrial Epithelial and Stromal Cells

The HES cells are a spontaneously immortalized human endometrial epithelial cell line (Fig. 3). The HES cells express both cytokeratin and vimentin, and secrete no prolactin after progesterone stimulation, which strongly support their epithelial phenotype (Desai et al., 1994).

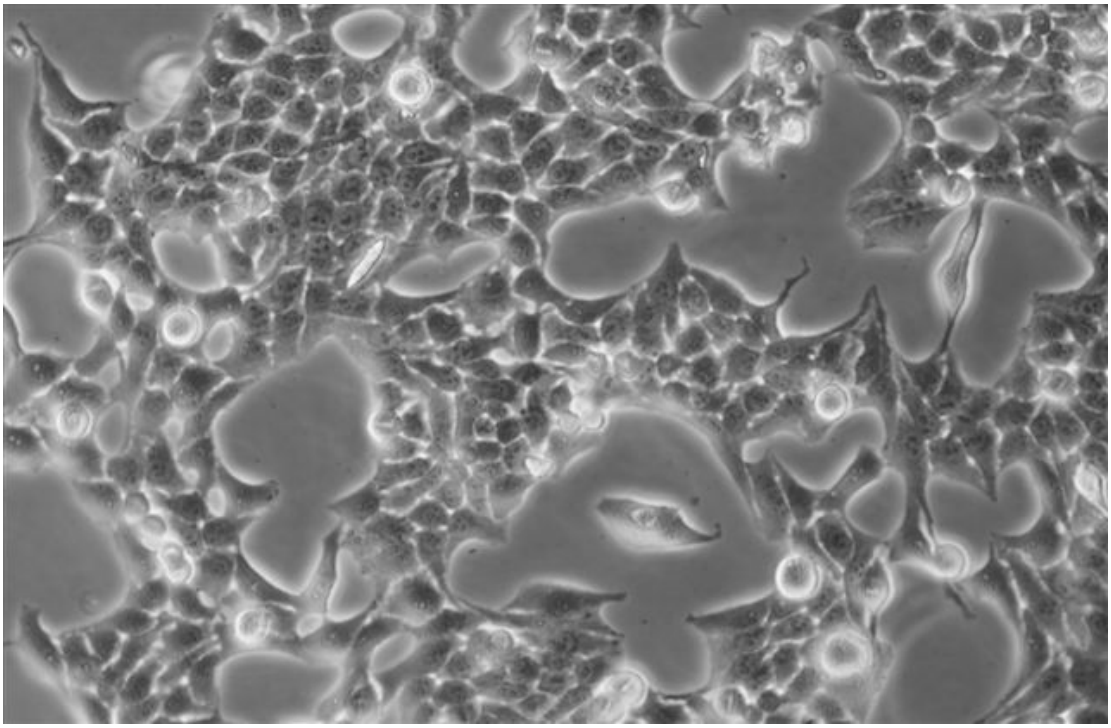

Figure 3. Human endometrial epithelial HES cells. The HES cells show a typical shape of epithelial cells in vitro. The cells appear as triangular, flat plates and aggregate closely, which indicates epithelial cell-to-cell contacts. The HES cells normally form flat monolayers in vitro.

The THESC cells (CRL-4003, ATCC) (Fig. 4) are telomerase immortalized endometrial stromal cells (Krikun et al., 2004). The estrogen receptor and the progesterone receptor are strongly expressed in THESC (Banu et al., 2008). The cells undergo decidualization when exposed to MPA (medroxyprogesterone 17-acetate) plus estrogen (Krikun et al., 2004).

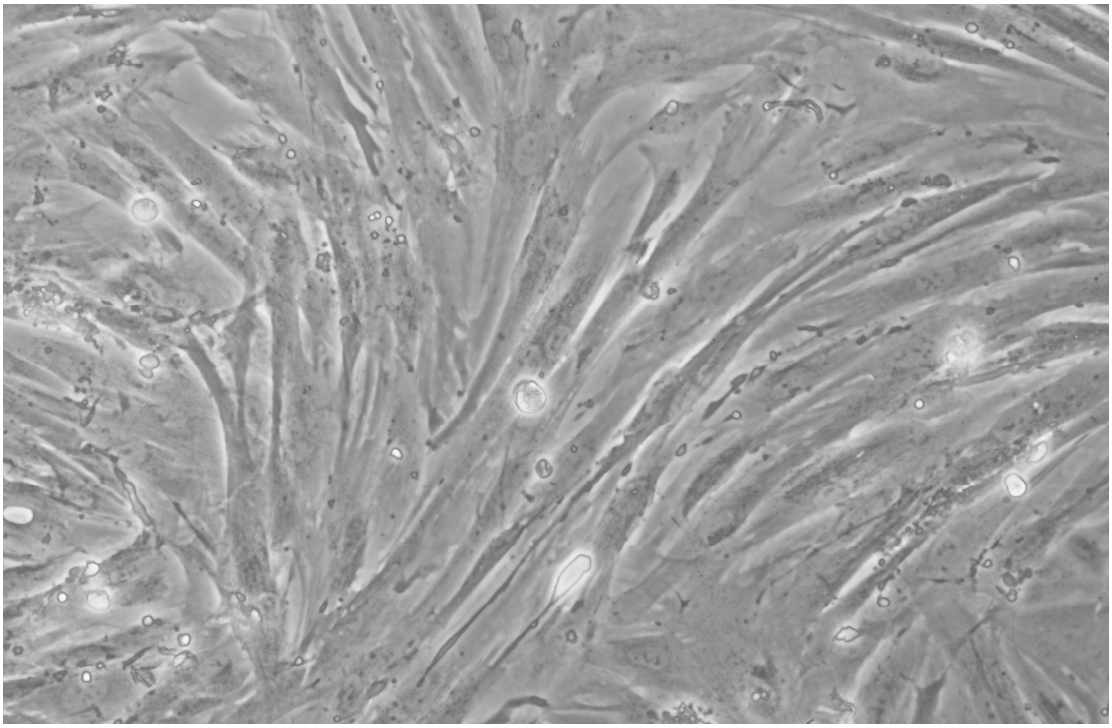

Figure 4. Human endometrial stromal THESC cells. The THESC cells are elongated spindle-shaped in vitro. Their size is larger compared to epithelial cells. The cells are often disjointed and scattered when their numbers are few. However, when crowded, they often aggregate in parallel clusters. Unlike HES cells, THESC cell do not form flat monolayers in vitro.

2.1.2 Endometriotic Epithelial and Stromal Cells

The 12Z cells are SV40 immortalized (Fig. 5). They are epithelial-like endometriotic cells expressing cytokeratins, vimentin and E-Cadherin that are typically found in the endometrial tissue (Zeitvogel et al., 2001) Furthermore, 12Z cells express the estrogen receptor α and β, as well as the progesterone receptor (Banu et al., 2008).

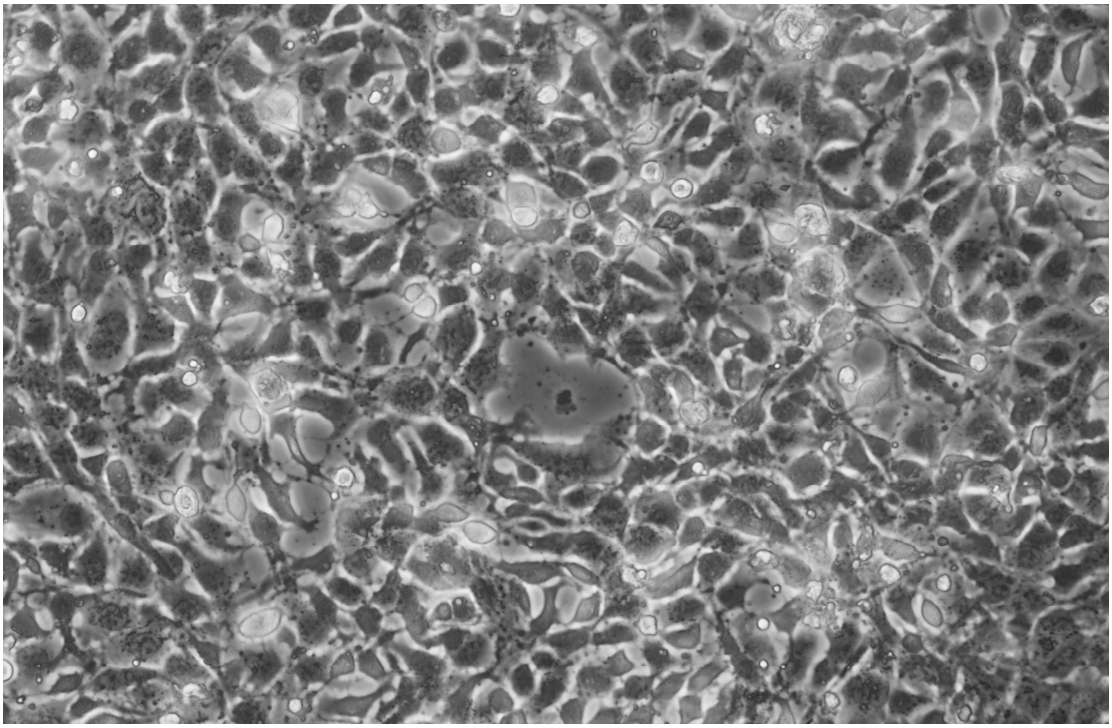

Figure 5. Human endometriotic epithelial 12Z cells. The 12Z cells have a typical epithelial cell shape in vitro. The cells have a similar appearance as HES cells - triangular, flat plates aggregating closely to each other, which suggests epithelial cell-to-cell contacts. The 12Z cells normally form a flat monolayer in vitro.

The 22B cells are SV40 immortalized (Fig. 6). They are stromal-like endometriotic cells and are vimentin-positive, but cytokeratin and E-Cadherin negative (Zeitvogel et al., 2001). The estrogen receptors α and β are strongly expressed in 22B cells. However, the progesterone receptor mRNA is nearly undetectable (Banu et al., 2008).

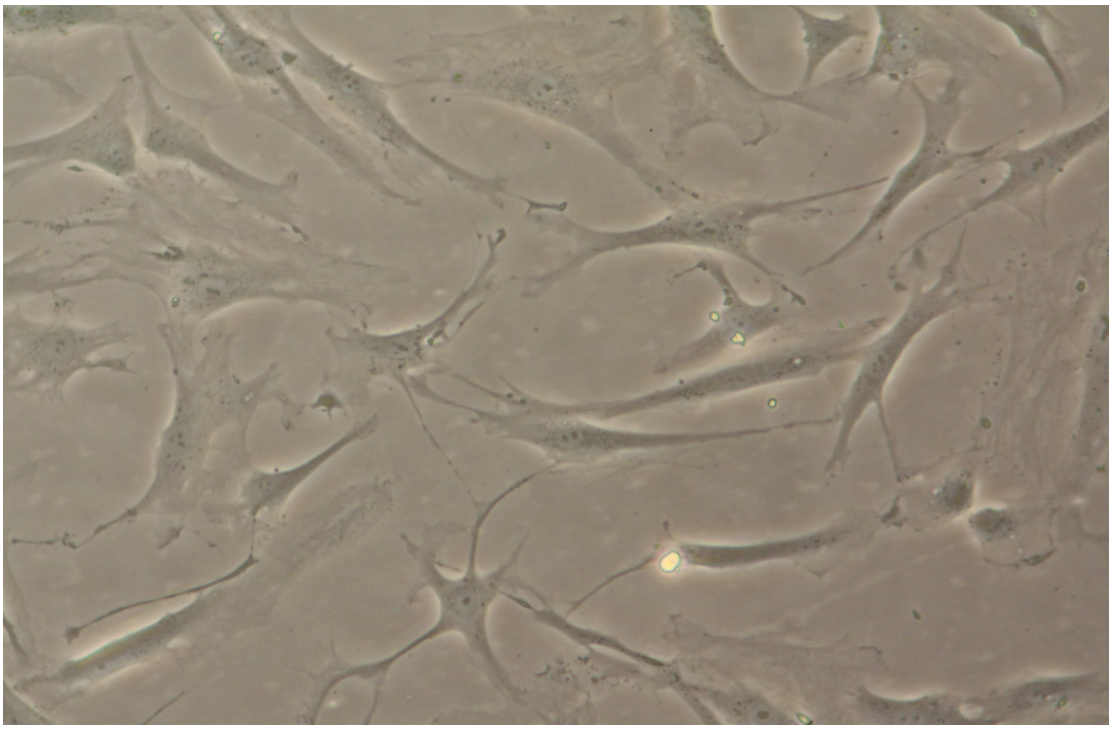

Figure 6. Human endometriotic stromal 22B cells. The 22B cells are elongated spindle-shaped cells. Some of them have a branched cytoplasm. The size of 22B is larger compared to THESC cells. Like THESC cells, the 22B cells are disjointed and scattered when their numbers are few and often aggregate in parallel clusters when crowded.

2.2 Cell Culture with Endometrial and Endometriotic Cell Lines

2.2.1 Aseptic Techniques for Cell Culture

All equipments and solutions needed for cell culture were either purchased sterile or sterilized in an autoclave. Solutions which are not thermoduric were sterilized using sterile filters with a pore size of 0.22 µm (disposable filter units, Millipore). The surface of the laminar flow bench was wiped with 70% alcohol. Then the UV light in the laminar was put on for 20 minutes before starting to work. All handling was performed under the laminar flow bench in order to guarantee sterile conditions. Equipment was also cleaned with 70% alcohol before taken into the laminar flow bench. Hands were protected with latex gloves and cleaned with 70% alcohol, before starting to work at the laminar flow bench.

The media used in cell culture were supplemented with 1% pen/strep (100X, PAA, Austria) and 1% plasmocin in order to protect cells against bacteria and mycoplasms. To minimize contamination, medium transfer was performed using sterile disposable pipettes. The bottle necks of the cell culture flasks were cleaned with sterile cotton swabs after medium transfer.

2.2.2 Medium Preparation

Cell culture medium provided the environment and necessary nutrients required in cell culture. In general, media were supplemented with an adjusted amount of serum, antibiotics and nutrients. Ready-to-use media were prepared previous of the work with cells. To ensure the medium retaining sterile, all procedures were done under the laminar flow bench.

- Medium for endometrial and endometriotic epithelial cells:

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

- Medium for endometrial stromal cells:

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

- Medium for endometriotic stromal cells:

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

The ready-to-use medium was stored at 4℃ by a maximum of 45 days. Aliquots were pre-warmed to 37℃ in a water bath before use.

2.2.3 Medium Change

The medium in the cell culture flask was replaced with fresh medium every 2-3 days under the laminar flow bench. Medium was pre-warmed in a water bath (37℃) for 20 minutes prior to use. Sterilized and disposable pipette tips were used during the whole process. The old medium was discarded and fresh medium was added. The date of medium change was recorded on the flask after replacement. Then the flasks were taken back to the cell culture incubator.

2.2.4 Cell Passage

Cell passage is a technique that keep cells alive and growing under cultured conditions for extended periods of time. Cells were passaged when they are 70%-80% confluent. Medium and accutase (PAA, Austria) were pre-warmed in a 37℃ water bath for 20 minutes. Old medium was discarded with a disposable pipette tip. Then 10ml accutase was added and cells were incubated in the cell culture incubator (37℃, 5% CO_2) for 5 to 10 minutes. After that, a microscope was used to ensure that cells were completely detached from the cell culture flask. Then 10 ml fresh medium was added to neutralize the accutase. The cell suspension was transferred into a 50

ml falcon tube, centrifuged for 5 minutes at 1500 x g. The liquid phase was discarded. Fresh medium (5 ml) was added to re-suspend cell pellets. One part of the cell suspension (500 µl to 2 ml) was transferred into a new cell culture flask with 25 ml fresh medium. The date of passage was written down on the flask. Then the flask was taken back into the cell culture incubator.

2.2.5 Cell Freezing

Cryopreservation was performed to maintain the cells for longer periods. The cell pellet was obtained by following the process of cell passage (2.2.4). Instead of resuspending the cells in culture medium, cells were resuspended in medium with Filoceth^{plus} (Procryoptect, Germany), cryo-medium with low DMSO which protected cells from the extremely low temperature and was less toxic to the cells compared to older standard procedures with 20% DMSO. Aliquots of 1ml cell suspensions are transferred into cryotubes. These cryotubes are then stored at -80℃ for 4-6 hours before finally preserved in liquid nitrogen.

2.2.6 Cell Thawing

Cells were stored with 1 ml Filoceth^{plus} in cryotubes in liquid nitrogen (-196℃) for long term preservation. For thawing, the cryotubes were taken out of the liquid nitrogen and placed into a 37℃ water bath. As soon as the ice in the cryotube was completely thawed (approx. 2 to 5 minutes), cells were transferred to a 15 ml falcon tube with 9 ml fresh cell culture medium. The liquid phase was discarded after centrifugation (1500 x g, 5 min) to remove the DMSO. The remaining pellet was resuspended with 1 ml medium. Then the cell suspension was 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 microorganisms. The cell culture flasks were incubated in a cell culture incubator (37℃, 5% CO_2).

2.2.7 Cell Counting

When measuring the cell numbers in cell culture flasks, cells were detached and resuspended as described in section 2.2.4. Then one part of the cell suspension (100 µl) was transferred to a CASY tube with 10 ml CASY-ton solution and mixed well. Then the cells were measured with the CASY-counter (Schaerfe System, Germany).

When measuring the cell number from cell culture plates, accutase was given according to the size of the plates (500 µl for 6-well plates, 200 µl for 24-well plates). After cells were completely detached from the plate, equal volumes of fresh medium was added and cells were resuspended thoroughly. Then 10 µl cell suspension was transferred to a CASY tube with 10 CASY-ton solution and mixed well. Then the cell numbers were determined with the CASY-counter.

2.2.8 Cell Seeding

After the cell numbers from the cell culture flasks were determined, cells were transferred into cell culture plates or dishes. In the experiments described here, $3x10^4$ cells were seeded into each well of a 24-well cell culture plate (TPP, Switzerland) with 1 ml medium containing 10% FCS, $3x10^5$ cells were seeded into each well of a 6-well cell culture plate (TPP, Switzerland) with 4 ml medium containing 10% FCS, and $1x10^6$ cells were seeded in a 60 mm cell culture dish (TPP, Switzerland) with 5 ml medium containing 10% FCS. The plates or dishes were cultured overnight (37℃, 5% CO_2).

2.2.9 Cell Starving

After culturing overnight, the old medium was discarded. Then fresh medium containing 1% FCS was added to starve the cells.

2.2.10 Stimulations with TGF-βs

- rh-TGF-β1 and rh-TGF-β2 (Promokine, Germany)
  stock concentration=25 µg/ml

Medium containing 1% FCS with the desired concentration of TGF-β1 or TGF-β2, respectively, was prepared. Then the old medium was removed and the medium with TGF-β1 or TGF-β2 was added into the appropriate wells, respectively. PBS was added into the control well. After that, cells were cultured in the cell culture incubator (37℃, 5% CO_2).

2.3 Determination of Cell Numbers after Stimulation with TGF-βs

To analyze how TGF-β1 and TGF-β2 affect the cell numbers, the cell numbers were measured after the stimulation with TGF-β1 or TGF-β2. Three different concentrations of TGF-β1 or TGF-β2 (1 ng/ml, 5 ng/ml, 10 ng/ml) were used to test the dose-dependency. The procedure is described briefly in Figure 7. Three independent experiments were performed. Statistical analysis was done by using a statistic software (GraphPad InStat 3).

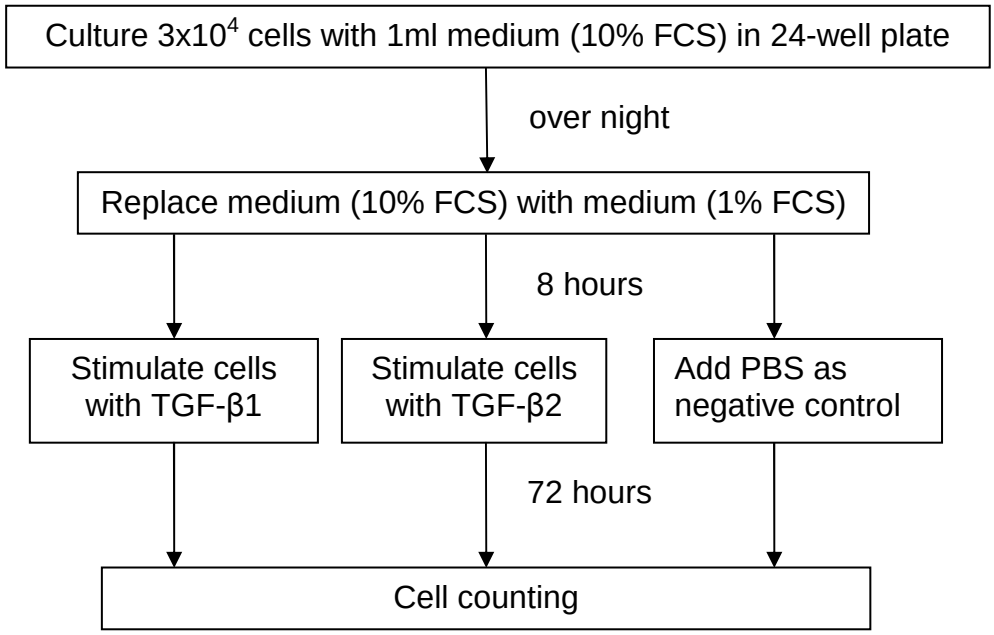

Figure 7. Determination of cell numbers after stimulation with TGF-βs

2.4 Analysis of MMP-2, MMP-9 and PAI-1 Secretion

To analyze how TGF-β1 and TGF-β2 influence MMP-2, MMP-9 and PAI-1 secretion, MMP-2, MMP-9 and PAI-1 ELISAs were performed. The procedure is described briefly in Figure 8. Three independent experiments were performed. Statistical analysis was done by using a statistic software (GraphPad InStat 3).

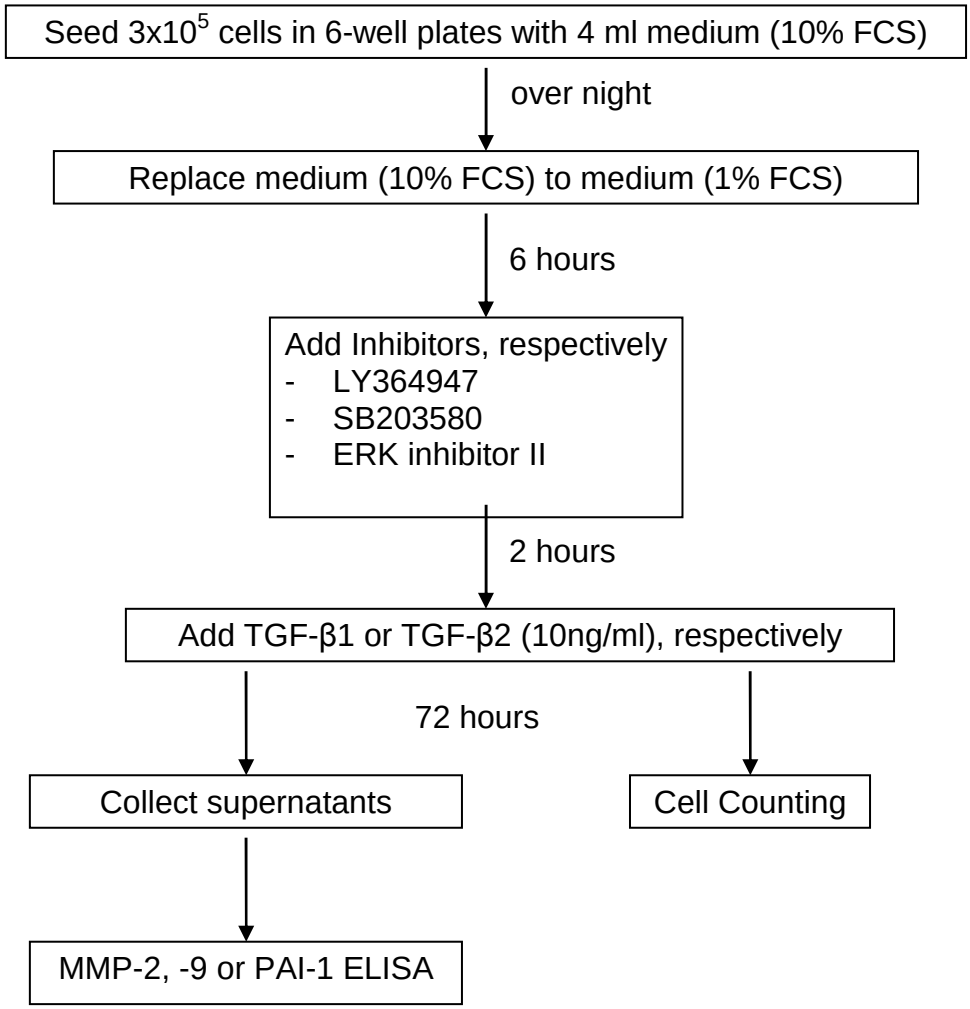

Figure 8. Scheme for analyzing the secretion of MMP-2 and PAI-1

2.4.1 Cell Culture with Intracellular Pathway-specific Inhibitors

To investigate the pathways that might be involved in TGF-β signaling, three inhibitors targeting different pathways were used.

- TGF-β receptor type I inhibitor: LY364947 (Sigma-Aldrich, USA) (Sawyer et al., 2003)
- p38/MAPK inhibitor: SB203580 (Cayman, USA) (Roux and Blenis, 2004)
- ERK inhibitor: ERK inhibitor II (Merck, Germany) (Ohori et al., 2005)

Media with 1% FCS containing 5 μM LY364947, 5 μM SB203580 or 20 μM ERK inhibitor II, respectively, were prepared. Media in 6-well culture plates were removed. Then 2ml medium with or without inhibitor was added into the corresponding wells, respectively. DMSO was added into wells without inhibitor as vehicle, because all inhibitors are dissolved in DMSO. Then cells were incubated for 2 hours (37℃, 5% CO_2). Then cells were stimulated with 10 ng/ml TGF-β1 or TGF-β2 as described in section 2.2.10.

2.4.2 Collection of supernatants and Cell Counting

Supernatant (1 ml) from each well was collected in a 1.5 ml microcentrifuge tube. Then 7μl Protease Inhibitor cocktail (Sigma-Aldrich, USA) was added into each tube in order to protect proteins against proteases. The supernatants were centrifuged (5000 x g, 10 min. at 4℃). Then 900 μl supernatant from each tube was collected and stored as three aliquots at -20℃.

After the collection of supernatants, cell numbers of each well were determined as described in section 2.2.7.

2.4.3 MMP-2 and MMP-9 ELISAs

MMP-2 or MMP-9 levels in the supernatant were measured by using MMP-2 or MMP-9 ELISA kits (R&D Systems) detecting total (active and inactive) MMPs, respectively. The kits contained:

- **Total MMP-2 or MMP-9 Microplate** – 96-well polystyrene microplate (12 strips of 8 wells) coated with a polyclonal antibody against human MMP-2 or MMP-9
- **Total MMP-2 or MMP-9 Conjugate** – polyclonal antibody against human MMP-2 or MMP-9 conjugated to horseradish peroxidase, with preservatives

- **Total MMP-2 or MMP-9 Standard** – recombinant human pro-MMP-2 or pro-MMP-9 in a buffered protein with preservatives, lyophilized
- **Assay Diluent RD1-74** – a buffer with preservatives
- **Calibrator Diluent RD5-32** – a buffer with preservatives
- **Wash Buffer Concentrate** – a 25-fold concentrated solution of buffered surfactant with preservatives
- **Color Reagent A** – stabilized hydrogen peroxide
- **Color Reagent B** – stabilized chromogen
- **Stop Solution** – 2M sulfuric acid

Reagent Preparation

All reagents were brought to room temperature before use.

- **Wash Buffer** – 20 ml of Wash Buffer Concentrate is diluted with 480 ml deionized water.
- **Substrate Solution** – Color Reagent A and B are mixed together in equal volumes and protected from light within 15 minutes of use.
- **Standard** – The MMP-2 or MMP-9 standard was reconstituted with 1ml deionized water to get a stock solution of 100 ng/ml. The standard was kept at room temperature for at least 15 minutes with gentle agitation. Meanwhile, 7 EP tubes with 200 µl Calibrator Diluent RD5-32 were prepared. Then 200 µl standard stock solution was used to produce dilution series (50 ng/ml, 25 ng/ml, 12.5 ng/ml, 6.25 ng/ml, 3.12 ng/ml, 1.56 ng/ml and 0.78 ng/ml). The Calibrator Diluent RD5-32 served as standard of 0 ng/ml.
- **Supernatant Sample** – Supernatants stored at -20℃ were thawed at room temperature. Supernatants collected from THESC and 22B were 5-fold diluted with Calibrator Diluent RD5-32 for MMP-2 measurement because the concentration of MMP-2 in these supernatants was outside the detectable range. The samples from the other cell lines were not diluted. All samples were mixed thoroughly before use.

Assay Procedure

All reagents and samples were brought to room temperature before use. All samples, standards and controls were assayed in duplicate. The procedure of the assay is described in Figure 9.

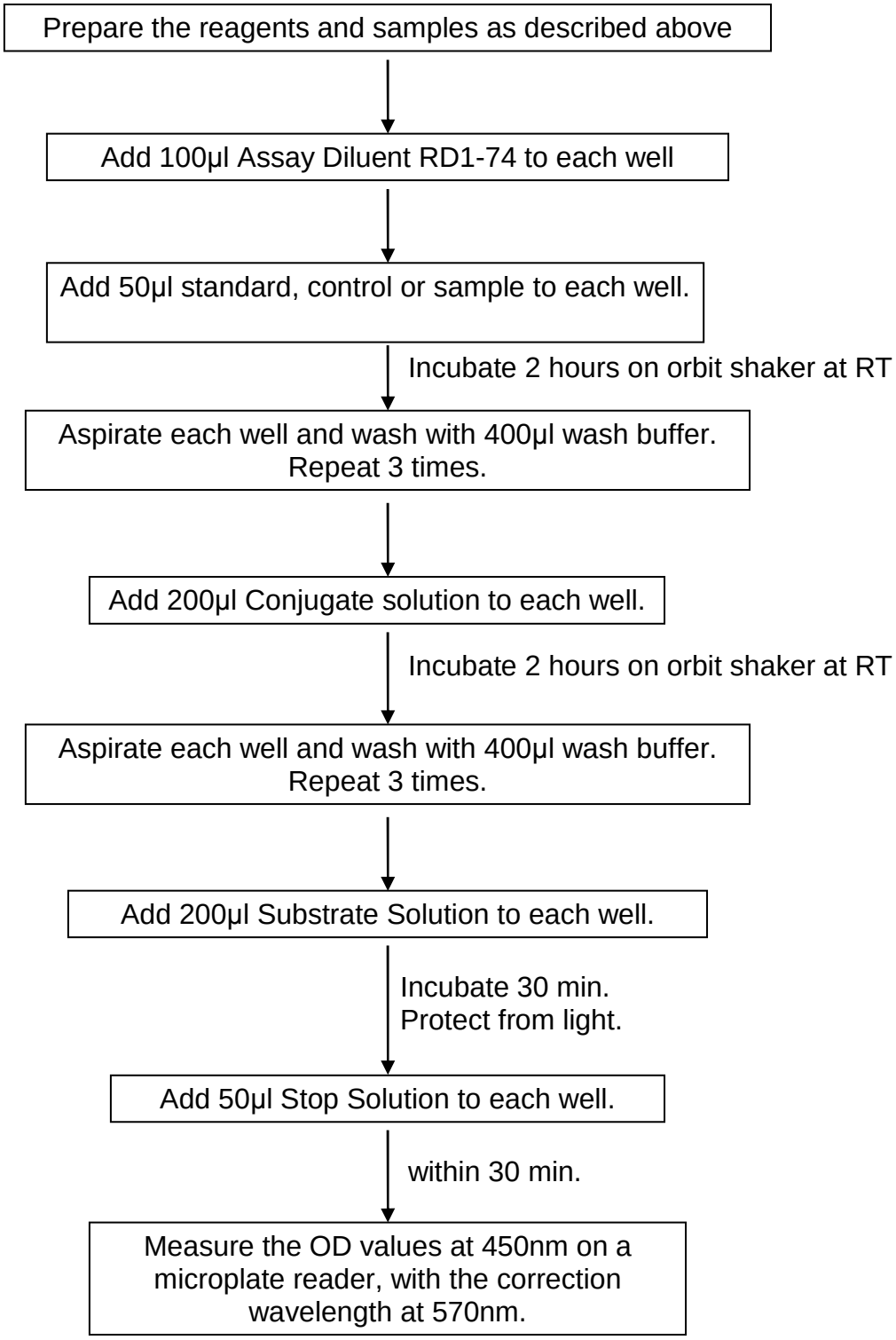

Figure 9. Scheme of MMP-2 or MMP-9 ELISAs

2.4.4 PAI-1 ELISAs

The PAI-1 level in the supernatants was measured by using a TECHNOZYM® PAI-1 Antigen ELISA Reagent Kit (Technoclone, Germany).

The kit contained:

- **Coating Antibody** – 500 µg lyophilized monoclonal anti-PAI-1
- **Conjugate monoclonal anti-PAI-1**
- **Calibrator**

Reagents Preparation

All reagents were brought to room temperature before use.

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

Test Preparation

- **Calibrator Reconstitution** – The calibrator was reconstituted with 500µl distilled water to get a stock solution of 132.7 ng/ml. Then it was kept at room temperature for at least 15 minutes with gentle agitation. Meanwhile, 4 microcentrifuge tubes with 100 µl incubation buffer were prepared. Then 100µl stock solution was used to make a series of dilution (66.35 ng/ml, 33.18 ng/ml, 16.59 ng/ml and 8.29 ng/ml). The incubation buffer served as a standard of 0 ng/ml.
- **Supernatant Sample** – Supernatants stored at -20℃ were thawed at room temperature. Supernatants collected from HES, 12Z, THESC and 22B were diluted 10-fold, 20-fold, 25-fold and 50-fold with incubation buffer, respectively.
- **Coating Plate** – The coating antibody was reconstituted with 500 µl distilled water. Then 100 µl of coating antibody was mixed thoroughly with 10 ml coating buffer. This

solution (100 µl) was pipetted into each well of a 96-well ELISA plate. The plate was incubated over night (at least for 16 hours) at 4℃ .

- **Diluting Conjugate Solution** – The conjugate solution was stored as a 50x concentrated solution. The conjugate solution was diluted 1:50 with incubation buffer prior to use.

Assay Procedure

All reagents and samples were brought to room temperature before use. All samples, standards and controls were assayed in duplicate. The procedure of the assay is described in Figure 10.

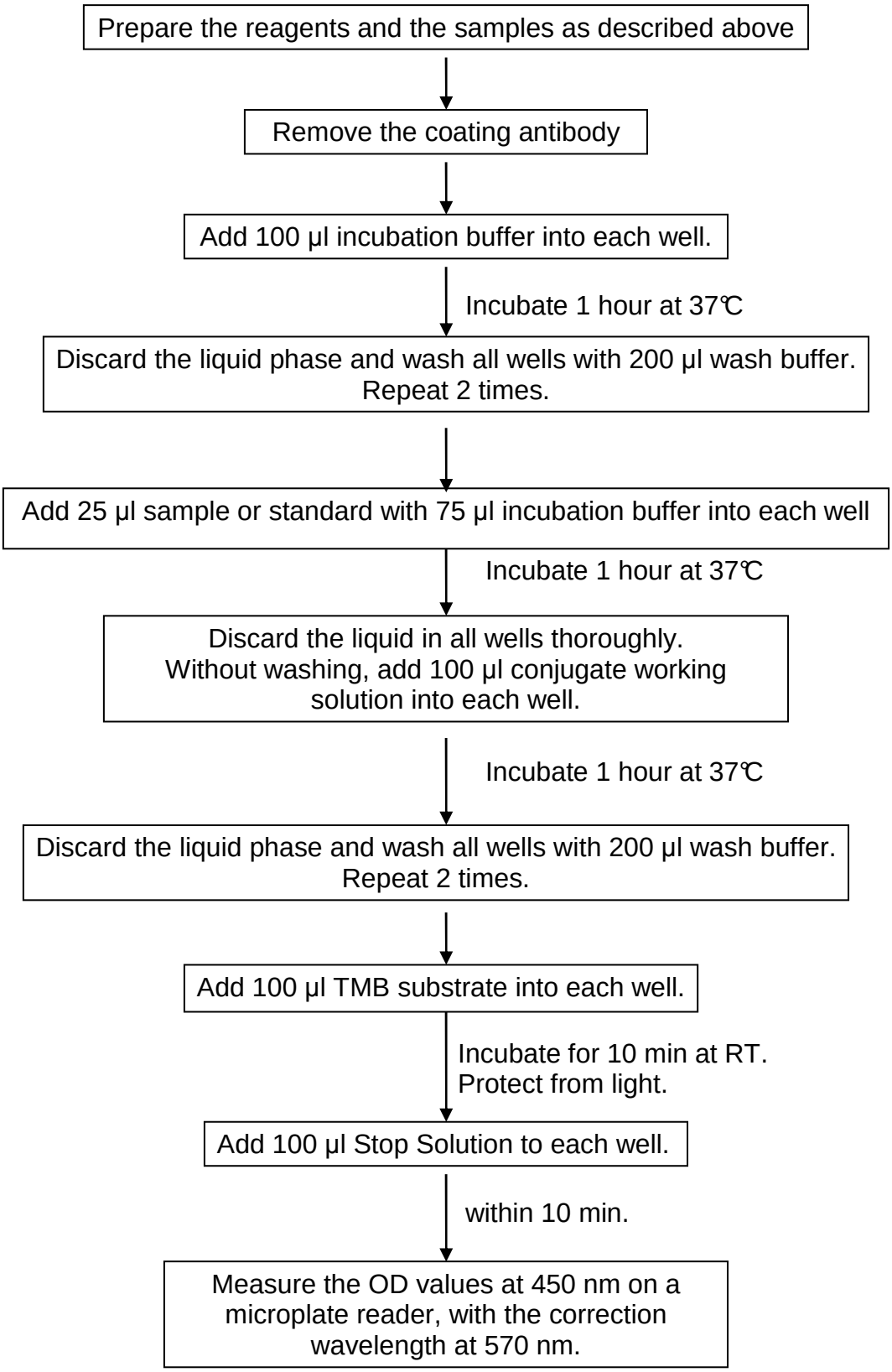

Figure 10. Scheme of PAI-1 ELISAs

2.5 Cell Adhesion Assay

To analyse the effect of PAI-1 on cell adhesion, a cell adhesion assay was performed. The procedure of this assay is described briefly in Figure 11. Three independent experiments were performed.

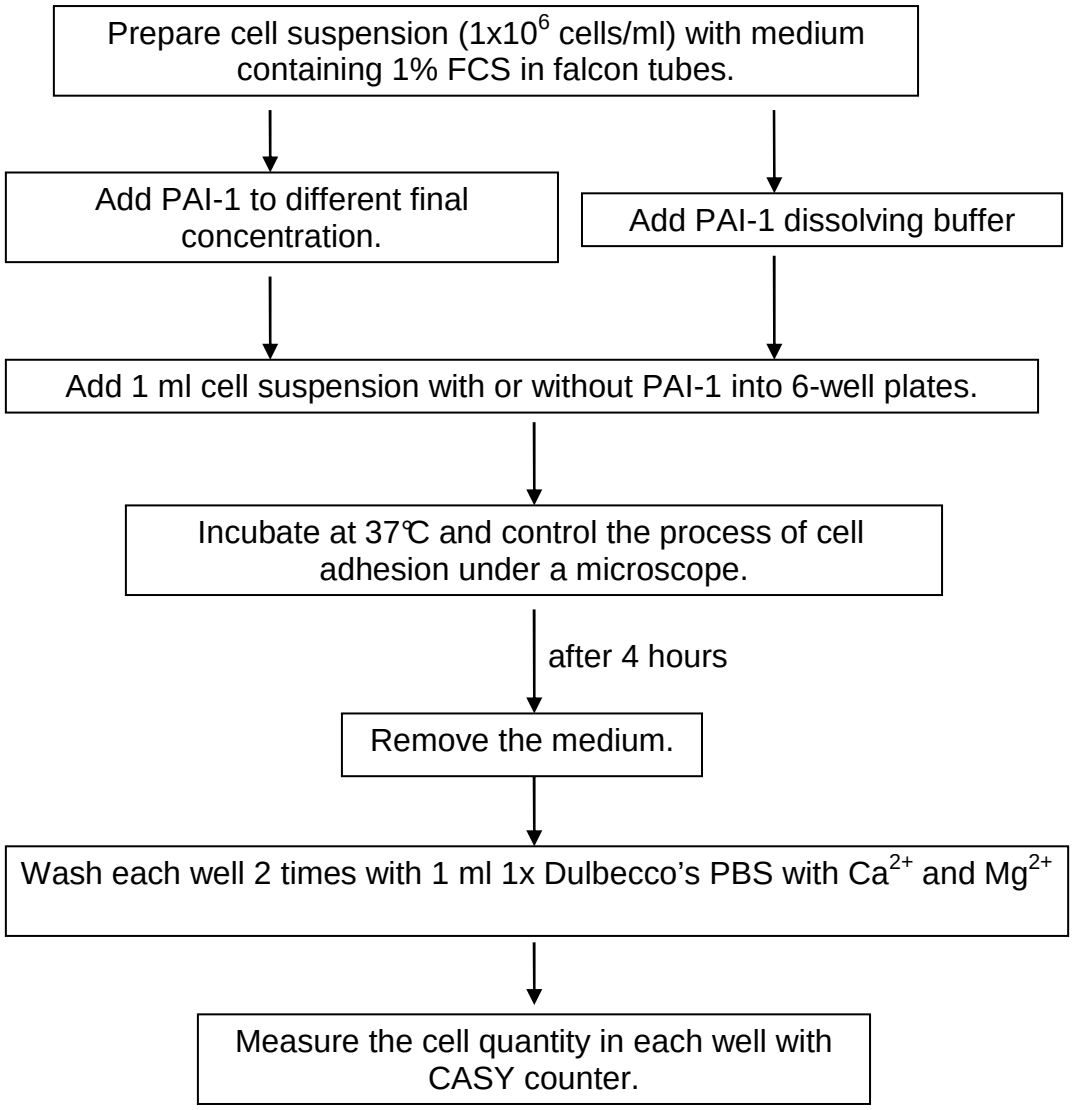

Figure 11. Procedure of the cell adhesion assay after treating cells withPAI-1

2.5.1 Cell Stimulation with PAI-1

- recombinant active mutant human PAI-1 (American diagnostica, U.S.A.)
  stock concentration=40 µM

The cell pellets of all four cell types studied were obtained by following the procedure which was described in section 2.2.4. Cell pellets were re-suspended with medium containing 1% FCS. Then the cell numbers were measured and several tubes of cell suspensions containing $1X10^6$ cells/ml were prepared. Then active rh-PAI-1 was added into the corresponding cell suspension at final concentrations of 40 nM, 20 nM, 10 nM or 5 nM, respectively. Buffer (50 mM NaH_2PO_4, 150 mM NaCl, 1 mM EDTA, pH=6.6) used to dissolve PAI-1 was used as a negative control. Then 1 ml cell suspension with or without PAI-1 was transferred into 6-well cell culture plates, respectively. The cell culture plates were cultured (37℃, 5% CO_2) for 4 hours.

2.5.2 Determining the Number of Adherent Cells

The process of cell adhesion was controlled under a microscope every hour. After 4 hours, nearly all cells in the control wells were attached on the culture plates. Then the medium was removed. Dulbecco's PBS with Ca^{2+} and Mg^{2+} (1x, PAA, Austria) was used to wash 2 times in order to remove the residual medium and detached cells. Then 500 µl accutase was added into each well and the cell culture plates were incubated at 37℃ until all cells were detached. The detachment of cells was controlled microscopically. Then 500 µl fresh and warm medium was added into each well to neutralize the accutase. Thereafter, 10 µl cell suspension was transferred to a CASY tube with 10 ml CASY ton solution and mixed thoroughly. Then the cell numbers were measured with the CASY-counter.

2.6 Analysis of the Phospho-EGF Receptor Level after TGF-β Stimulation

To investigate whether the EGF receptor is phosphorylated by TGF-βs in endometrial and endometriotic cells, PathScan® ELISAs were performed. The procedure is described briefly in Figure 12.

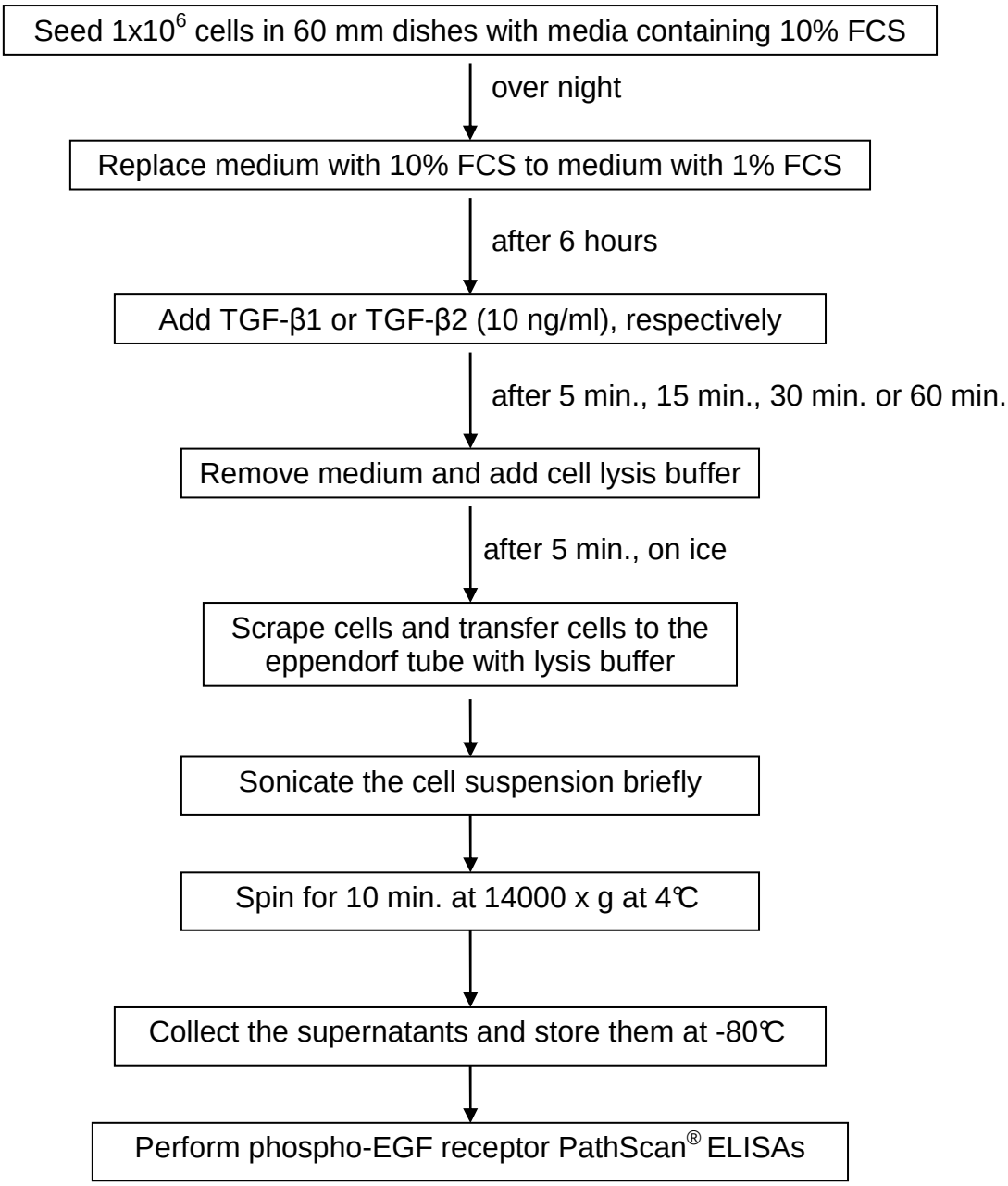

Figure 12. Scheme for analyzing EGF receptor phosphorylation.

2.6.1 Cell Homogenate

• **1x Cell Lysis Buffer (Cell Signaling Technology®)** : 20 mM Tris (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na_3VO_4 and 1 µg/ml leupeptin.

After stimulation with TGF-βs, medium was removed from the cell culture dishes and all procedures afterwards were performed on ice. Ice cold PBS was added to remove the residual medium. The cell lysis buffer was kept on ice and mixed with 1 mM phenylmethylsulfonyl fluoride (PMSF) prior to use. Cell lysis buffer (300 µl) was added into each dish. After incubating on ice for 5 minutes, cells were scraped off and transferred into a 2 ml eppendorf tube with cell lysis buffer. Then the cell lysates were sonicated for 1 minute (pulsed, 40% of max power, 40% duty cycle) by using Sonifier® cell disruptor B15 (Branson Ultralsonics Corp., USA). After that, the cell lysates were centrifuged (14000 x g, 10 minutes at 4℃). Then the supernatant from each tube was transferred into a new eppendorf tube and stored at -80℃.

2.6.2 PathScan® Phospho-EGF Receptor ELISAs

PathScan® ELISA kits targeting three different phosphorylation sites of the EGF receptor (Tyr845, Tyr1068, Tyr1173) were used to test the level of phospho-EGF receptor in the cell lysates (Hackel et al., 1999; Zwick et al., 1999).

The kits contained:

- **EGF Receptor mAb coated microwells (96 tests)**
- **P-EGF Receptor (Tyr845, Tyr1068, Tyr1173) Detection Antibodies**
- **Anti-rabbit IgG, HRP-linked Antibody**
- **TMB Substrate**
- **Stop Solution**
- **Sample Diluent Buffer**
- **ELISA Wash Buffer (20X):** This buffer was diluted to 1X buffer with purified water prior to use.
- **Cell Lysis Buffer (10X)**

Assay Procedure

All reagents and samples were brought to room temperature before use. The 1X cell lysis buffer was used as the blank sample. All samples were assayed in duplicate. The procedure of the assay is described in Figure 13.

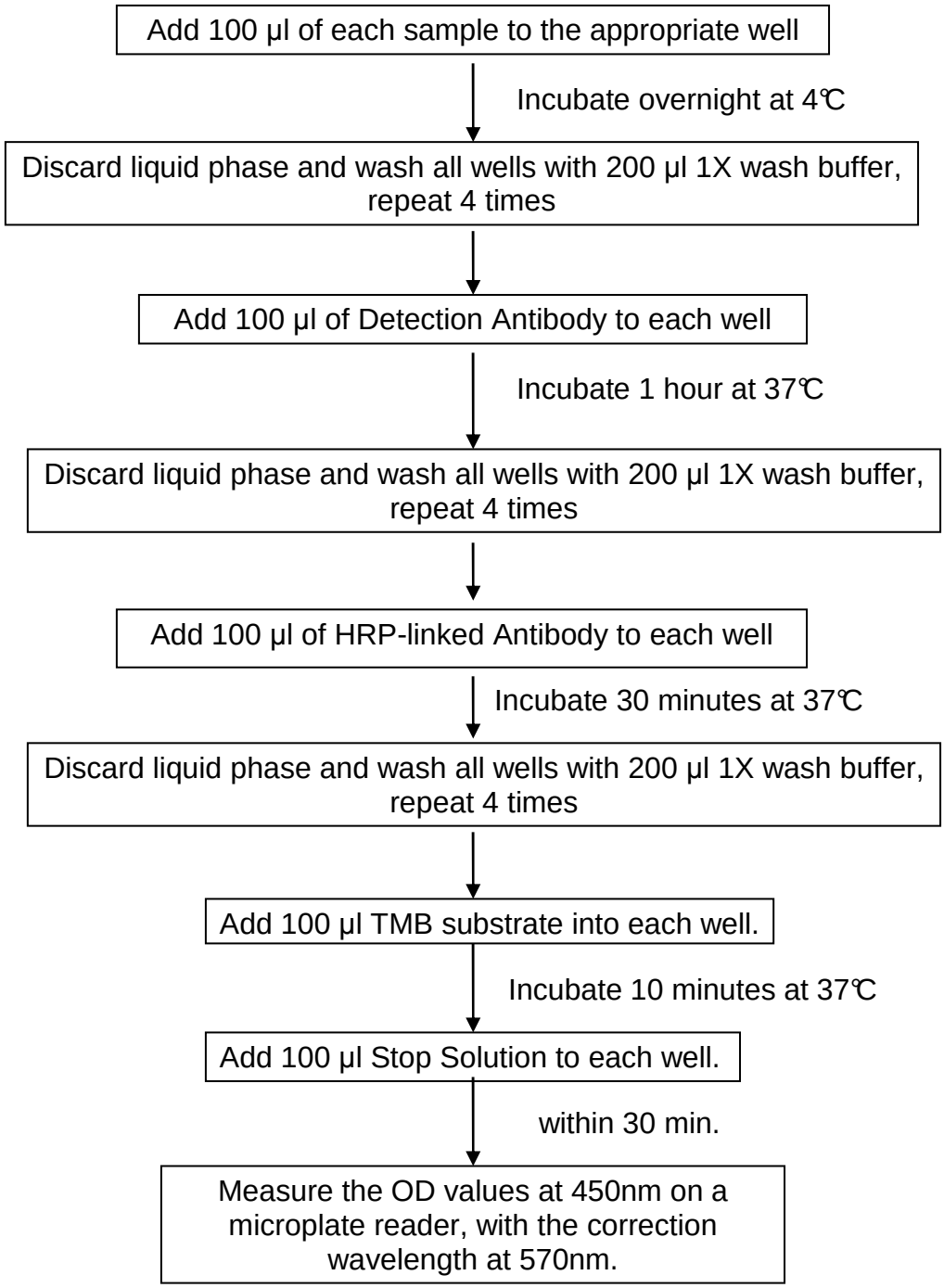

Figure 13. Scheme of PathScan® Phospho-EGF Receptor ELISAs

2.7 Analysis of the Localization of Phospho-ERK1/2 after Stimulation with TGF-βs in Endometrial and Endometriotic Cells

To investigate whether TGF-βs stimulate phosphorylation of ERK1/2, the intracellular localization of phospho-ERK1/2 in endometrial and endometriotic cells was evaluated. The procedure is described briefly in Figure 14.

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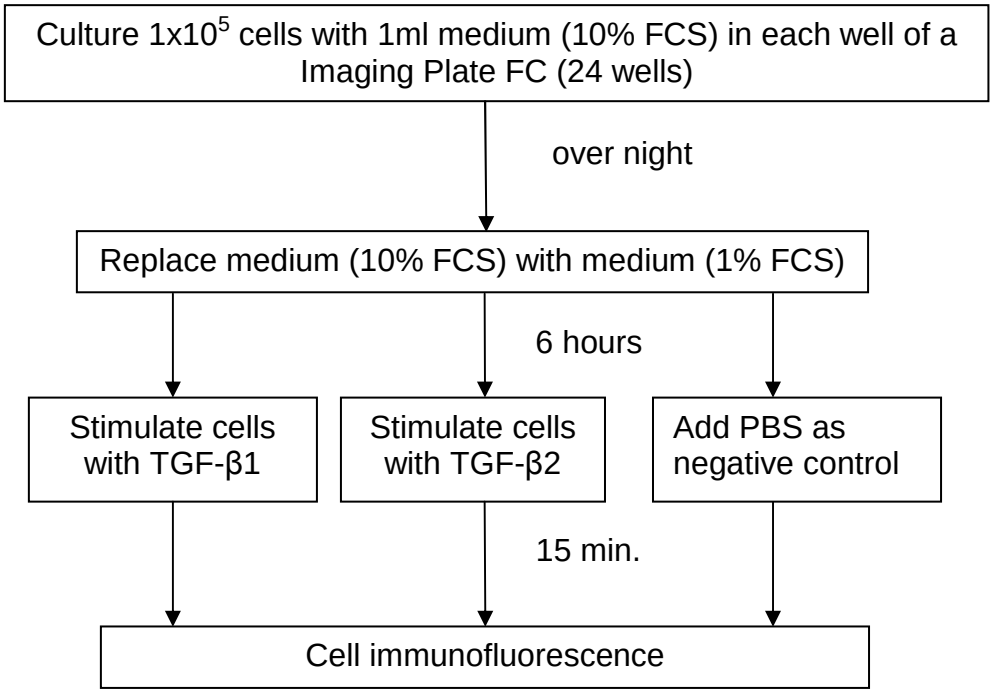


Figure 14. Procedure of phospho-ERK1/2 assay with stimulation with TGF-βs

2.7.1 Pre-treatment with TGF-βs

Cells were counted as described in 2.2.7. Then $1x10^5$ cells were seeded into each well of a 24-well Imaging Plate FC (Zell-Kontact, Germany), which is designed specially for immunofluorescence, with 1 ml medium containing 10% FCS. The cells were cultured (37℃, 5% CO_2) overnight. Cells were then starved as described in 2.2.9 for 6 hours. After that, cells were stimulated with 10 ng/ml TGF-β1 or TGF-β2 as described in 2.2.10, for 15 minutes.

2.7.2 Cell Immunofluorescence

After stimulating with TGF-βs for 15 minutes, the medium was removed. Then a cell immunofluorescence assay was performed. The procedure is described in Figure 15. The image was evakyated under a confocal microscope (Olympus, Germany).

Reagents preparation:

- **Fixation buffer** – ice cold acetone and methanol mixture (1:1)
- **Phosphate buffered saline (PBS)** – 8.0 g NaCl, 0.2 g KCl, 0.2 g KH_2PO_4, 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 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
- **Blocking buffer** – 3% bovine serum albumin (BSA) in TBST
- **Diluting buffer** – 1% bovine serum albumin (BSA) in TBST
- **First antibody** – phospho-ERK1/2 antibody (Cell Signaling Technology®), rabbit source, 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-nuclei staining dye (Invitrogen), 1:1000 diluted in PBS

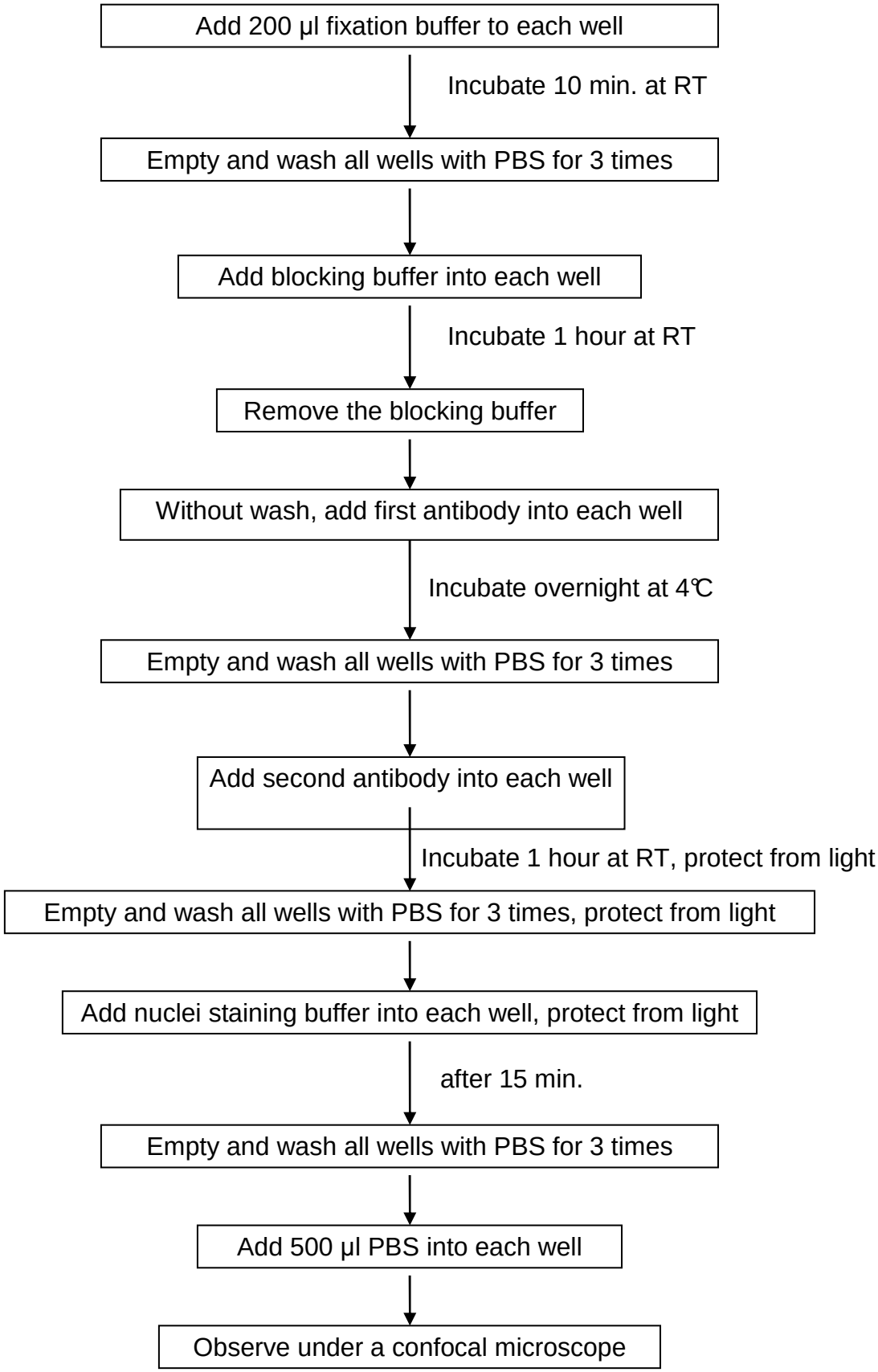


Figure 15. Scheme of cell immunofluorescence.

2.8 Statistical Methods

To analyse the data from the results, statistical analysis was performed. All experiments were done at least three times in duplicate, and the data are shown as means±SEM. To analyse the significance of the data, we used the statistic software like InStat Graphpad® (www.graphpad.com) and Microsoft Excel and performed the test of Kruskal-Wallis, a non-parametric test.

3 Results

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

Endometrial and endometriotic cells were treated with TGF-β1 or TGF-β2 (10ng/ml), respectively, in order to find out how TGF-βs affect cell numbers and to compare endometrial and endometriotic cells. Furthermore, cell numbers were needed to standardize the cytokine secretion afterwards.

The results showed that either TGF-β1 or TGF-β2 reduced the proliferation of both endometrial (Fig. 16) and endometriotic cells (Fig. 17) within 72 hours. Although the reduction in cell numbers was modest, it was significant.

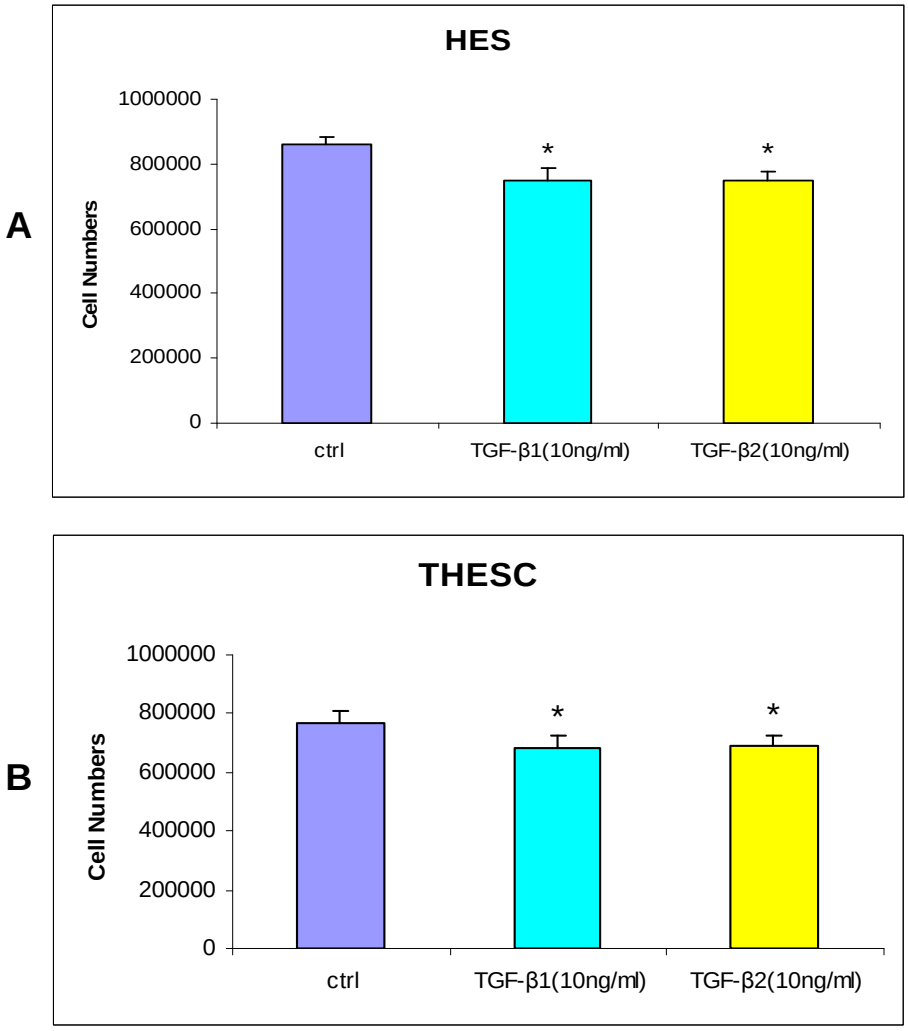


Figure 16. Quantification of endometrial cells with/without stimulation by TGF-βs. The quantity of endometrial epithelial cells **(A)** was reduced by approximately 13%, while the quantity of endometrial stromal cells **(B)** was reduced by approximately

10%, after being treated for 72 hours with TGF-β1 or TGF-β2 (10 ng/ml), respectively (P<0.05, n=9).

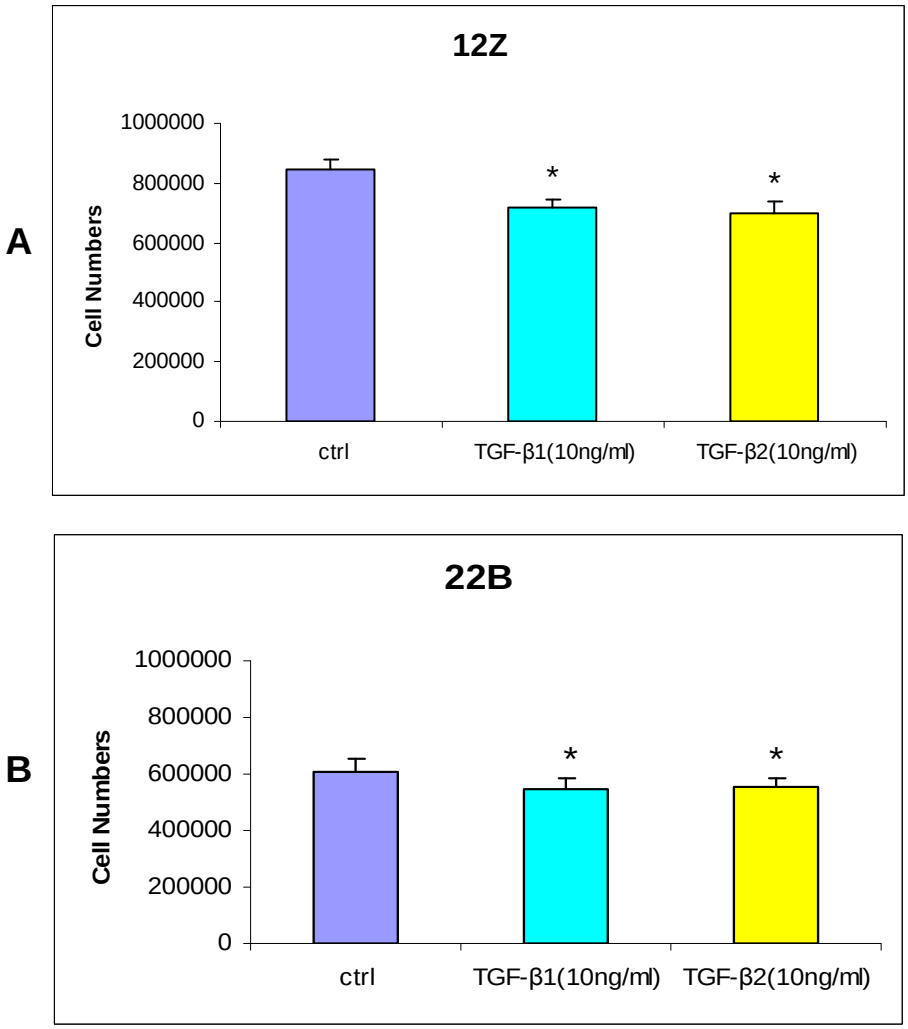


Figure 17. Quantification of endometriotic cells with/without stimulation by TGF-βs. The quantity of endometriotic epithelial cells **(A)** was reduced by approximately 15%, while the quantity of endometriotic stromal cells **(B)** was reduced by approximately 11% after being treated for 72 hours with TGF-β1 or TGF-β2 (10 ng/ml), respectively (P<0.05, n=9).

In summary, either TGF-β1 or TGF-β2 reduced the cell numbers of four different cell types in a range from 10% to 15%. However, both TGF-β1 and TGF-β2 did not show different effects on cell numbers in endometrial cells and endometriotic cells, neither in epithelial cells nor in stromal cells.

In the experiments described above, $3x10^5$ cells/well have been seeded initially. Interestingly, when the initial cell quantity was lower ($3x10^4$ cells/well), TGF-βs demonstrated a different effect. TGF-βs increased the cell numbers of endometriotic epithelial cells (12Z), in a dose-dependent manner (Fig. 18). Similar results were observed in endometrial epithelial cells (HES), endometrial stromal cells (THESC) and endometriotic stromal cells (22B) (data not shown).

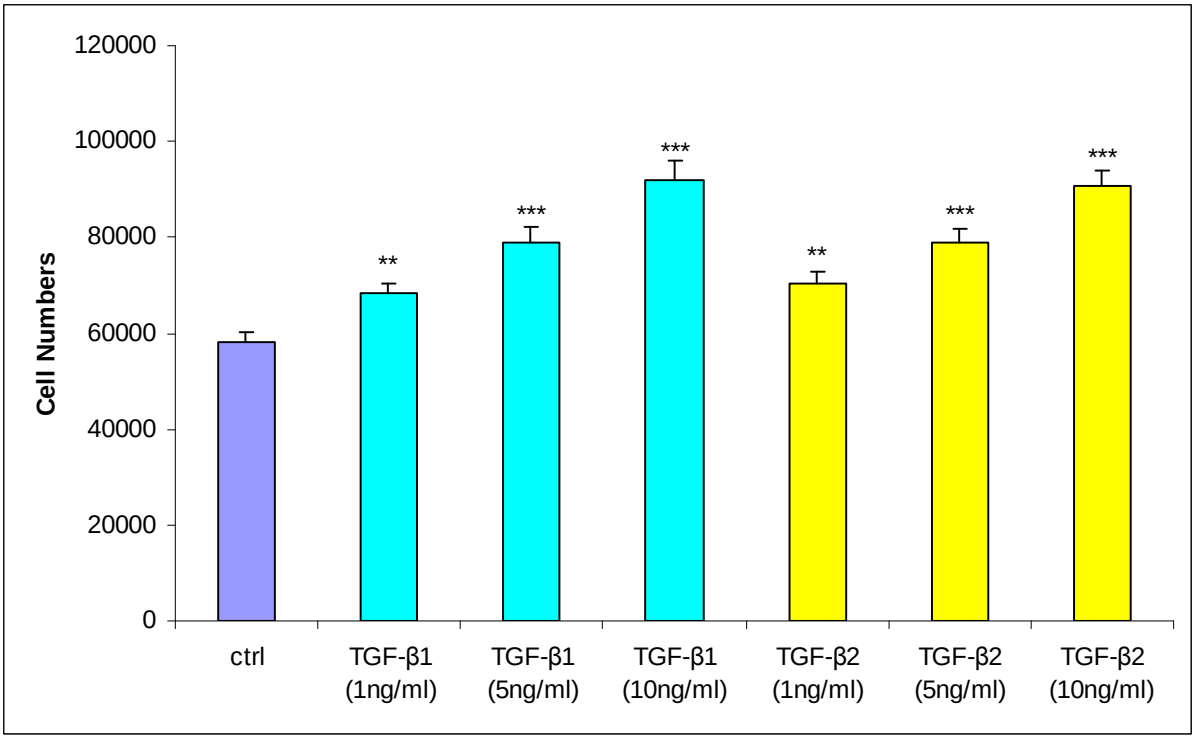


Figure 18. Quantification of cell numbers with different concentrations of TGF-βs or without TGF-βs. Endometriotic epithelial cells (12Z) were treated for 72 hours with TGF-β1 or TGF-β2 (1 ng/ml, 5 ng/ml, 10 ng/ml), respectively. Cell numbers were increased either by TGF-β1 or TGF-β2 dose-dependently (**=P<0.01, ***=P<0.001, n=9).

3.2 Influence of TGF-β1 or TGF-β2 on MMP-2 Secretion

MMPs are important in tissue breakdown and cell shedding during menstruation and may be involved in the invasiveness of ectopic endometrial tissue (Juhasz-Böss et al., 2009). TGF-β1 suppresses the production of MMP-3 and MMP-7 in endometrium (Bruner et al., 1995) but increases the expression of MMP-2 and MMP-9 (Hirata et al., 2003). MMP-2 expression is also detected at higher level in the endometrium of patients with endometriosis compared to endometrium of women without

endometriosis (Chung et al., 2002). Thus, it is important to study the secretion of gelatinases and whether TGF-βs affect the production in endometrial or endometriotic cells.

3.2.1 Basal Secretion of MMP-2 and MMP-9 in Endometrial and Endometriotic Cells

To compare the basal MMP-2 secretion by endometrial and endometriotic cells, ELISAs have been performed to quantify the MMP-2 levels of each cell type after cell culture for 72 hours. The concentration of MMP-2 was standardized as ng/ml per $1x10^7$ cells.

Results showed that endometrial epithelial cells (HES) secreted only very low MMP-2 levels (8 ng/ml), while endometriotic epithelial cells (12Z) secreted 116 ng/ml MMP-2. Endometrial stromal cells (THESC) secreted 1403 ng/ml MMP-2 which was much higher compared to the epithelial cells. Endometriotic stromal cells (22B), which secreted 1726 ng/ml MMP-2, were the most productive of all cell lines studied (Fig. 19).

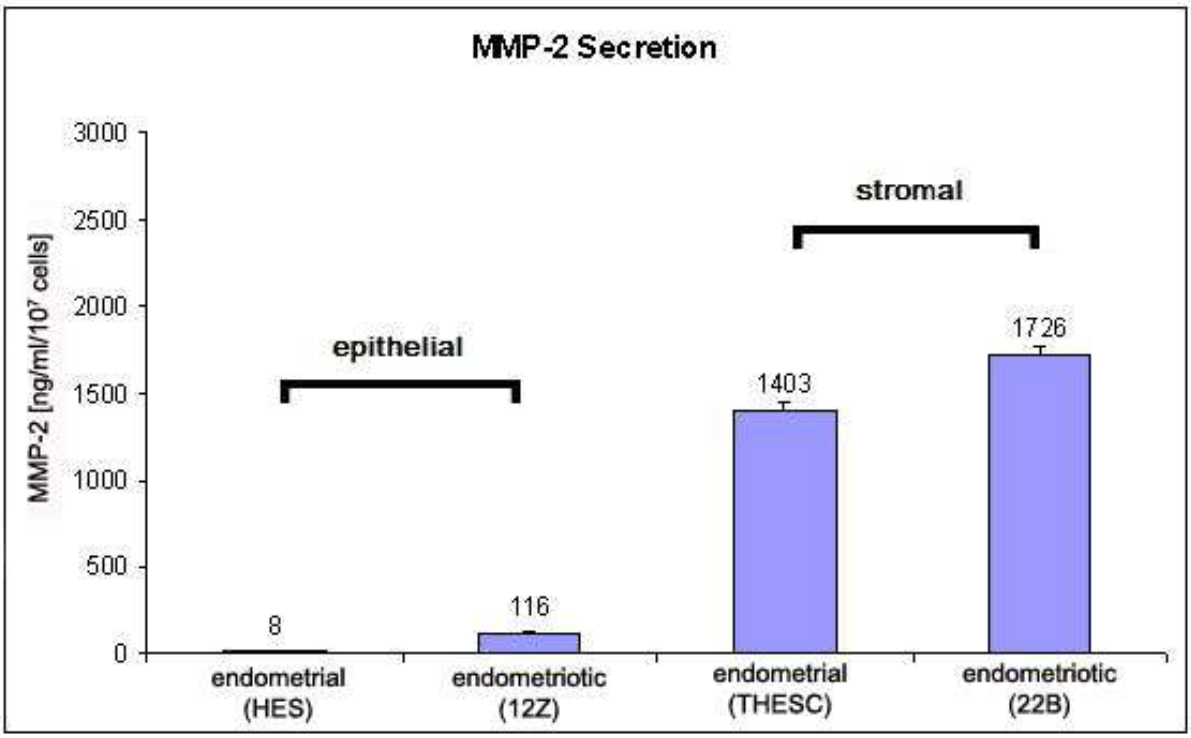


Figure 19. Basal secretion of MMP-2 by endometrial and endometriotic cells. Cells were cultured for 72 hours without any treatment. Endometriotic epithelial cells (12Z) secreted approximately 15-fold more MMP-2 than endometrial epithelial cells (HES). Endometriotic stromal cells secreted more MMP-2 compared to endometrial stromal

cells (THESC). In general, the stromal cells (THESC and 22B) secreted much more MMP-2 compared to the epithelial cells (HES and 12Z).

The MMP-9 secretion was also measured with ELISAs. However, MMP-9 was not detected in endometrial cells and also rarely detectable in endometriotic cells after being cultured for 72 hours (Fig. 20). Endometriotic epithelial cells (12Z) secreted 5.7 ng/ml MMP-9 while endometriotic stromal cells (22B) secreted 34.8 ng/ml. However, both endometrial epithelial cells (HES) and stromal cells (THESC) secreted no MMP-9.

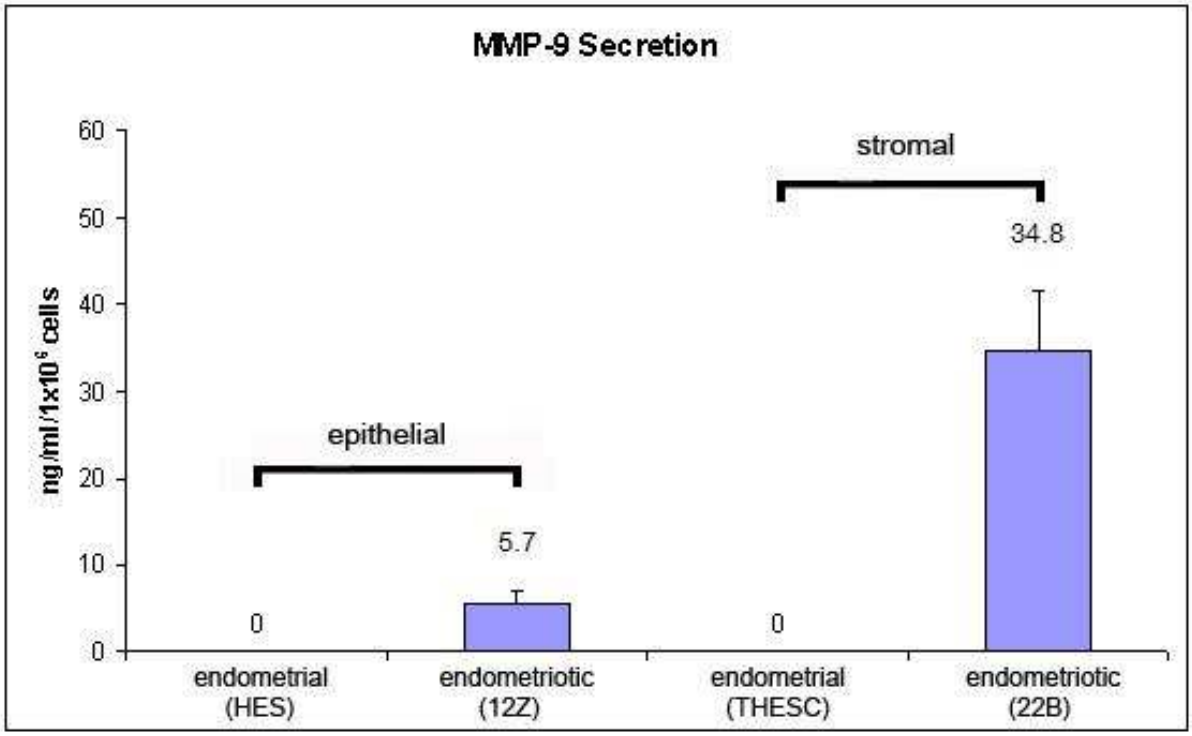


Figure 20. Basal secretion of MMP-9 by endometrial and endometriotic cells. Cells were cultured for 72 hours without any treatment. Endometrial epithelial cells (HES) and endometrial stromal cells (THESC) secreted no MMP-9, while endometriotic stromal cells (22B) secreted 6-fold more MMP-9 compared to endometriotic epithelial cells (12Z), but secretion was quite low.

In summary, endometriotic cells generally secreted more MMP-2 compared to normal endometrial cells. Stromal cells still contributed the most to MMP-2 secretion in normal endometrial cells as well as in endometriotic cells. However, MMP-9 was much less secreted compared to MMP-2. MMP-9 was even not detectable in endometrial cells.

3.2.2 Analysis of Smad-dependent and Smad-independent TGF-β-induced MMP-2 Secretion

To investigate whether TGF-βs regulate MMP-2 secretion, endometrial and endometriotic cells were treated for 72 hours with 10 ng/ml TGF-β1 or TGF-β2, respectively. In the inhibition group, three different inhibitors which target Smad-dependent or non-Smad-dependent pathways were added 2 hours before adding the TGF-βs. In all cell types studied, MMP-2 secretion was increased by TGF-β1 or TGF-β2, approximately 5 to 6-fold in epithelial cells and 2-fold in stromal cells, compared to the control group ($P<0.01$, $n=24$) (Figs. 21–26).

3.2.2.1 Analysis of the Smad Pathway in MMP-2 Secretion

The inhibitor LY364947 was used to investigate the role of the Smad-dependent pathway in MMP-2 secretion. The inhibitor blocks the phosphorylation of Smad2/3 by selectively inhibiting TβR1. LY364947 (5 μM) was added 2 hours before adding the TGF-βs (10 ng/ml). Then cells were cultured for 72 hours. The MMP-2 level in the supernatants was quantified with ELISAs for MMP-2.

The results showed that TGF-β1 and TGF-β2 both increased MMP-2 secretion, and this elevation was completely blocked by LY364947 in all cell lines. The inhibitor also moderately reduced the basal MMP-2 secretion because the FCS in the medium contains a small amount of TGF-β1. All results are statistically significant (Figs. 21, 22).

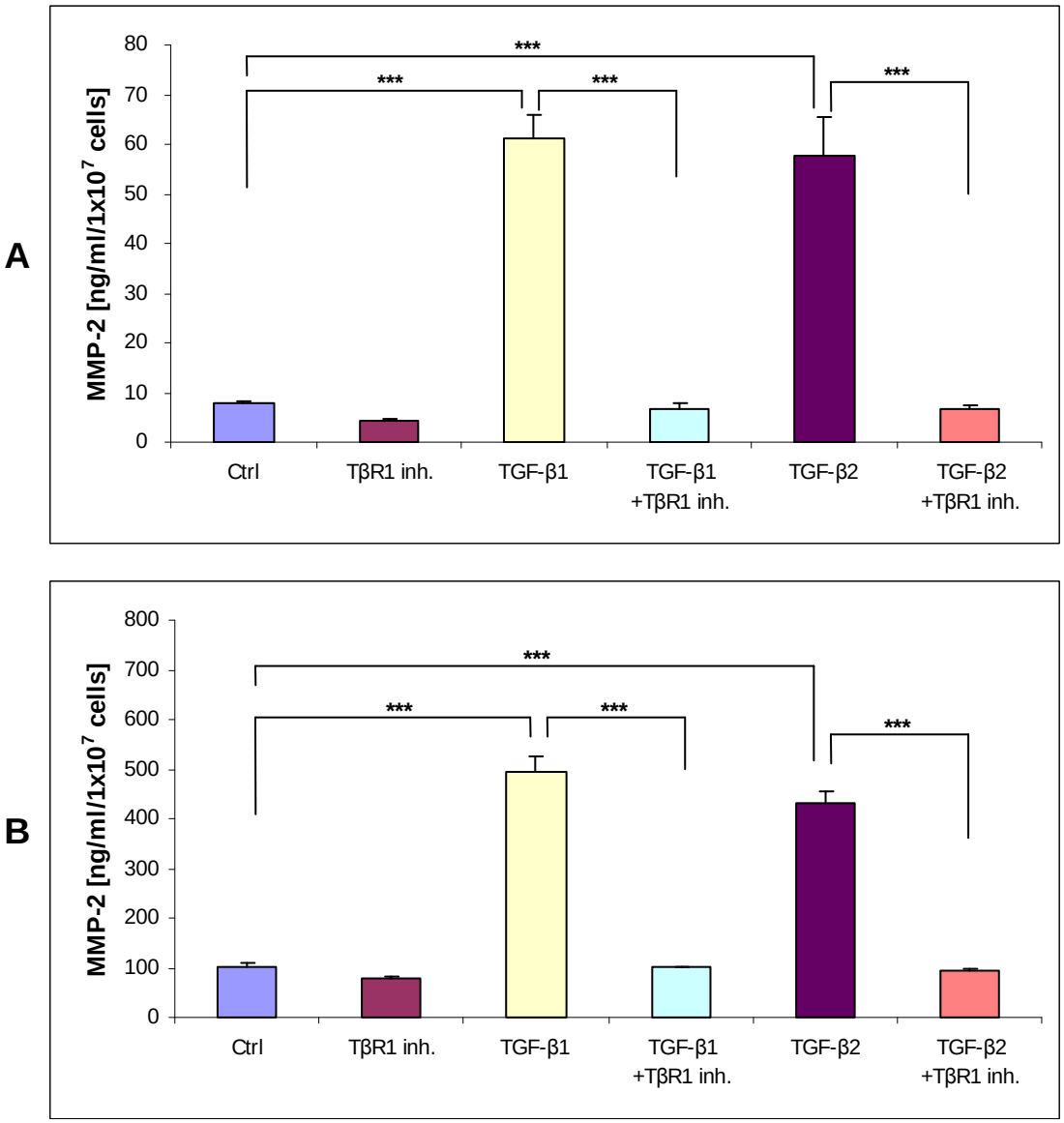


Figure 21. The TβR1 inhibitor (LY364947) completely inhibited the TGF-β1 or TGF-β2 induced MMP-2 secretion in endometrial and endometriotic epithelial cells.

(A) The level of MMP-2 in endometrial epithelial cells (HES) was significantly increased 6-fold after treatment for 72 hours with TGF-β1 or TGF-β2 (10 ng/ml), respectively ($P<0.001$, $n=8$). **(B)** The level of MMP-2 in endometriotic epithelial cells (12Z) was significantly increased 5-fold after treatment for 72 hours with TGF-β1 or TGF-β2 (10 ng/ml), respectively ($P<0.001$, $n=8$). LY364947 reduced the TGF-β1 or TGF-β2 induced secretion of MMP-2 to the control level in both endometrial and endometriotic epithelial cells.

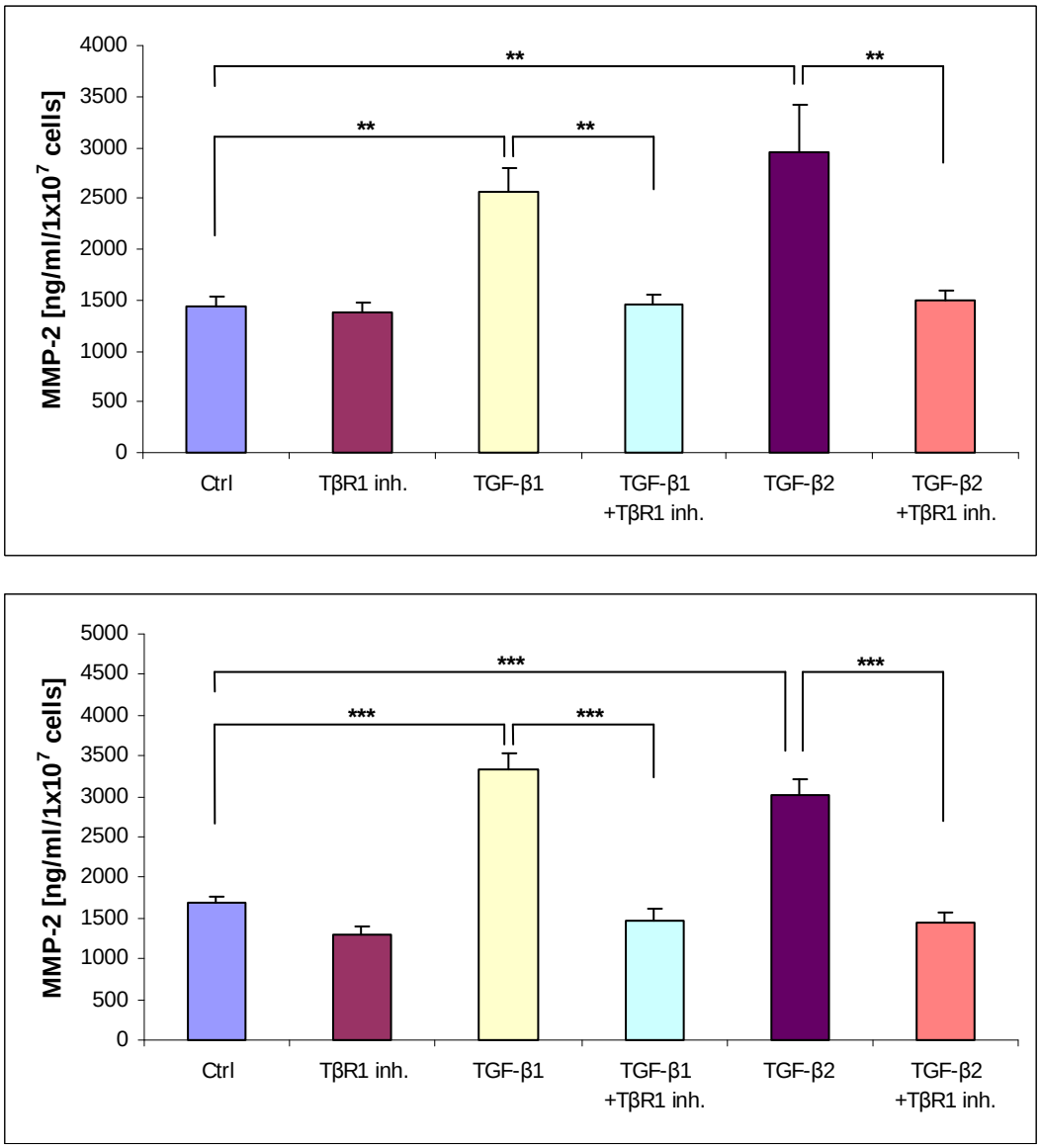


Figure 22. The TβR1 inhibitor (LY364947) completely inhibited the TGF-β1 or TGF-β2 induced MMP-2 secretion in endometrial and endometriotic stromal cells.

(A) The level of MMP-2 in endometrial stromal cells (THESC) was significantly increased 2-fold after treatment for 72 hours with TGF-β1 or TGF-β2 (10 ng/ml), respectively ($P<0.01$, n=8). **(B)** The level of MMP-2 in endometriotic stromal cells (22B) was significantly increased 2-fold after treatment for 72 hours with TGF-β1 or TGF-β2 (10 ng/ml), respectively ($P<0.001$, n=8). LY364947 reduced the TGF-β1 or TGF-β2 induced secretion of MMP-2 to control level in both endometrial and endometriotic stromal cells.

3.2.2.2 Analysis of the ERK Pathway in MMP-2 Secretion

A highly selective ERK1/2 inhibitor (ERK inhibitor II) was used to study the role of the ERK pathway in MMP-2 secretion. The ERK inhibitor II (20 μM) was added before adding the TGF-βs (10 ng/ml). Then cells were cultured for 72 hours. The MMP-2 level in the supernatants was quantified with ELISAs for MMP-2.

The results showed that TGF-β1 and TGF-β2 both increased MMP-2 secretion. The ERK inhibitor II inhibited the TGF-βs-induced MMP-2 secretion by approximately 15-25% in all cell lines studied (Figs. 23, 24).

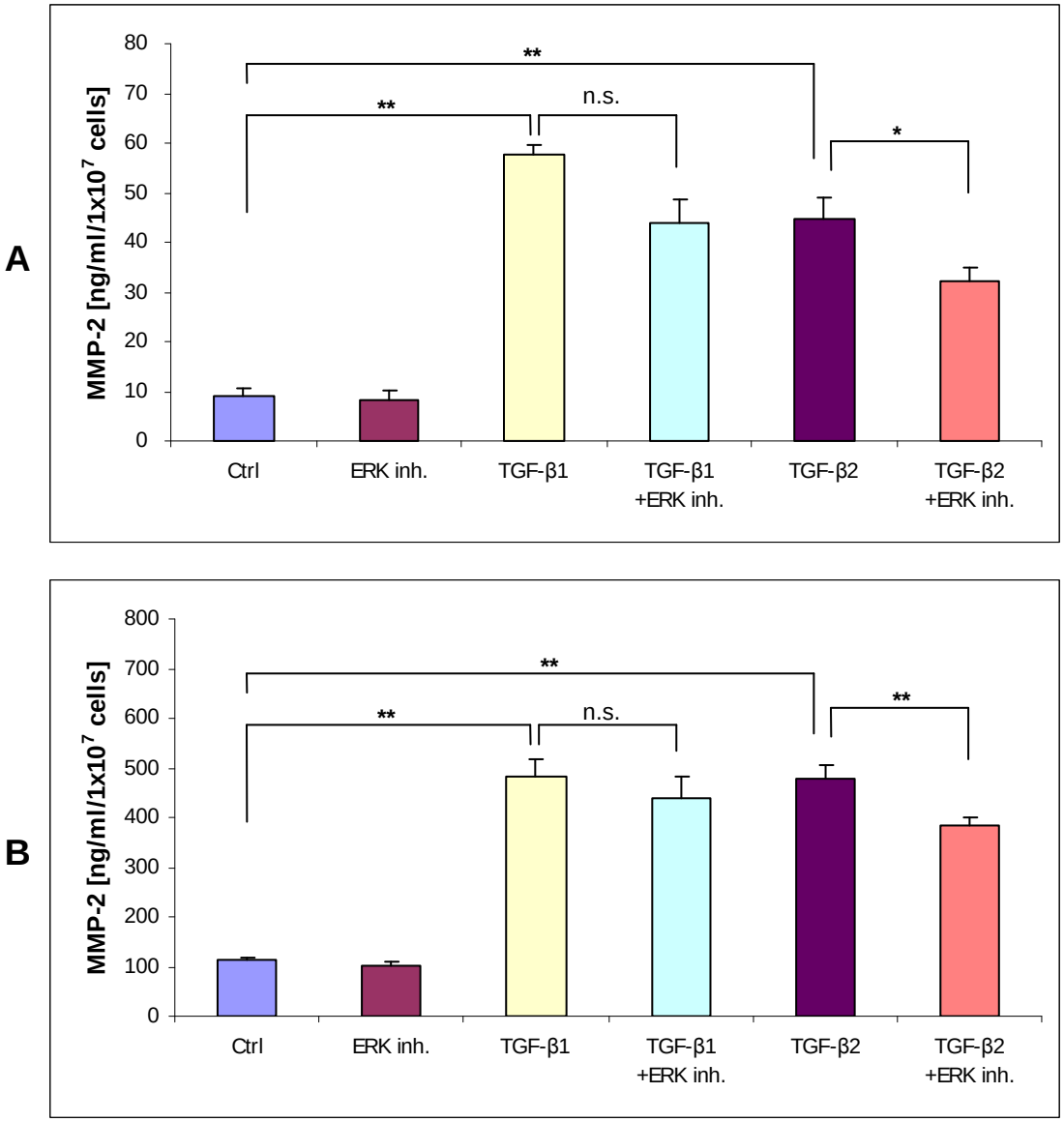


Figure 23. The ERK inhibitor (ERK inhibitor II) moderately inhibited the TGF-β1 or TGF-β2 induced MMP-2 secretion in endometrial and endometriotic epithelial cells.

(A) The level of MMP-2 in endometrial epithelial cells (HES) was significantly increased 6-fold after treatment for 72 hours with TGF-β1 or TGF-β2 (10 ng/ml),

respectively (P<0.01, n=8). The ERK inhibitor moderately inhibited the TGF-β1 or TGF-β2-induced MMP-2 secretion in HES (TGF-β1, n.s., n=8; TGF-β2, P<0.05, n=8). **(B)** The level of MMP-2 in the endometriotic epithelial cells (12Z) was significantly increased 5-fold after treatment for 72 hours with TGF-β1 or TGF-β2 (10 ng/ml), respectively (P<0.01, n=8). The ERK inhibitor slightly inhibited the TGF-β1-induced MMP-2 secretion (approx. 10%, n. s., n=8) and moderately inhibited the TGF-β2-induced MMP-2 secretion in 12Z (approx. 20%, P<0.05, n=8).

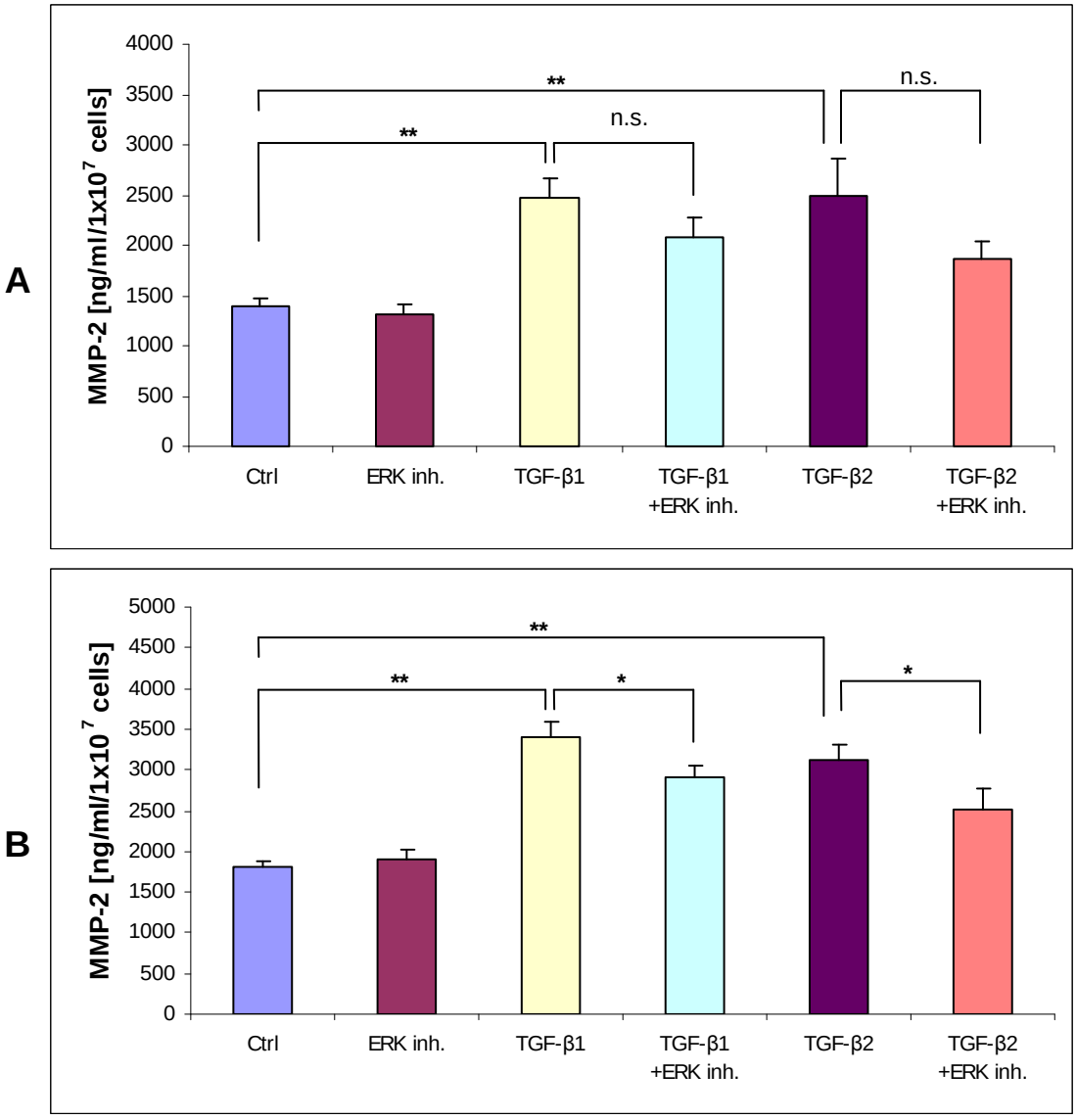


Figure 24. The ERK inhibitor (ERK inhibitor II) moderately inhibited the TGF-β1 or TGF-β2 induced MMP-2 secretion in endometrial and endometriotic stromal cells. **(A)** The level of MMP-2 in endometrial stromal cells (THESC) was significantly increased 2-fold after treatment for 72 hours with TGF-β1 or TGF-β2 (10 ng/ml),

respectively (P<0.01, n=8). The ERK inhibitor II moderately inhibited the TGF-β1 or TGF-β2 induced MMP-2 secretion in THESC (approx. 20%, n. s., n=8). **(B)** The level of MMP-2 in endometriotic stromal cells (22B) was significantly increased 2-fold after treatment for 72 hours with TGF-β1 or TGF-β2 (10 ng/ml), respectively (P<0.01, n=8). The ERK inhibitor II moderately inhibited the TGF-β1 or TGF-β2-induced MMP-2 secretion in 22B (approx. 20%, P<0.05, n=8).

3.2.2.3 Analysis of the p38 MAPK Pathway in MMP-2 Secretion

A highly selective p38 MAP kinase inhibitor (SB203580) was used to study the role of the p38 MAPK pathway in MMP-2 secretion. SB203580 (5 μM) was added 2 hours before adding the TGF-βs (10 ng/ml). Then cells were cultured for 72 hours. The MMP-2 level in supernatants was quantified with ELISAs for MMP-2.

The results showed that TGF-β1 and TGF-β2 both increased MMP-2 secretion. However, SB203580 showed a slight but non-significant effect on inhibition of the TGF-β induced MMP-2 secretion in epithelial cells (Fig. 25) and had no effects on the stromal cells (Fig. 26).

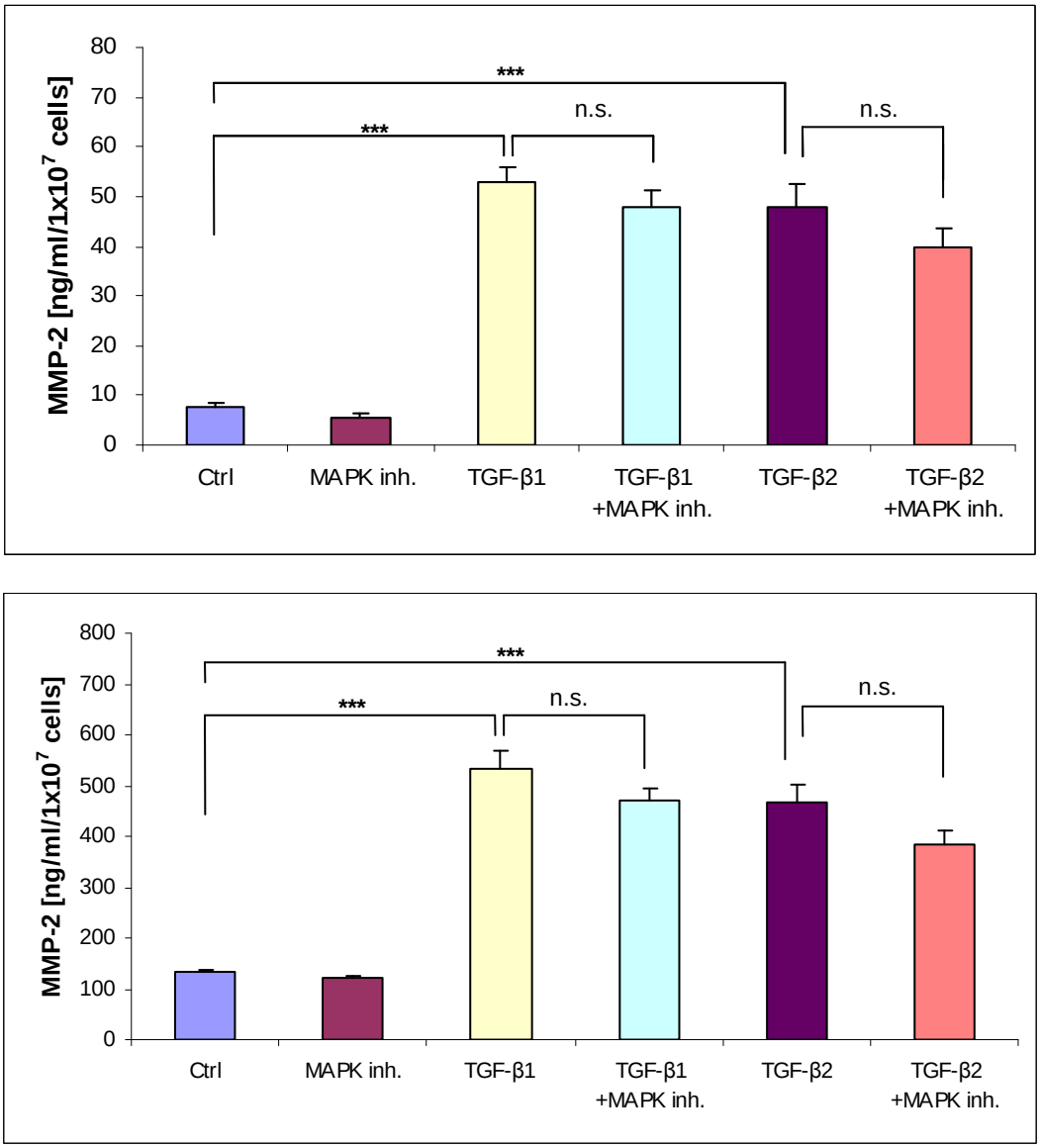


Figure 25. The p38 MAPK inhibitor (SB203580) slightly inhibited the TGF-β1 or TGF-β2 induced MMP-2 secretion in endometrial and endometriotic epithelial cells.

(A) The level of MMP-2 in endometrial epithelial cells (HES) was significantly increased 6-fold after treatment for 72 hours with TGF-β1 or TGF-β2 (10 ng/ml), respectively ($P<0.001$, n=8). **(B)** The level of MMP-2 in endometriotic epithelial cells (12Z) was significantly increased 5-fold after treatment for 72 hours with TGF-β1 or TGF-β2 (10 ng/ml), respectively ($P<0.001$, n=8). SB203580 slightly reduced (approx. 10%) the TGF-β1 or TGF-β2 induced secretion of MMP-2 in both endometrial and endometriotic epithelial cells. However, it was not significant.

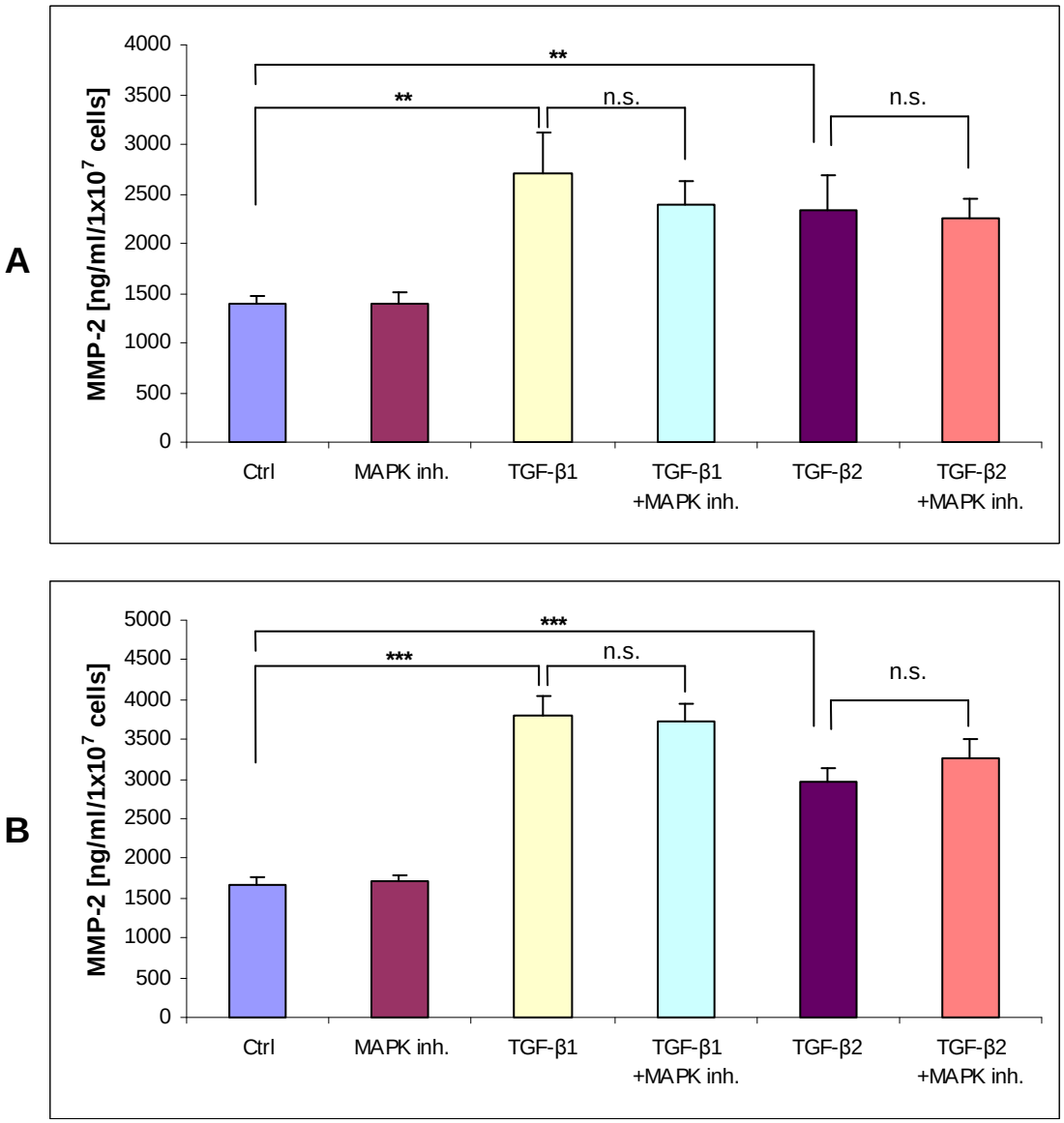


Figure 26. The p38 MAPK inhibitor (SB203580) did not inhibit the TGF-β1 or TGF-β2 induced MMP-2 secretion in endometrial and endometriotic stromal cells.

(A) The level of MMP-2 in endometrial stromal cells (THESC) was significantly increased 2-fold after treatment for 72 hours with TGF-β1 or TGF-β2 (10 ng/ml), respectively ($P<0.01$, n=8). **(B)** The level of MMP-2 in endometriotic stromal cells (22B) was significantly increased 2-fold after treatment for 72 hours with TGF-β1 or TGF-β2 (10 ng/ml), respectively ($P<0.001$, n=8). SB203580 showed no effect on the TGF-β1 or TGF-β2 induced MMP-2 secretion in both endometrial and endometriotic stromal cells.

In summary, both TGF-β1 and TGF-β2 increased the secretion of MMP-2 dramatically in all four cell types studied. However, the extent of elevation was quite similar between endometrial and endometriotic cells (HES versus 12Z, THESC versus 22B). The TβR1 inhibitor (LY364947) repressed the TGF-β1 or TGF-β2-induced MMP-2 secretion down to the control level while the p38 MAPK inhibitor (SB203580) showed no effect. The ERK inhibitor II showed a moderate effect on inhibition of the MMP-2 secretion, which indicated that the Smad pathway was the main pathway, but the ERK pathway is also required in order to achieve high level secretion of MMP-2.

3.3 Influence of TGF-β1 or TGF-β2 on PAI-1 Secretion

PAI-1 is considered to be one of the gold standards for studying the effects of TGF-βs on gene expression. Moreover, PAI-1 might play an important role in cell migration because PAI-1 detaches cells from the extracellular matrix by inactivating integrins (Czekay et al., 2003), which might contribute to the migration of retrograde endometrial tissue during the pathological process of endometriosis.

3.3.1 Basal Secretion of PAI-1 in Endometrial and Endometriotic Cells

To compare the basal PAI-1 secretion by endometrial and endometriotic cells, ELISAs have been performed to quantify the PAI-1 level of each cell line after cell culture for 72 hours. The concentration of PAI-1 was standardized as ng/ml per $1x10^6$ cells.

Results showed that endometrial epithelial cells (HES) secreted only 3 ng/ml PAI-1 which was almost undetectable, while endometriotic epithelial cells (12Z) secreted \621 ng/ml PAI-1. Endometrial stromal cells (THESC) secreted 2992 ng/ml PAI-1 which was much higher compared to both epithelial cells. The endometriotic stromal cells (22B) which produced huge amounts of PAI-1 (11994 ng/ml) were the most productive among the four cell lines (Fig. 27).

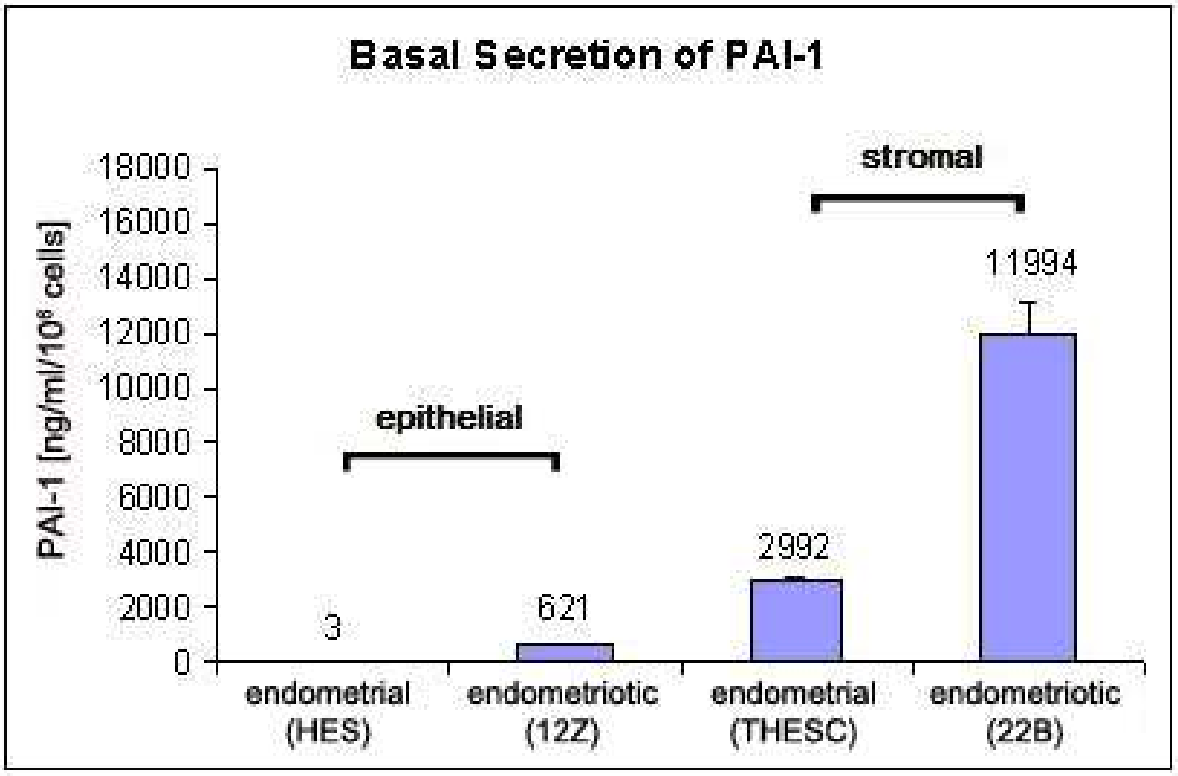


Figure 27. Basal secretion of PAI-1 by endometrial and endometriotic cells. Cells were cultured for 72 hours without any treatment. Endometriotic epithelial cells (12Z) secreted approximately 200-fold more PAI-1 compared to endometrial epithelial cells (HES), while HES nearly secreted no PAI-1. Endometriotic stromal cells (22B) secreted 4-fold more PAI-1 compared to endometrial stromal cells (THESC).

In summary, stromal cells (THESC and 22B) secreted much more PAI-1 compared to epithelial cells (HES and 12Z). Moreover, endometriotic cells generally secreted more PAI-1 compared to normal endometrial cells (12Z versus HES; 22B versus THESC).

3.3.2 Analysis of Smad-dependent and Smad-independent TGF-β-induced PAI-1 Secretion

To investigate how TGF-βs affect PAI-1 secretion, endometrial and endometriotic cells were treated with 10 ng/ml of TGF-β1 or TGF-β2, respectively.

In all cell types studied, PAI-1 secretion was increased significantly by TGF-β1 or TGF-β2, approximately 50-fold in endometrial epithelial cells (HES), 7-fold in endometriotic epithelial cells (12Z), two to three-fold in stromal cells (THESC and 22B), compared to the control group. TGF-β1 was more effective on stimulating PAI-1 secretion compared to TGF-β2 in epithelial cells but not in stromal cells (Figs. 28–33).

3.3.2.1 Analysis of the Smad Pathway in PAI-1 Secretion

The inhibitor LY364947 was used to investigate the role of the Smad-dependent pathway in MMP-2 secretion. The inhibitor blocks the phosphorylation of Smad2/3 by selectively inhibiting TβR1. LY364947 (5 μM) was added 2 hours before adding the TGF-βs (10 ng/ml). Cells were cultured for 72 hours after stimulation with the TGF-βs. The results showed that TGF-β1 and TGF-β2 both increased PAI-1 secretion, and LY364947 was able to block the TGF-β induced secretion of PAI-1 to control levels in all cell lines studied (Figs. 28, 29).

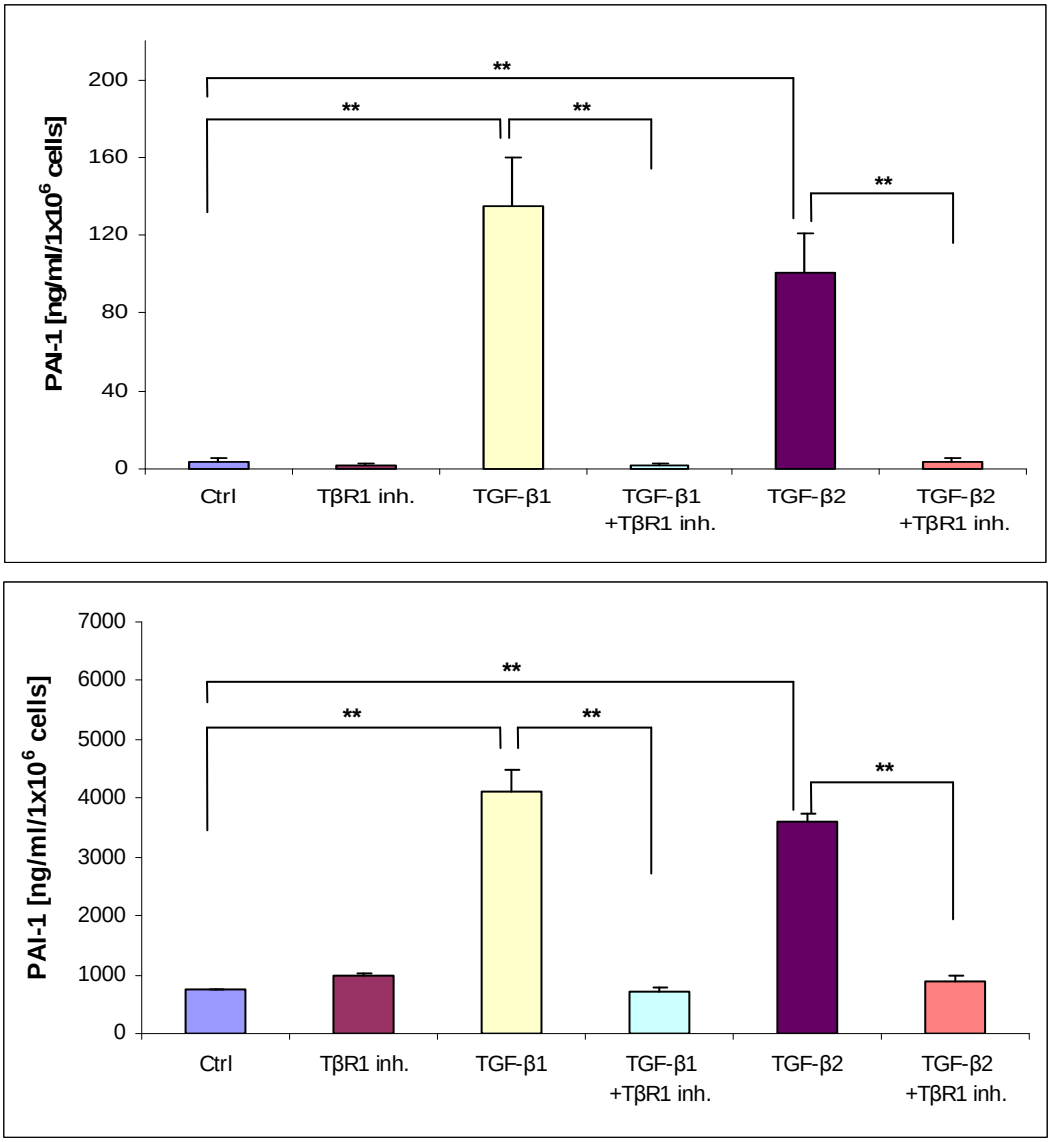


Figure 28. The TβR1 inhibitor (LY364947) completely inhibited the TGF-β1 or TGF-β2 induced PAI-1 secretion in endometrial and endometriotic epithelial cells.

(A) The level of PAI-1 in endometrial epithelial cells (HES) was significantly increased 50-fold after treatment for 72 hours with TGF-β1 or TGF-β2 (10 ng/ml), respectively ($P<0.01$, n=6). **(B)** The level of MMP-2 in endometriotic epithelial cells (12Z) was significantly increased 6-fold after treatment for 72 hours with TGF-β1 or TGF-β2 (10 ng/ml), respectively ($P<0.01$, n=6). LY364947 reduced the TGF-β1 or TGF-β2-induced secretion of PAI-1 to control levels in both endometrial and endometriotic epithelial cells.

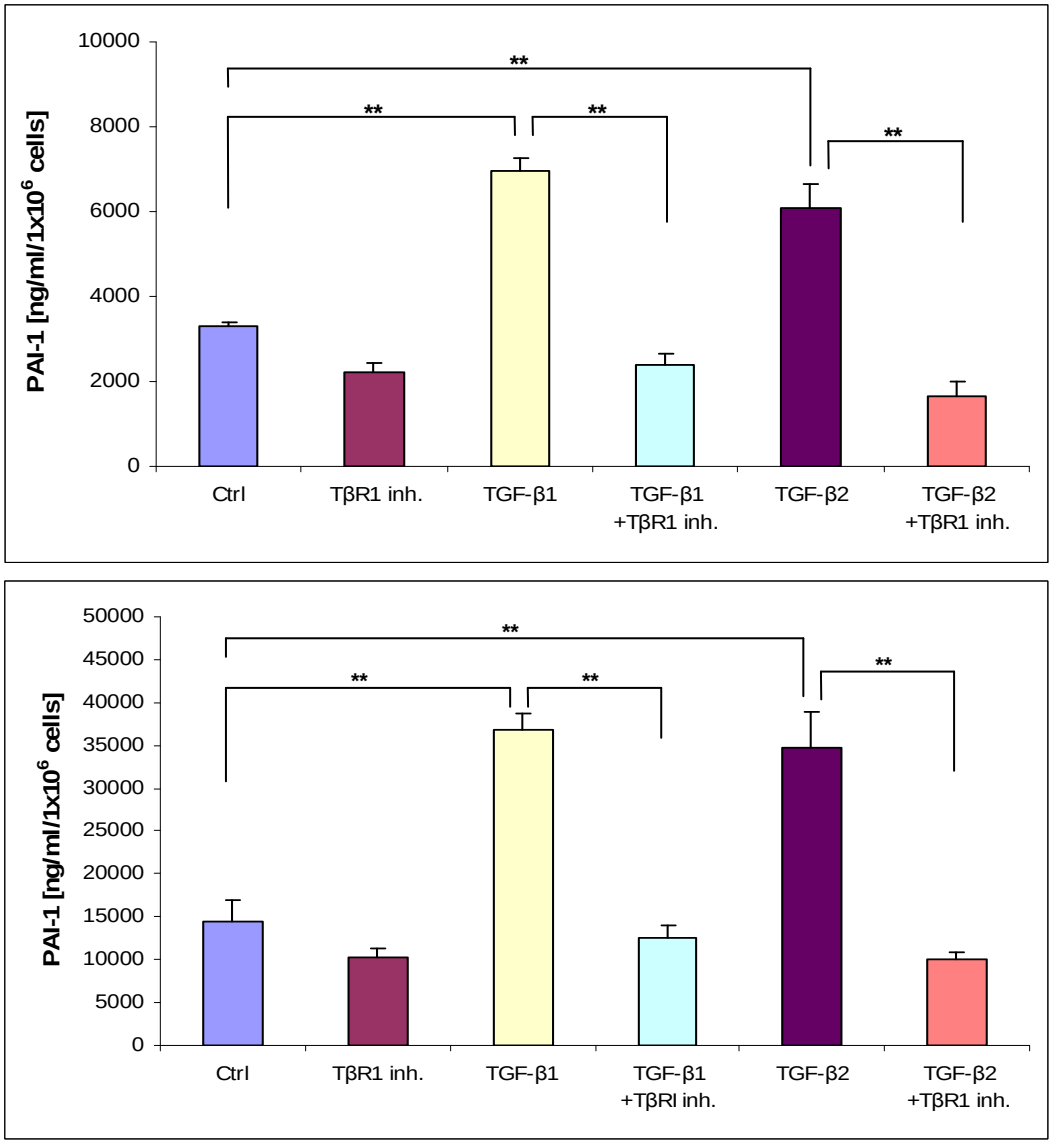


Figure 29. The TβR1 inhibitor (LY364947) completely inhibited the TGF-β1 or TGF-β2 induced PAI-1 secretion in endometrial and endometriotic stromal cells.

(A) The level of MMP-2 in endometrial stromal cells (THESC) was significantly increased 2-fold after treatment for 72 hours with TGF-β1 or TGF-β2 (10 ng/ml), respectively ($P<0.01$, n=6). **(B)** The level of MMP-2 in endometriotic stromal cells (22B) was significantly increased 3-fold after treatment for 72 hours with TGF-β1 or TGF-β2 (10 ng/ml), respectively ($P<0.01$, n=6). LY364947 reduced the TGF-β1 or TGF-β2 induced secretion of PAI-1 to control levels in both endometrial and endometriotic stromal cells.

3.3.2.2 Analysis of the ERK Pathway in PAI-1 Secretion

A highly selective ERK1/2 inhibitor (ERK inhibitor II) was used to study the role of the ERK pathway in PAI-1 secretion. The ERK inhibitor II (20 μM) was added before adding the TGF-βs (10 ng/ml). Cells were cultured for 72 hours after stimulation with the TGF-βs.

The results showed that TGF-β1 and TGF-β2 both increased PAI-1 secretion in all cell lines studied. The ERK inhibitor II demonstrated a modest repression (approx. 35% to 50%) of PAI-1 secretion which was induced by TGF-β1 or TGF-β2 in all cell lines studied (Figs. 30, 31).

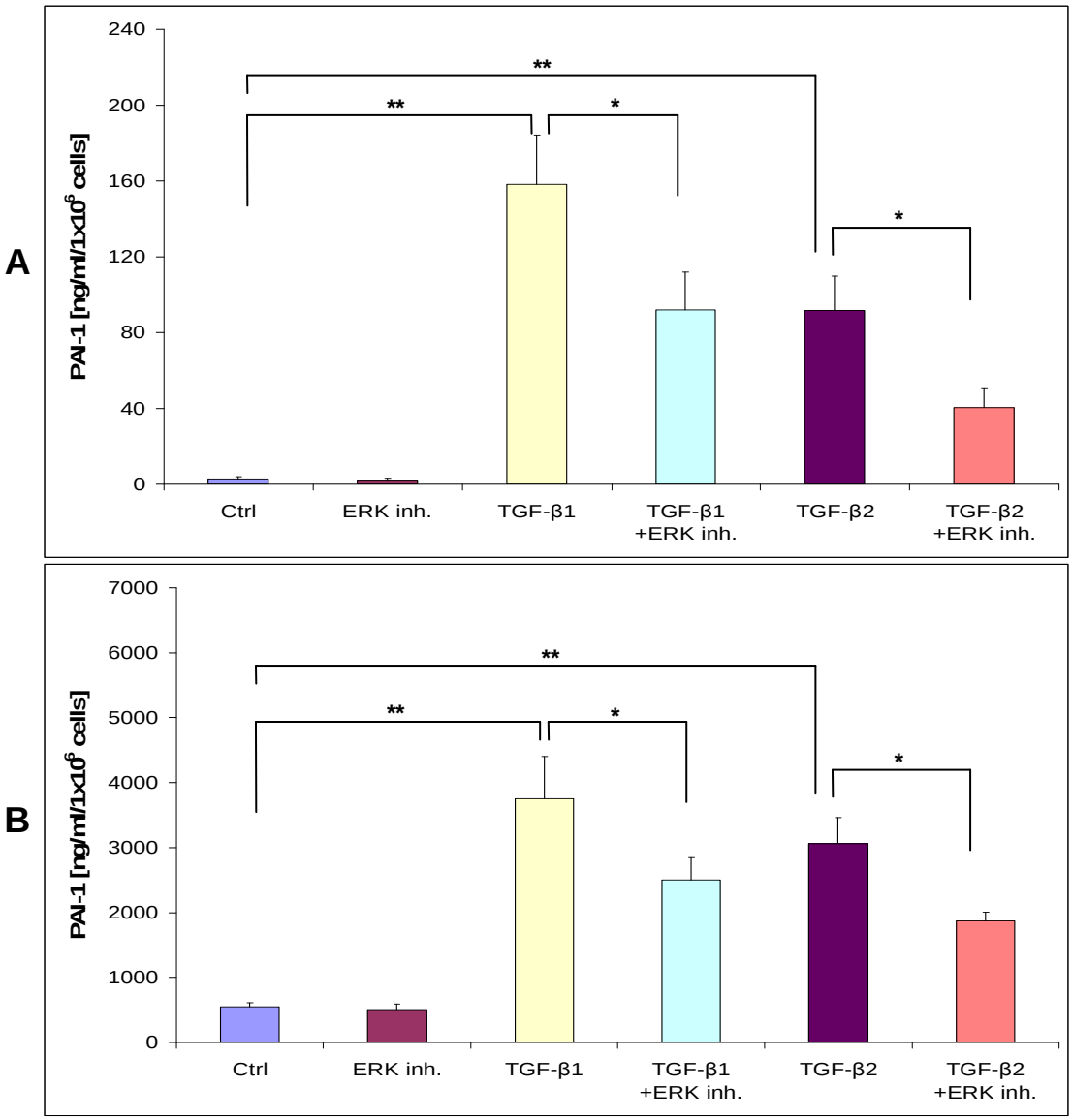


Figure 30. The ERK inhibitor (ERK inhibitor II) moderately inhibited the TGF-β1 or TGF-β2 induced PAI-1 secretion in endometrial and endometriotic epithelial cells.

(A) The level of PAI-1 in endometrial epithelial cells (HES) was significantly increased 50-fold after treatment for 72 hours with TGF-β1 or TGF-β2 (10 ng/ml), respectively (P<0.01, n=6). The ERK inhibitor moderately inhibited the TGF-β1 or TGF-β2-induced PAI-1 secretion in HES (approx. 50%, P<0.05, n=6). **(B)** The level of PAI-1 in endometriotic epithelial cells (12Z) was significantly increased 7-fold after treatment for 72 hours with TGF-β1 or TGF-β2 (10 ng/ml), respectively (P<0.01, n=6). The ERK inhibitor moderately inhibited the TGF-β1 or TGF-β2 induced PAI-1 secretion in 12Z (appro. 40%, P<0.05, n=6).

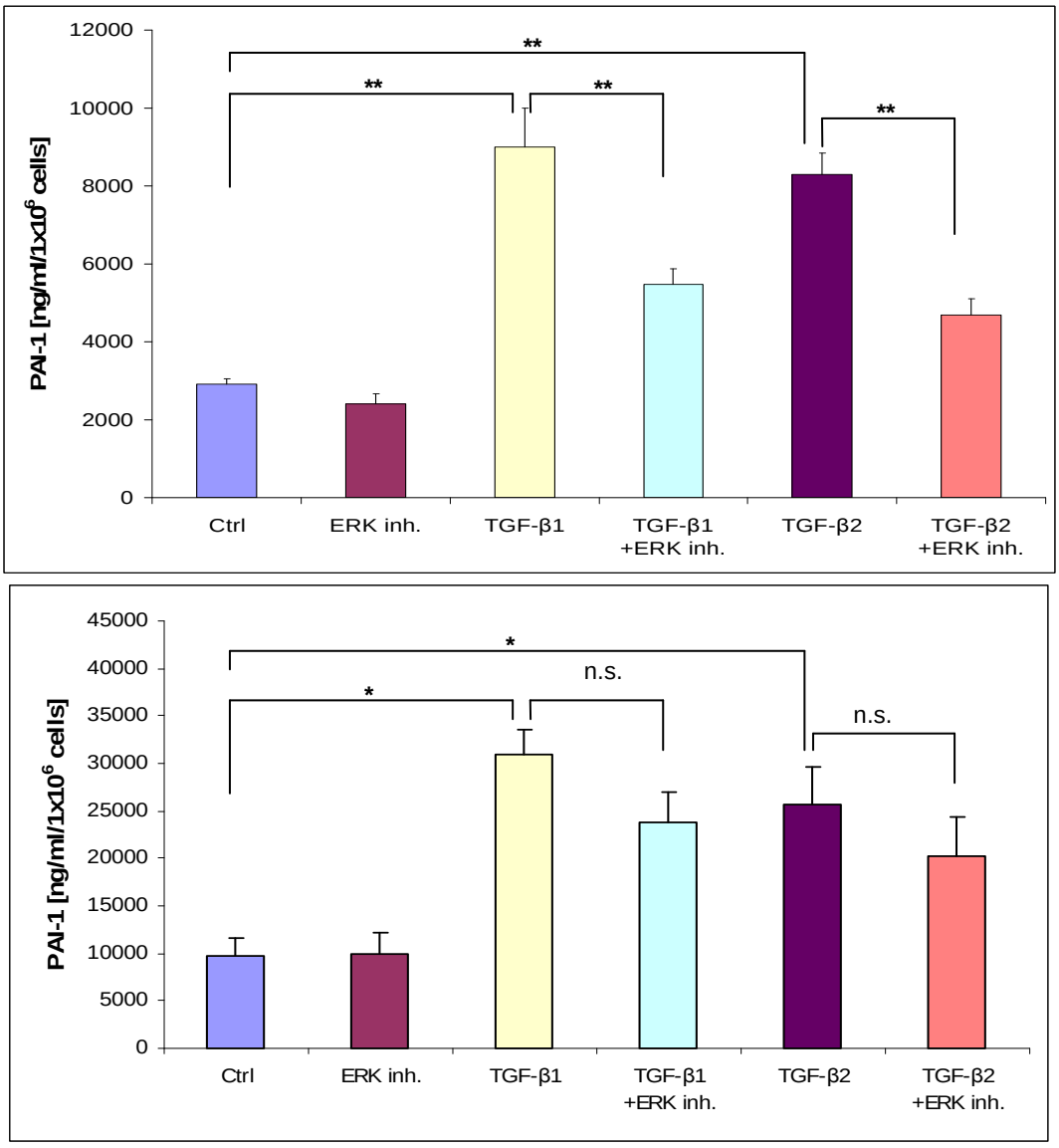


Figure 31. The ERK inhibitor (ERK inhibitor II) moderately inhibited the TGF-β1 or TGF-β2 induced PAI-1 secretion in endometrial and endometriotic stromal cells.

(A) The level of PAI-1 in endometrial stromal cells (THESC) was significantly increased 3-fold after treatment for 72 hours with TGF-β1 or TGF-β2 (10 ng/ml), respectively ($P<0.01$, n=6). The ERK inhibitor moderately inhibited the TGF-β1 or TGF-β2-induced PAI-1 secretion in THESC (approx. 50%, $P<0.01$, n=6). **(B)** The level of PAI-1 in endometriotic stromal cells (22B) was significantly increased 3-fold after treatment for 72 hours with TGF-β1 or TGF-β2 (10 ng/ml), respectively ($P<0.05$, n=6). The ERK inhibitor slightly inhibited the TGF-β1 or TGF-β2 induced PAI-1 secretion in 22B (approx. 30%, not significant, n=6).

3.3.2.3 Analysis of the p38 MAPK Pathway in PAI-1 Secretion

A highly selective p38 MAP kinase inhibitor (SB203580) was used to study the role of the p38 MAPK pathway in PAI-1 secretion. SB203580 (5 μM) was added 2 hours before adding the TGF-βs (10 ng/ml). Cells were cultured for 72 hours after stimulation with TGF-β1 or TGF-β2.

The results showed that either TGF-β1 or TGF-β2 increased the PAI-1 secretion. In the epithelial cells (HES and 12Z), SB203580 showed a moderate inhibiting effect which was not significant (Fig. 32). However, SB203580 did not inhibit the TGF-β induced secretion in the two stromal cell lines studied (THESC and 22B, Fig. 33).

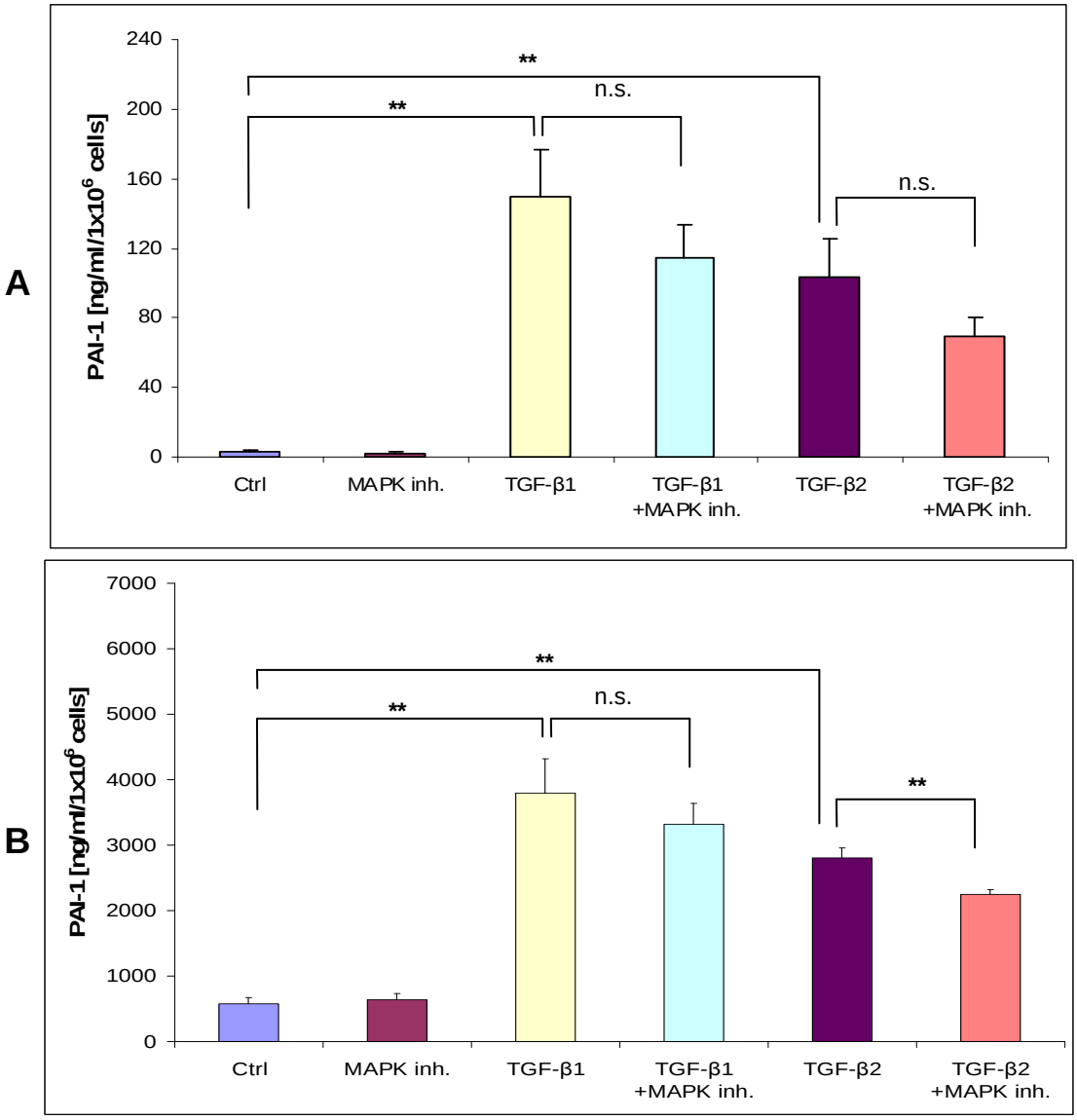


Figure 32. The p38 MAPK inhibitor (SB203580) moderately inhibited the TGF-β1 or TGF-β2 induced PAI-1 secretion in endometrial and endometriotic epithelial cells.
(A) The level of PAI-1 in endometrial epithelial cells (HES) was significantly increased 50-fold after treatment for 72 hours with TGF-β1 or TGF-β2 (10 ng/ml), respectively ($P<0.05$, n=6). SB203580 moderately reduced (approx. 25%) the TGF-β1 or TGF-β2-induced secretion of PAI-1 in HES. However it was not significant. **(B)** The level of PAI-1 in endometriotic epithelial cells (12Z) was significantly increased 6-fold after treatment for 72 hours with TGF-β1 or TGF-β2 (10 ng/ml), respectively ($P<0.05$, n=6). SB203580 reduced the TGF-β2 induced secretion of PAI-1 (approx. 20%, $P<0.01$, n=6) and the TGF-β1 induced secretion of PAI-1 (approx. 15%, n. s., n=6) in 12Z.

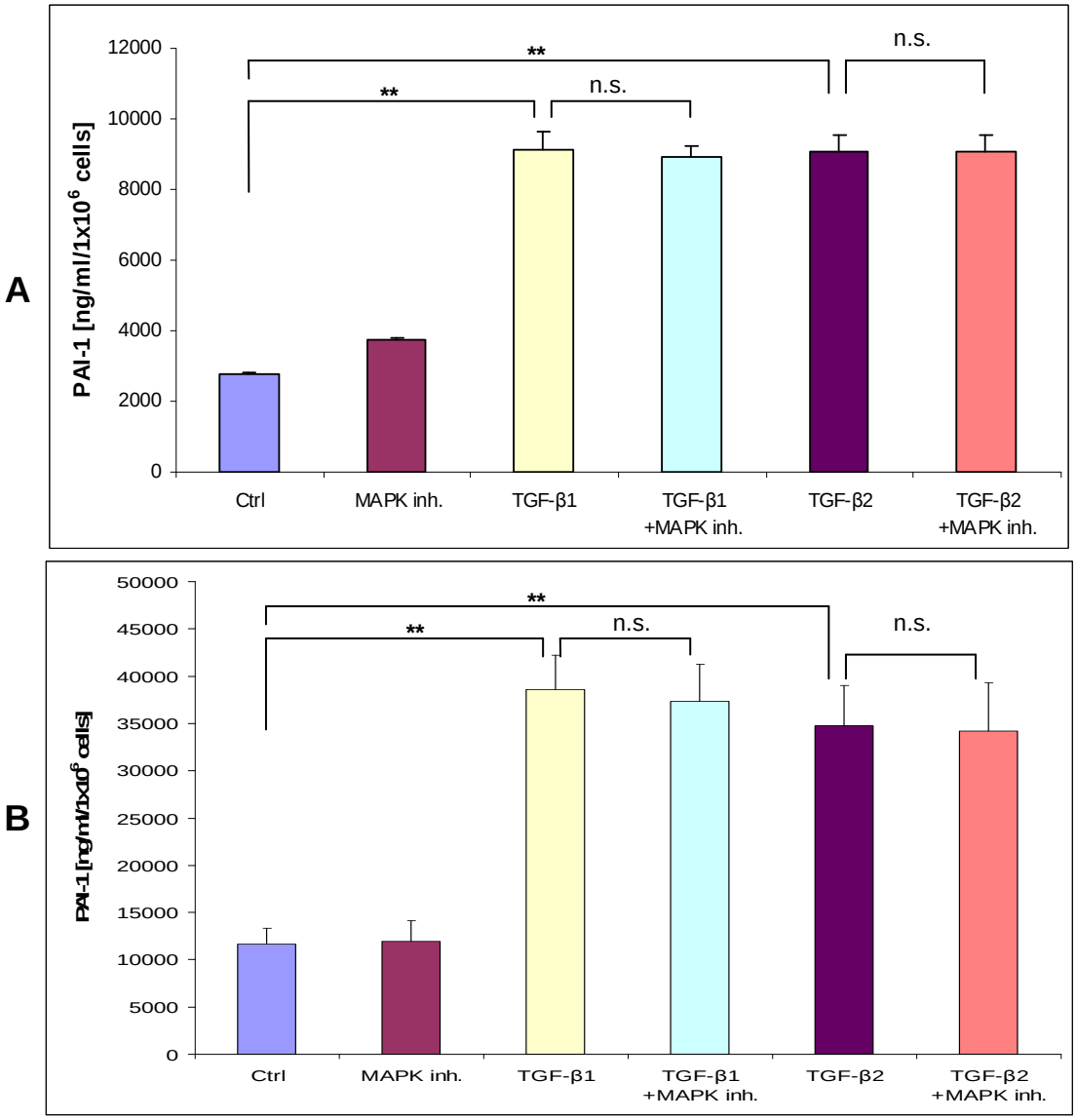


Figure 33. The p38 MAPK inhibitor (SB203580) did not inhibit the TGF-β1 or TGF-β2 induced PAI-1 secretion in endometrial and endometriotic stromal cells.

(A) The level of PAI-1 in endometrial stromal cells (THESC) was significantly increased 3-fold after treatment for 72 hours with TGF-β1 or TGF-β2 (10 ng/ml), respectively ($P<0.01$, n=6). **(B)** The level of PAI-1 in endometriotic stromal cells (22B) was significantly increased 3-fold after treatment for 72 hours with TGF-β1 or TGF-β2 (10 ng/ml), respectively ($P<0.01$, n=6). SB203580 showed no effect on the TGF-β1 or TGF-β2 induced PAI-1 secretion in both endometrial and endometriotic stromal cells.

In summary, both TGF-β1 and TGF-β2 increased the secretion of PAI-1 dramatically in all four cell types studied. The extent of elevation was similar between endometrial and endometriotic stromal cells (THESC versus 22B), but quite different between endometrial and endometriotic epithelial cells (HES=50-fold versus 12Z=7-fold). The TβR1 inhibitor (LY364947) repressed the TGF-β1 or TGF-β2-induced PAI-1 secretion down to the control level while the ERK inhibitor II moderately inhibited the TGF-β1 or TGF-β2-induced PAI-1 secretion by about 35 to 50% in all four cell lines studied. The p38 MAPK inhibitor (SB203580) showed a slight inhibiting effect on TGF-β1 or TGF-β2-induced PAI-1 secretion in epithelial cells (HES and 12Z), but did not affect the stromal cells (THESC and 22B).

3.4 Analysis of the ERK1/2 Pathway after Stimulation with TGF-β1 or TGF-β2

By using ELISAs, we have shown that the ERK1/2 inhibitor decreased the TGF-β-induced MMP-2 or PAI-1 secretion. However, it is not clear which component of the ERK pathway is involved. Therefore, it was important to elucidate the connection of the ERK and TGF-β pathways.

3.4.1 EGF Receptor Phosphorylation after Stimulation with TGF-β1 or TGF-β2 in Endometrial and Endometriotic Cells

The ERKs are generally phosphorylated by activated EGF receptor (Ramos, 2008). Samarakoon et al. (2008) reported that TGF-β1 induced the phosphorylation of the EGF receptor leading to PAI-1 expression in vascular smooth muscle cells.

To investigate whether the EGF receptor is phosphorylated by TGF-βs in endometrial and endometriotic cells, PathScan® ELISAs were performed. Cells were treated with 10ng/ml TGF-β1. Considering that the phosphorylation of the EGF receptor might be a fast process, several time points less than 1 hour were chosen (5 min., 15 min., 30 min., and 60 min.). Cells were collected after treatment and homogenized with cell lysis buffer (Cell Signaling Technology®). PathScan® ELISAs targeting three different phosphorylating sites of the EGF receptor (Tyr845, Tyr1068, Tyr1173) were then performed with the cell homogenates.

The results showed that the phospho-EGF receptor was not detectable in THESC cells at any time point analyzed after treatment with 10 ng/ml TGF-β1 (Fig. 34).

Similar experiments were performed with the other three cell lines (HES, 12Z and 22B) and also with the stimulation of 10 ng/ml TGF-β2. However, the results also did not reveal any phosphorylation of the EGF receptor by TGF-βs.

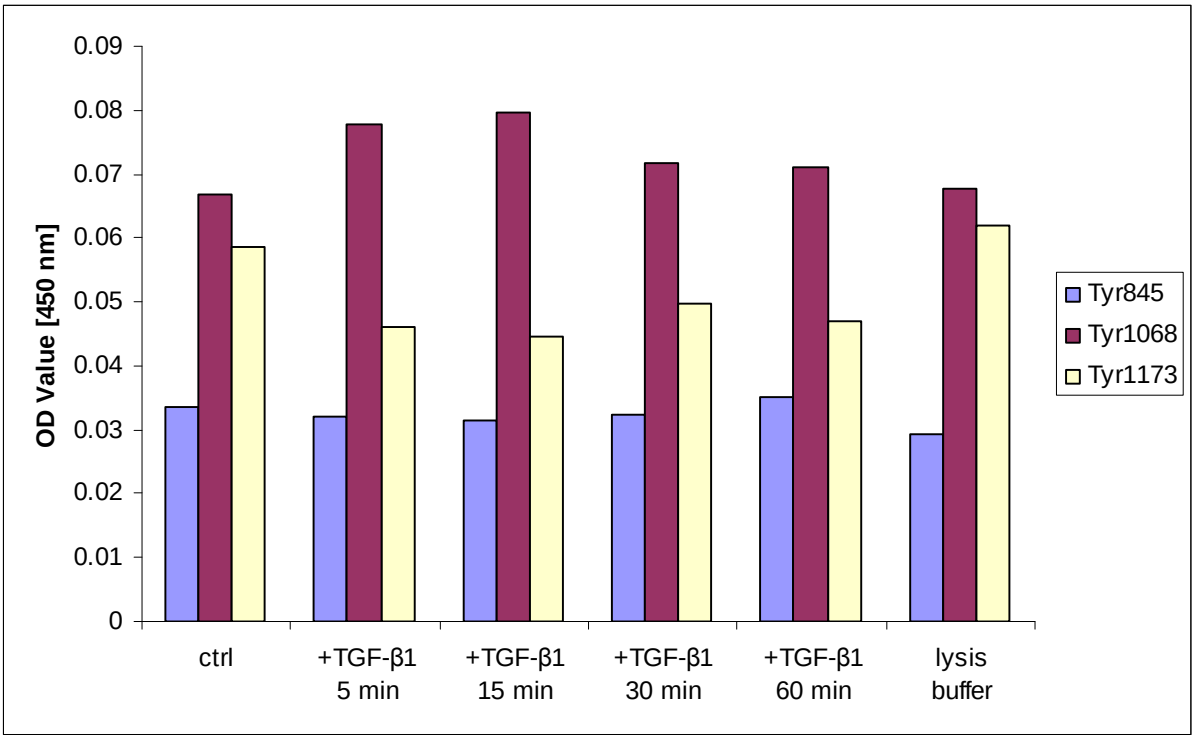


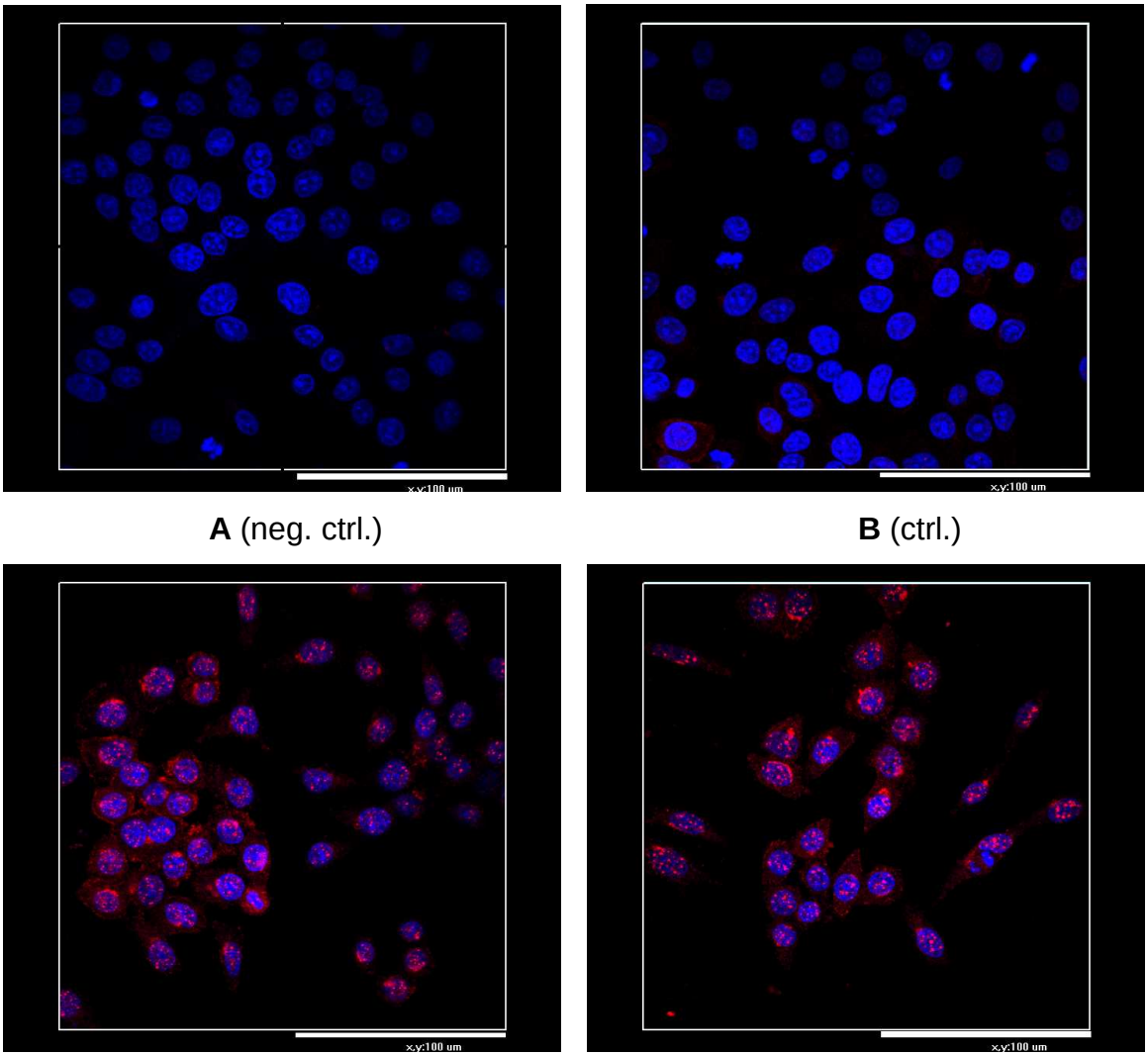
Figure 34. Treatment of THESC cells with TGF-β1 did not stimulate phosphorylation of the EGF receptor (Tyr845, Tyr1068 or Tyr1173). The THESC cells were treated with 10 ng/ml TGF-β1 for 5 min., 15 min., 30 min., or 60 min., respectively. The OD values are shown in the figure with blue (Tyr845), red (Tyr1168) and yellow (Tyr1173) bars. The OD values of all samples were close to the blank (<0.1). No phospho-EGF receptor was detected and TGF-β1 showed no effect on inducing the phosphorylation of the EGF receptor at all time points.

3.4.2 Localization of ERK1/2 Phosphorylation after Stimulation with TGF-β1 or TGF-β2 in Endometrial and Endometriotic Cells

Samarakoon et al. (2008) summarized the role of ERKs in the non-Smad signalling in TGF-β-induced PAI-1 transcription. Our results also showed that the ERK inhibitor moderately repressed the TGF-β-induced PAI-1 and MMP-2 secretion in endometrial and endometriotic cells. This indicates that TGF-βs might initiate activation of ERK.

To investigate whether TGF-βs stimulate the phosphorylation of ERK1/2, the intracellular localization of phospho-ERK1/2 in endometrial and endometriotic cells was tested with immunofluorescence.

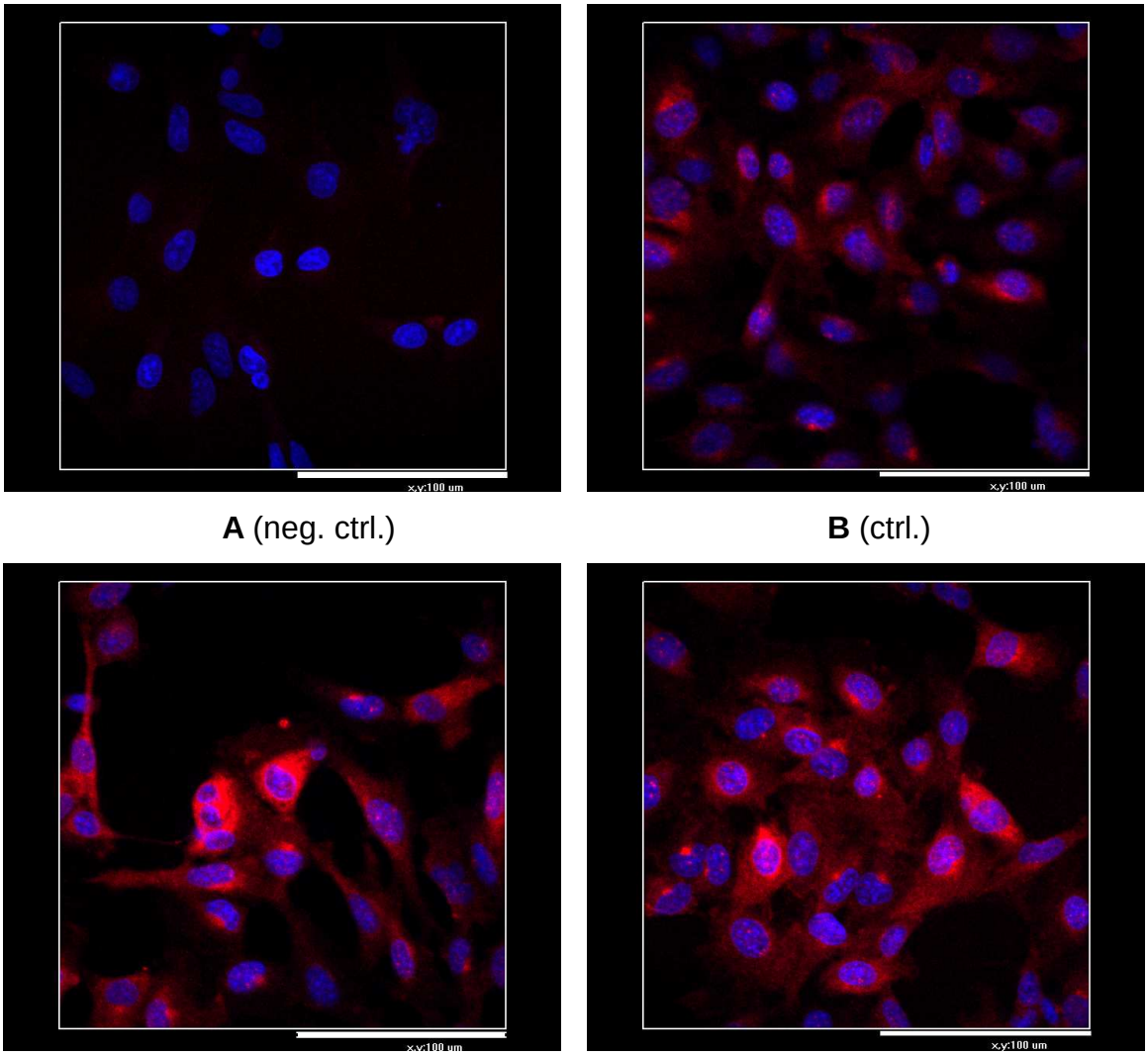
Cells were treated for 15 minutes with 10 ng/ml TGF-β1 or TGF-β2, respectively. Then cells were fixed with ice-cold acetone/methanol (1:1). A high affinity phospho-ERK1/2 antibody (Cell Signaling Technology®) was used as first antibody. A goat anti-rabbit IgG labelled with Alexa Fluor® 546 dye (Invitrogen) was used as second antibody. Hoechst dye (Invitrogen) was used to stain the nuclei. All pictures were taken with a confocal microscope. The results are shown in the figures below (Figs. 35, 36, 37, 38).



A (neg. ctrl.) **B** (ctrl.)

C (+ TGF-β1) **D** (+ TGF-β2)

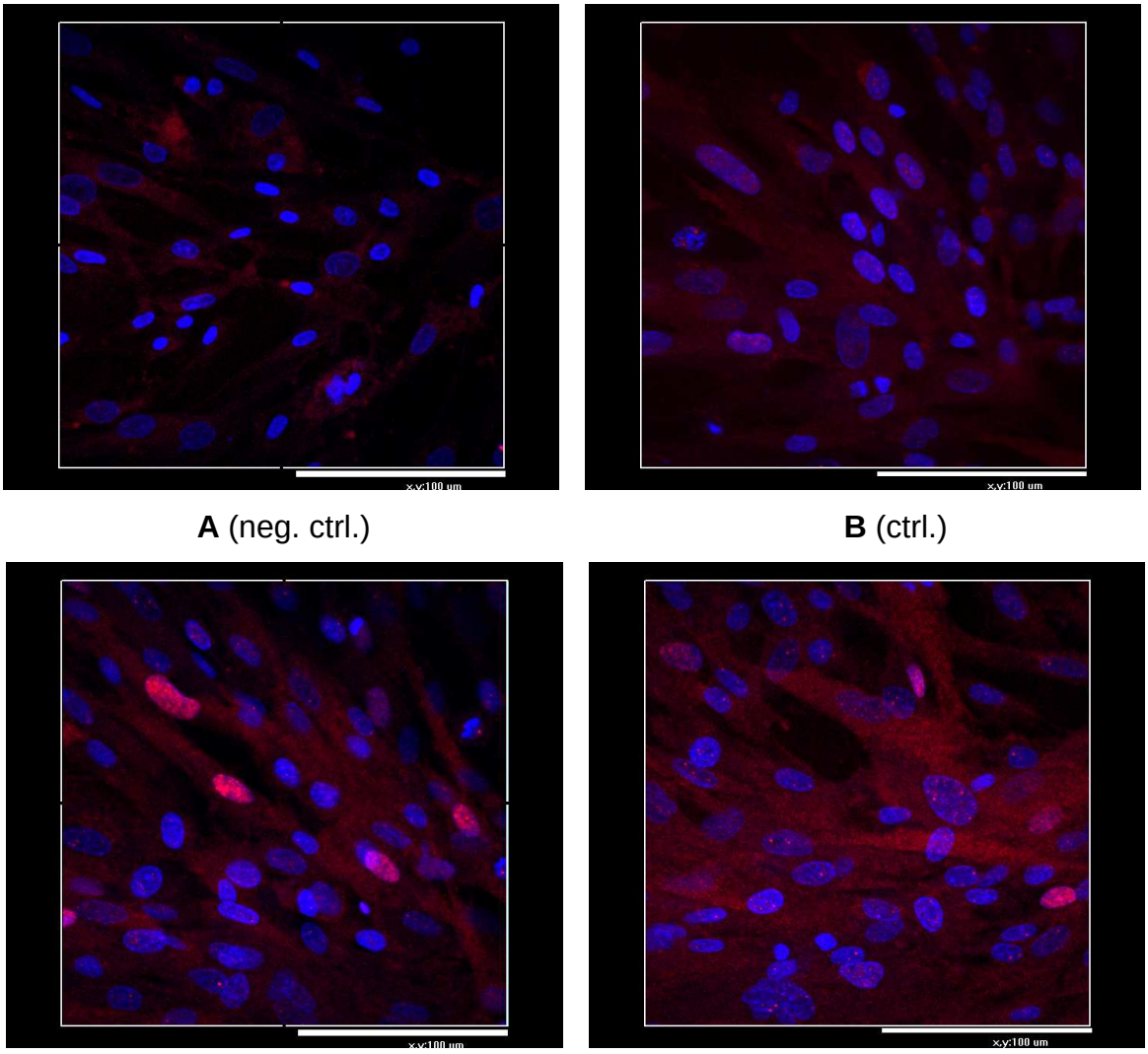
Figure 35. Treatment with TGF-β1 or TGF-β2 resulted in the phosphorylation of ERK1/2 in endometrial epithelial HES cells. Cells were treated for 15 minutes with 10 ng/ml TGF-β1 or TGF-β2, respectively. No staining was observed in the negative control **(A)**. The cytoplasm of very few cells was stained weakly in the untreated control group **(B)**. In the TGF-β1 **(C)** or the TGF-β2 **(D)** stimulation group, the cytoplasm of cells was much stronger stained compared to the untreated control group. Moreover, a strong staining was observed both inside and next to the nuclei.



A (neg. ctrl.) **B** (ctrl.)

C (+ TGF-β1) **D** (+ TGF-β2)

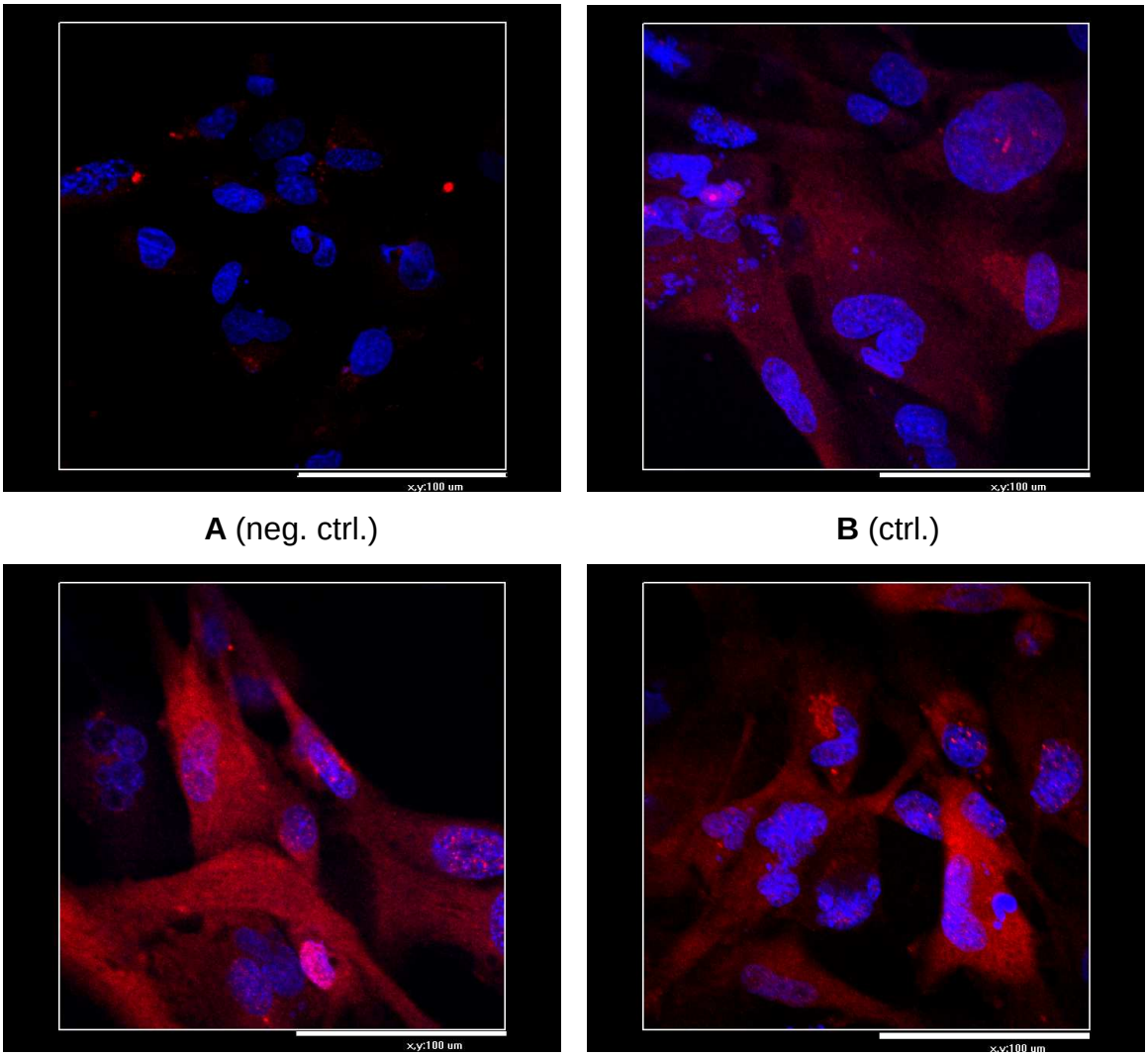
Figure 36. Treatment with TGF-β1 or TGF-β2 resulted in the phosphorylation of ERK1/2 in endometriotic epithelial 12Z cells. Cells were treated for 15 minutes with 10 ng/ml TGF-β1 or TGF-β2, respectively. No staining was observed in the negative control **(A)**. In the cells without TGF-β treatment **(B)**, there was a moderate staining in the cytoplasm and the nuclei. However, the cells were much stronger stained in the cytoplasm and nuclei either in the TGF-β1 **(C)** or the TGF-β2 **(D)** stimulation group, compared to the untreated control group. The staining was mostly concentrated in the nuclei and the cytoplasm next to the nuclei.



A (neg. ctrl.) **B** (ctrl.)

C (+ TGF-β1) **D** (+ TGF-β2)

Figure 37. Treatment with TGF-β1 or TGF-β2 resulted in the phosphorylation of ERK1/2 in endometrial stromal THESC cells. Cells were treated for 15 minutes with 10 ng/ml TGF-β1 or TGF-β2, respectively. Weak background staining was observed in the negative control **(A)**. In the cells without TGF-β treatment **(B)**, there was a weak staining in the cytoplasm and the nuclei. However, the cells were much stronger stained in the cytoplasm and nuclei either in the TGF-β1 **(C)** or the TGF-β2 **(D)** stimulation group, compared to the untreated control group and the staining was more intense in some nuclei.



A (neg. ctrl.) **B** (ctrl.)

C (+ TGF-β1) **D** (+ TGF-β2)

Figure 38. Treatment with TGF-β1 or TGF-β2 resulted in the phosphorylation of ERK1/2 in endometriotic stromal 22B cells. Cells were treated for 15 minutes with 10 ng/ml TGF-β1 or TGF-β2, respectively. Weak background staining was observed in the negative control **(A)**. In the cells without TGF-β treatment **(B)**, there was a moderate staining in the cytoplasm and weak staining in the nuclei. However, the cells were much stronger stained in both cytoplasm and nuclei either in the TGF-β1 **(C)** or the TGF-β2 **(D)** stimulation group, compared to the untreated control group. The staining was more intense in some nuclei and the cytoplasm next to the nuclei.

In summary, without the stimulation of TGF-βs, phospho-ERK1/2 was slightly detectable in endometriotic epithelial 12Z cells, endometrial stromal THESC cells and endometriotic stromal 22B cells, but not in the endometrial epithelial HES cells. Most of the phospho-ERK1/2 dispersed slightly in the cytoplasm and was rarely found inside the nuclei. After TGF-β1 or TGF-β2 stimulation, phosphorylation of ERK1/2 in all four cell types studied was strongly enhanced and the localization of phospho-ERK1/2 was mostly detected in the nuclei and the cytoplasm next to the nuclei.

3.5 Influence of PAI-1 on Cell Adhesion

One report showed that PAI-1 inhibited the cell attachment to the ECM (fibronectin and type I collagen) by inhibiting urokinase receptor-vitronectin and integrin-vitronectin interaction (Czekay et al., 2003). Considering that TGF-βs induce a dramatic elevation of PAI-1 in endometrial and endometriotic cells, we hypothesized that they might influence cell adhesion via PAI-1.

To prove our hypothesis, a cell adhesion assay was performed. Cells were stimulated with medium containing 5 nM, 10 nM, 20 nM or 40 nM recombinant PAI-1, respectively. After 4 hours only the attached cells were quantified with a cell counter.

The results showed that PAI-1 decreased the number of adherent cells of all cell lines studied after 4 hours in a dose-dependent manner (Figs. 39, 40). Already at a concentration of recombinant PAI-1 of 20 nM, a substantial effect on cell detachment was observable. The numbers of HES cells were reduced by 38% (Fig. 39), while THESC cells were reduced by 35% (Fig. 40), 22B cells were reduced by 28% (Fig. 40) and 12Z cells were reduced by 19% (Fig. 40).

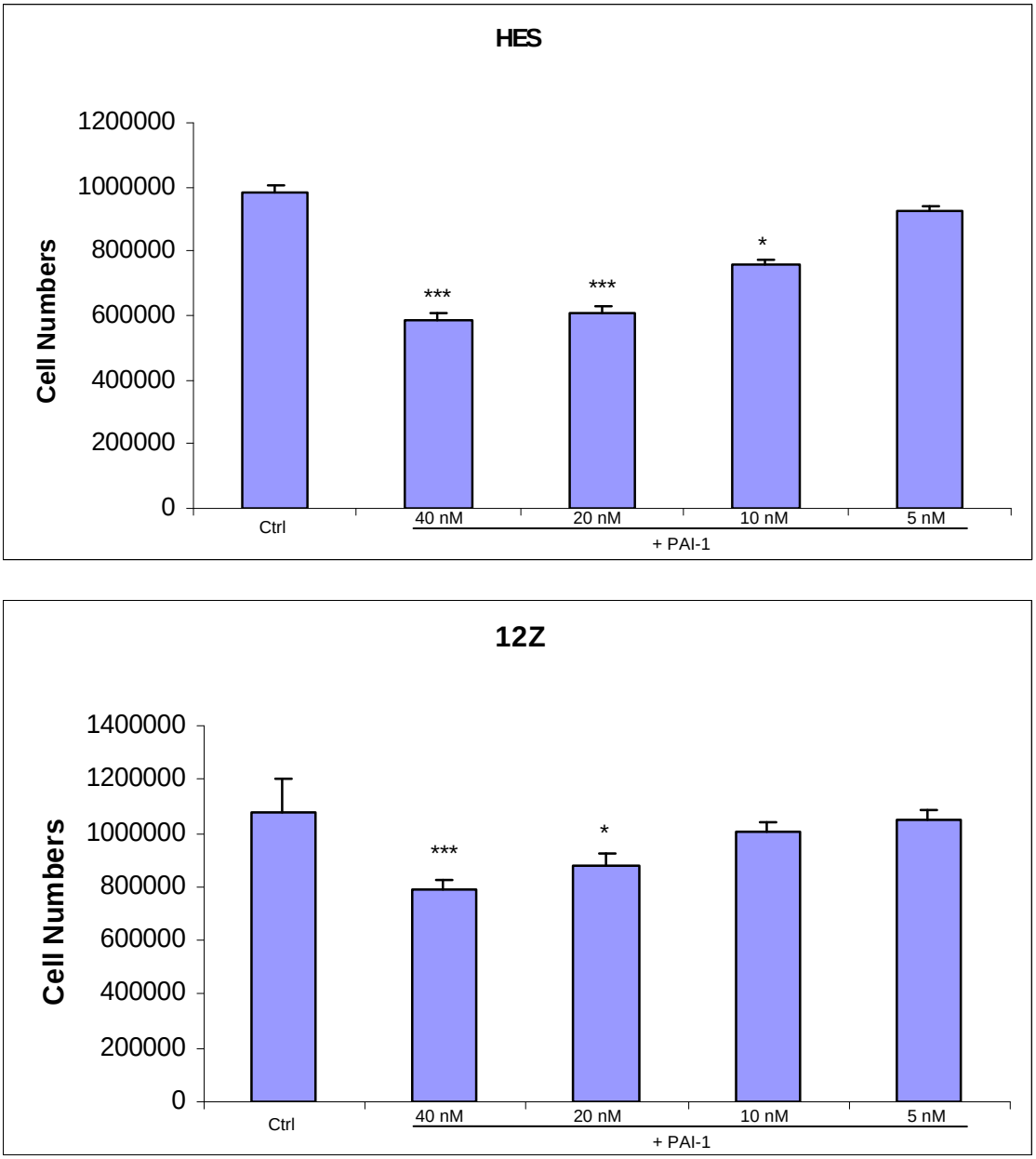


Figure 39. PAI-1 dose-dependently inhibited cell adhesion of endometrial and endometriotic epithelial cells to the cell culture plate. **(A)** The numbers of attached endometrial epithelial (HES) cells were decreased by 40%, 38%, 23% or 6% after treatment for 4 hours with 40 nM, 20 nM, 10 nM or 5 nM PAI-1, respectively. **(B)** The numbers of attached endometriotic epithelial (12Z) cells were decreased by 26%, 19%, 7% or 2% after treatment for 4 hours with 40 nM, 20 nM, 10 nM or 5 nM recombinant PAI-1, respectively.

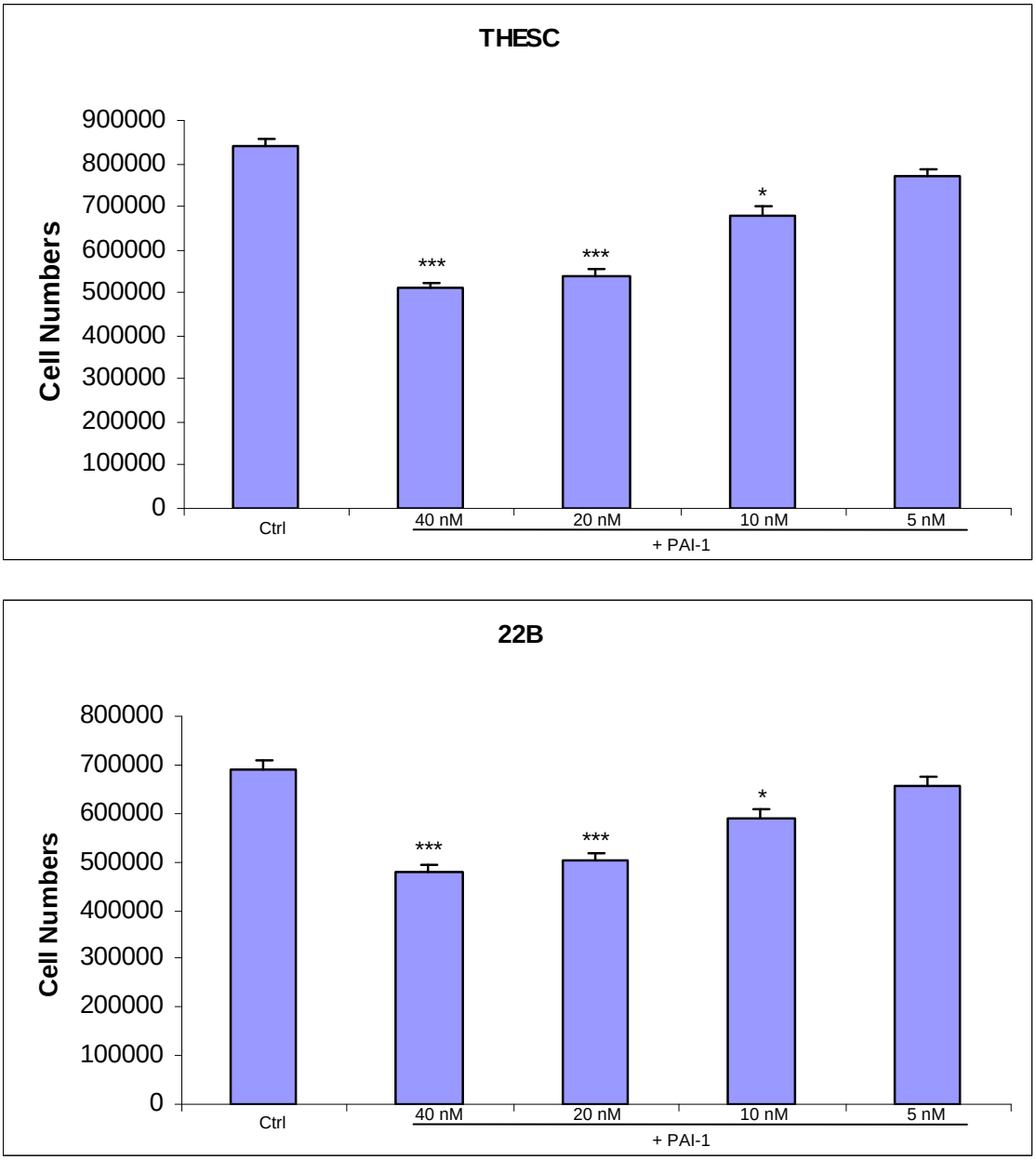


Figure 40. PAI-1 dose-dependently inhibited cell adhesion of endometrial and endometriotic stromal cells to the cell culture plate. **(A)** The numbers of attached endometrial stromal (THESC) cells were decreased by 39%, 35%, 19% or 8% after treatment for 4 hours with 40 nM, 20 nM, 10 nM or 5 nM PAI-1, respectively. **(B)** The numbers of attached endometriotic stromal (22B) cells were decreased by 31%, 28%, 15% or 5% after treatment for 4 hours with 40 nM, 20 nM, 10 nM or 5 nM PAI-1, respectively.

4 Discussion

As one of the most important growth factors, TGF-βs are involved in cell proliferation, differentiation, apoptosis, and motility in various cell types. They also play important roles in many biological processes, e.g. protein synthesis, morphogenesis, embryonic development, wound healing, inflammation, immune regulation, and tumorigenesis (Kaminska et al., 2005; Padua and Massagué, 2009; Taylor, 2009; Santibañez et al., 2011).

Because of the increasing importance of TGF-βs in endometriosis and menstruation (Oosterlynck et al., 1994; Pizzo et al., 2002; Gaide Chevronnay et al., 2008), TGF-βs and their high-affinity receptors are involved in the development and manifestion of endometriosis (Omwandho et al., 2010).

In this study, endometrial and endometriotic cells were compared. We investigated biological functions of TGF-β1 and TGF-β2 in endometrial and endometriotic cells. Furthermore, we studied the pathways of TGF-β signaling involved in the secretion of MMP-2 and PAI-1. Our study investigated not only the Smad pathway, but also the Smad-independent pathways of TGF-β signaling in endometrial and endometriotic cells for the first time.

4.1 Biological Functions of TGF-βs in Endometrial and Endometriotic Cells

4.1.1 Influence of TGF-βs on cell numbers of Endometrial and Endometriotic Cells

The cell number is determined by the rate of proliferation, apoptosis or necrosis (Sommer and Rao, 2002). However, the effects of TGF-βs on cell proliferation and apoptosis are extremely dependent on cell types and context. TGF-βs trigger apoptosis in epithelial cells, but induce proliferation in fibroblasts (Rahimi and Leof, 2007). Thus, TGF-βs provide signals for either cell survival or cell apoptosis.

So far, the interaction between TGF-βs pathway and apoptotic pathways still remains uncertain. The Smad pathway is the canonical pathway of TGF-β signaling (Roberts, 1999). Nevertheless, TGF-βs also cooperate with the death receptor apoptotic

pathway (Fas, TNF), the mitochondrial apoptotic pathway (Bcl-2) and several intracellular apoptotic modulators (JNK, NF-kappaB) (Sánchez-Capelo, 2005). Furthermore, the G1 phase of the cell cycle can be delayed by TGF-βs because they induce the synthesis of p15 and p21, which block the cyclin-dependent kinases (CDKs), and thus inhibit gene expression of c-myc, which is involved in cell cycle progression (Hanahan and Weinberg, 2000).

In endometrial cells, TGF-βs exerted different effects on cell proliferation and apoptosis, compared to cells from other organs. Meresman et al. (2003) showed that TGF-β1 stimulated the growth of endometrial epithelial cells at low cell numbers, but inhibited it at high cell numbers in women with or without endometriosis. In addition, Nasu et al. (2005) demonstrated that TGF-βs inhibited proliferation of endometrial stromal cells in a dose-dependent manner. However, these two studies reported the influence of TGF-βs on cell proliferation by only using a ^{3}H-thymidine incorporation assay (Meresman et al., 2003) or an MTT assay (Nasu et al., 2005), but did not count the cells directly.

In our experiments, we directly counted the number of cells. Our results showed that TGF-β1 and TGF-β2 influenced the cell number differently according to the initial cell number. TGF-βs increased the cell number when the initial cell number was low, whereas decreased the cell number when the initial cell number was high. Our results are similar to the results provided by Meresman et al. (2003), showing that the influence of TGF-βs on cell proliferation might depend on the initial cell numbers. However, the influences of TGF-βs on cell numbers are similar in all four cell types studied.

In addition to cell proliferation, apoptosis or necrosis, also cell adhesion could influence cell numbers as will be discussed in the following paragraph.

4.1.2 The role of TGF-βs in retrograde menstruation

According to Sampson's theory of retrograde menstruation, endometriotic tissues originate from retrograde endometrial tissues (Sampson, 1922; 1927). The process includes the contraction of the uterus, cell shedding, migration, ectopic cell survival and invasion (Omwandho et al., 2010).

The highest expression of TGF-βs in endometrial tissues was described during the late secretory phase and menses (Gaide Chevronnay et al., 2008). Furthermore,

TGF-β1 induced an impaired contraction of the uterus (Nasu et al., 2005). These results suggest that overexpression of TGF-βs might cause an increased menstrual tissue breakdown and retrograde movement of endometrial tissue fragments.

Endometrial cells are adherent cells that bind to components of the ECM (Klemmt et al., 2007). The process of cell-to-ECM adhesion is regulated by specific cellular adhesion molecules (CAMs). Typical examples for CAMs are integrins, a group of transmembrane proteins, which connect cells to the ECM, such as fibronectin and collagens (Witz et al., 2003). Preliminary results obtained in the present study with recombinant proteins coated on cell culture plates indicated that all four cell lines studied adhered primarily onto fibronectin, collagen I and collagen IV (data not shown).

Koth et al. (2007) showed that integrins can activate latent TGF-β1. However, so far there is no evidence that TGF-βs could affect cell adhesion directly in the endometrium. The regulation might be indirect. High levels of MMP-2 might trigger shedding of endometrial cells by reducing the attachment of cells to the ECM because MMP-2 degrades the ECM (Matrisian and Hogan, 1990). Czekay et al. (2003) reported that PAI-1 inhibited the cell attachment to the ECM components, fibronectin and type I collagen, by inhibiting uPAR-vitronectin and integrin-vitronectin interaction. These data suggest that PAI-1 and MMP-2 negatively affect the cell-to-ECM connection.

In our experiments, we demonstrated that secretion of both, PAI-1 and MMP-2, was dramatically increased by TGF-βs in endometrial and endometriotic cells. Thus, we supposed that TGF-βs might reduce cell adhesion and increase cell shedding by elevating the levels of MMP-2 and PAI-1. In this case, together with inducing the contraction of the uterus, TGF-βs might facilitate retrograde movement of the endometrial tissue fragments.

On the other hand, high levels of TGF-βs might increase the survival potential of ectopic endometrial cells. TGF-β1 suppressed the immune system, which was demonstrated by lethal multi-organ inflammation in gene knockout mice (Shull et al., 1992). Furthermore, TGF-β1 suppressed proliferation and differentiation of lymphocytes including cytolytic T cells, natural killer cells and macrophages (Pardali and Moustakas, 2007). Blocking TGF-β1 function increased the levels of IFN-γ and IL-10, which are secreted by natural killer cells derived from human endometrium

(Eriksson et al., 2004). Thus, high levels of TGF-βs may trigger immune escape, which might decrease the response of natural killer cells to the ectopic tissues.

4.2 Differences between Endometrial and Endometriotic Cells

Sampson's theory of retrograde menstruation is the most widely accepted (Jensen and Coddington, 2010). However, this theory is not able to explain why most women can have retrograde menstruation, but not all of them develop endometriosis. The different characteristics of ectopic endometrial tissues and eutopic endometrial tissues might be one possibility to answer this question. Compared to normal endometrial tissues, endometriotic tissues generally show more capability in metastasis, implantation, invasiveness and proliferation (Liu and Lang, 2011).

Banu et al. (2008) analyzed the expression of several genes associated with steroid synthesis, cell cycle regulation, ECM degradation, angiogenesis, cell growth, cell death and cytokine production. They showed that the genes, which are positively associated with metastasis, implantation, invasiveness and proliferation, are expressed stronger in endometriotic cells, compared to endometrial cells.

In our study, we tested the basal secretion of MMP-2, MMP-9 and PAI-1 in vitro. Our results also showed clear differences between endometrial and endometriotic cells. As shown in the following paragraphs, endometriotic cells produced more MMP-2, MMP-9 and PAI-1, compared to endometrial cells, either in epithelial cells as well as in stromal cells.

4.2.1 MMP-2 and MMP-9 Secretion in Endometrial and Endometriotic cells

The function of MMPs is mainly the degradation of extracellular matrix, which is involved in the shedding process of cells during menstruation and contributes to the invasiveness of ectopic endometrial cells (Juhasz-Böss et al., 2009). However, TGF-βs, which are highly expressed during menses (Gaide Chevronnay et al., 2008), mediate the suppression of mRNA expression of stromelysin (MMP-3) and matrilysin (MMP-7) in the endometrium (Bruner et al., 1995), but increase the expression of gelatinases, MMP-2 and MMP-9 (Hirata et al., 2003). High expression of MMP-2 was also detected in the endometrium of patients with endometriosis (Chung et al., 2002) and in an endometriosis model with transplanted human endometrial tissue into rat

peritoneum (Sotnikova et al., 2010). These data suggest an important role of gelatinases in the pathogenesis of endometriosis. However, they did not test MMP-2 or MMP-9 expression in epithelium or stroma separately.

In this study, we measured the basal secretion of MMP-2 and MMP-9 in endometrial and endometriotic cells in vitro. We found that endometriotic cells generally secreted more MMP-2 and MMP-9, compared to endometrial cells. Similar results were shown by other investigators in mRNA and activity levels. Banu et al. (2008) showed that the mRNA expression and the activity of MMP-2 and MMP-9 in endometriotic cells were stronger, compared to normal endometrial cells. Since MMP-2 and MMP-9 have been well documented to be involved in cell invasiveness and metastasis (Giannelli et al., 2002; Giannelli and Antonaci, 2002), we suppose that endometriotic cells might be more migratory and invasive, compared to normal endometrial cells. In normal endometrial cells, epithelial cells nearly secrete no MMP-2, whereas stromal cells secrete a lot, thus suggesting that MMP-2, which influence tissue breakdown, is mainly secreted by stromal cells. However, in the case of endometriotic cells, the epithelial cells were able to secrete a moderate amount of MMP-2. It suggests that not only the endometriotic stromal cells, but also the endometriotic epithelial cells might also contribute to the ECM degradation and migration/invasion of endometriotic tissues during retrograde menstruation.

The MMP-9 secretion was much lower compared to MMP-2 secretion in all cell lines studied. It suggests that the role of MMP-9 might be limited in the pathogenesis of endometriosis. However, MMP-9 is detectable in endometriotic cells after all but not in endometrial cells, which might contribute to the different characteristics of endometriotic cells and endometrial cells.

Furthermore, MMP-2 and MMP-9 in the endometrium or endometriosis might not only be secreted by endometrial cells. Macrophages also secrete large amounts of various MMPs (Goetzl et al., 1996). As immune cells, macrophages take part in the development of endometriosis (Weiss et al., 2009). Thus, macrophages might be another possible source of MMP-2 and MMP-9 in the endometrium or endometriosis.

4.2.2 PAI-1 Secretion in Endometrial and Endometriotic cells

PAI-1 is the major inhibitor of both tissue-type plasminogen activator (tPA) and urokinase-type plasminogen activator (uPA). They are both involved in tissue

degradation and remodeling (Zorio et al., 2008). Bruse et al. (1998, 2003) showed that the expression of PAI-1 and uPA were much higher in the endometriotic and endometrial tissue of women with endometriosis, compared to endometrial tissue of women without endometriosis. The PAI-1 levels were higher in ectopic tissues than in eutopic tissues of the same woman (Bruse et al., 2003). These data strongly suggests an important role of PAI-1 in endometriosis. Czekay et al. (2003, 2011) showed that cell migration was regulated by uPA and its high affinity receptor, uPAR. They demonstrated that PAI-1 perturbed cell attachment to the ECM by inhibiting uPAR-vitronectin and integrin-vitronectin interactions, and suggested that PAI-1 facilitates cell migration by regulating ECM proteolysis.

In this study, we compared the basal secretion of PAI-1 in endometrial and endometriotic cells in vitro. We found that endometriotic cells generally secreted more PAI-1 than endometrial cells. Our results showed an increased PAI-1 synthesis in endometriotic cells compared to endometrial cells, which corresponds to the result of increased PAI-1 expression that was observed in endometriotic tissues (Bruse et al., 2003). It suggests that endometriotic cells might be more potent to migrate, compared to normal endometrial cells. Secretion of PAI-1 in normal endometrial tissue might mostly rely on stromal cells since the endometrial epithelial cells secrete nearly no PAI-1. However, in endometriotic tissues, PAI-1 secretion is contributed by both epithelial and stromal cells, because the endometriotic epithelial cell can secrete a moderate amount of PAI-1.

4.3 TGF-β Signaling in Endometrial and Endometriotic Cells

Among all the pathways, the Smad-dependent pathway is considered to be the primary pathway of TGF-β signaling, which is activated by the phosphorylation of TβRI on serine/threonine (Massagué and Wotton, 2000). After being phosphorylated, TβRI propagates the signal to downstream substrates, mostly Smad2/3 (Massagué and Wotton, 2000). In addition to the Smad-dependent pathway, there are Smad-independent pathways, which are also activated by the TGF-β receptors (Kretzschmar et al., 1997; Lee et al., 2007; Zhang et al., 2009).

In our studies, we demonstrated a TGF-β-induced MMP-2 and PAI-1 secretion in endometrial and endometriotic cells. Furthermore, by using specific inhibitors

targeting downstream cascades of TGF-β signaling, both Smad-dependent and Smad-independent pathways were studied.

4.3.1 The Smad-dependent Pathway in TGF-β Signaling

The transducers of the Smad-dependent pathway include the receptor regulated (R-) Smads (Smad1, 2, 3, 5 and 8), the common mediator (co-) Smad (Smad4) and inhibitory (I-) Smads (Smad6 and 7). Generally, signals from TGF-βs are mediated through Smad2 and Smad3 (Shimizu et al., 1998). However, Kang et al. (2009) reported that TGF-β can transiently activate Smad1 and Smad5 as well.

In our study on the Smad-dependent pathway, we used a TβRI inhibitor (LY364947) that specifically blocks the kinase activity of the TβRI and thus also the phosphorylation of Smad2 and Smad3 (Sawyer et al., 2003). Our results showed that LY364947 can completely inhibit the TGF-β-induced secretion of both MMP-2 and PAI-1 in endometrial and endometriotic cells. Unpublished results of our group showed that a specific Smad3 inhibitor (SiS3) inhibited the TGF-β-induced secretion of PAI-1, partially but not completely, thus, suggesting also the involvement of Smad2 in TGF-β signaling. These data support the fact that Smads are the most important mediators of TGF-β signaling from the receptors to the nucleus.

4.3.2 The Smad-independent Pathways in TGF-β Signaling

Zhang et al. (2009) summarized the non-Smad pathways, which reinforce or modulate the signal transduction of the TGF-βs. In several in vitro studies on cell models, the small GTPase Ras and the extracellular signal-regulated kinases (ERKs), p38 mitogen-activated protein kinase (p38 MAPK) and c-Jun N-terminal kinases (JNKs) are found to be implicated in TGF-β signaling (Yue and Mulder, 2000). Furthermore, they are suggested to interact with the Smad-dependent pathway (Massague et al., 2000; Moustakas and Heldin, 2005). The ERK pathway can lead to direct phosphorylation of R-Smads, blocking their nuclear translocation and transcriptional output (Kretzschmar et al., 1997). The p38 substrate kinase, mitogen and stress activated protein kinase-1 (MSK1) regulates the transcriptional activity of Smad3 by promoting its association with the co-activator p300 (Abécassis et al.,

2004). In addition, TGF-β-activated JNK phosphorylate Smad3 and induce its nuclear translocation and transcriptional activity (Engel et al., 1999).

In our studies on the non-Smad-dependent pathways, we used an ERK inhibitor, which specifically inhibits the activity of ERK1 and ERK2 (Ohori et al., 2005), and SB203580, a p38/MAPK inhibitor (Roux and Blenis, 2004). Our results showed that the ERK inhibitor moderately inhibited the TGF-β-induced secretion of MMP-2 and PAI-1 in endometrial and endometriotic cells. SB203580 showed a slight inhibiting effect on the TGF-β-induced secretion of MMP-2 and PAI-1 in epithelial cell, whereas it showed no effect on stromal cells. Our results suggest that ERKs are involved in the TGF-β-induced gene transduction in endometrial and endometriotic cells, whereas p38/MAPK seems to be only slightly involved in the TGF-β-induced gene transduction in epithelial cell, but not in stromal cells.

Furthermore, we studied the intracellular localization of phospho-ERK1/2 in endometrial and endometriotic cells. Our results showed that, without TGF-β stimulation, phospho-ERK1/2 is expressed slightly in endometrial stromal cells and endometriotic cells (22B>THESC>12Z), but not in endometrial epithelial cells. Furthermore, expression of phospho-ERK1/2 is enhanced rapidly by the stimulation of TGF-βs and accumulated in and around the nucleus, thus suggesting that phospho-ERK1/2 is translocated from the cytoplasm to the nucleus after TGF-β stimulation. These results corroborate the results obtained with the ERK inhibitor, that ERKs are involved in the TGF-β induced MMP-2 and PAI-1 gene transcription in endometrial and endometriotic cells.

Because our results demonstrated besides the Smad pathway, the involvement of the ERK pathway in TGF-β signaling in endometrial and endometriotic cell lines, it was important to elucidate the connection between the TGF-β-induced Smad pathway and ERK pathway. With respect to the Smad pathway, TGF-β signals through its cell surface receptor (TβRI), which phosphorylates Smad2 or Smad3. Then Smad2/3, together with Smad4, binds to the Smad binding elements (SBEs) or transcription factor E3 (TFE3) (Hua et al., 1999; Massagué and Wotton, 2000).

Unlike the Smads, the phosphorylation of ERKs requires the activation of tyrosine kinases on the cell surface receptors, such as the EGF receptor. The activated tyrosine kinases phosphorylate growth factor receptor-bound protein-2 (GRB2) and (son of sevenless) SOS, which then activate the Ras cascades including the ERKs (Dance et al., 2008). Samarakoon et al. (2008) demonstrated that the activation of

ERKs leading to PAI-1 transcription required the phosphorylation of the EGF receptor by TGF-β1 in vascular smooth muscle cells. Therefore, we performed PathScan® ELISAs targeting the phospho-EGF receptor on three different tyrosine sites. However, the phospho-EGF receptor was not detectable in endometrial and endometriotic cells after TGF-β stimulation. Hence, TGF-βs does not activate ERKs via the EGF receptor in endometrial and endometriotic cells.

Other possibilities were shown by Lee et al. (2007), who demonstrated that TβRI directly phosphorylated ShcA, which induced the association with GRB2 and SOS, then leading to a sequential activation of Ras, Raf, MEK1/2 and ERK1/2. Besides, Suzuki et al. (2007) demonstrated that TGF-β activated the Ras-dependent ERK phosphorylation through the activation of p21 activated kinase-2 (PAK2). These data showed that TGF-β could directly activate the ERKs without participation of the Smad pathway. Because we could not find any EGF receptor phosphorylation in endometrial and endometriotic cells, thus we suppose that the ERKs might be activated by TGF-βs via Grb2/SOS, but this needs to be explored further.

4.4 Conclusions

In this study, we demonstrated the influence of TGF-βs on endometrial and endometriotic cell lines in vitro. We found that the endometriotic cells secreted more MMP-2, MMP-9 and PAI-1, compared to normal endometrial cells. We showed that TGF-βs dramatically increased secretion of MMP-2 and PAI-1 in endometrial and endometriotic cells. We also showed that PAI-1 reduced the cell attachment of all four cell lines studied. From our data, we suppose that the TGF-β-induced increase in PAI-1 and MMP-2 secretion might play a role in increased tissue breakdown during menstruation and the increased invasiveness during ectopic endometrial implantation. We confirmed that the Smad pathway was the main pathway of TGF-β signaling in endometrial and endometriotic cells. Furthermore, we demonstrated for the first time the participation of the ERK pathway, a mainly Smad-independent pathway, in TGF-β signaling in endometrial and endometriotic cells. However, more experiments are still required to clarify the connection between ERKs and TGF-β, as well as the crosstalk between Smads and ERKs. Nevertheless, our findings might provide new clues on studying or understanding the role of TGF-βs in the pathogenesis of endometriosis.

5 Summary

Endometriosis is a chronic pathological disorder in which endometrial-like cells are found outside the uterine cavity. TGF-βs were observed to be highly expressed in the peritoneal fluid of patients with endometriosis, as well as in endometriotic sites. Thus, TGF-βs may play an important role in the pathogenesis of endometriosis. In this study, our aim was to investigate the biological function and signal transduction of TGF-βs in endometrial and endometriotic cells in vitro.

Four different cell lines, endometrial epithelial and stromal cells, endometriotic epithelial and stromal cells were used in this study. Cells were treated with or without TGF-β1 or TGF-β2, respectively. Then the cell numbers were counted and the secretion of MMP-2, MMP-9 or PAI-1 was measured with ELISAs. Also the effect of PAI-1 on cell adhesion was tested. By using specific inhibitors targeting downstream cascades of TGF-β signaling, both Smad-dependent and Smad-independent pathways were studied. In addition, the localization of phospho-ERK1/2 after stimulation with TGF-β1 or TGF-β2 was analyzed with immunofluorescence.

Our results showed in the four cell lines studied: (1) TGF-βs had a dual effect on the cell numbers, with increased cell numbers when the initial cell number was low, but decreased cell numbers when the initial cell number was high. (2) Endometriotic cells secreted more MMP-2, MMP-9 and PAI-1, compared to endometrial cells. TGF-β1 or TGF-β2 dramatically increased MMP-2 and PAI-1 secretion in all cell lines studied. (3) A TβRI inhibitor completely blocked the TGF-β-induced MMP-2 or PAI-1 secretion. An ERK1/2 inhibitor partly blocked it. The p38/MAPK inhibitor had only slight effects. (4) TGF-β1 or TGF-β2 enhanced accumulation of phospho-ERK1/2 in the nucleus. (5) PAI-1 reduced cell attachment in all four cell lines studied.

In this study, we demonstrated the influence of TGF-βs on endometrial and endometriotic cell lines in vitro. From our data, we suppose that the TGF-β-induced increase in PAI-1 and MMP-2 secretion might play a role in increased tissue breakdown during menstruation and increased invasiveness during ectopic endometrial implantation. We confirmed that the Smad pathway was the main pathway of TGF-β signaling also in endometrial and endometriotic cells. Furthermore,

we demonstrated for the first time the participation of the ERK pathway, a mainly Smad-independent pathway, in TGF-β signaling in endometrial and endometriotic cells. This finding might provide new options to comprehend the role of TGF-βs in the etiology of endometriosis. However, since it is only a first glimpse into the ERK pathway in TGF-β signaling in endometrial and endometriotic cells, more researches are required to elucidate the connection between ERKs and TGF-β, as well as the crosstalk between Smads and ERKs.

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7 Acknowledgement

Firstly, I would like to give my deepest gratitude to my supervisor, PD. Dr. Lutz Konrad, whose encouragement, supervision and support from the preliminary to the concluding level enabled me to develop an understanding of the subject. This project would not have been possible to be finished without his help and efforts.

Secondly, I am heartily thankful to Prof. Hans-Rudolf Tinneberg, the director of the Department of Gynecology and Obstetrics of UKGM, who invited me to Giessen and provided me the opportunity to study for the doctor's degree. He also gave me a lot of help and advice during my study in Giessen and opportunities to aquire more clinical skills. I am also grateful for my co-supervisor, Prof. Charles Omwandho, associate professor of the Departpent of Biochemistry of the University of Nairobi, who gave me a lot of advices and helps in the first year of my study. Aslo with great respect, I give my appreciation to Prof. Etienne Marbaix, Professor of Pathology at the Faculty of Medicine of the Université catholique de Louvain, who gave me a lot of advice in MMPs.

Thirdly, I would like to express my profound appreciation to Mrs. Zoltan, MTA of the Department of Gynecology and Obstetrics, who taught me most of the techniques in cell culture and ELISAs. Without her kindest help, it would not be possible for me to finish my experiments. I would also like to thank Dr. Hersemeyer and the Department of Biochemistry of the JLU Giessen, who kindly and technically supported me in some experiments of my project.

Last but not least, I am grateful for the help and assistance from the other members in our group. Thanks to Dr. Stammler, who helped me to correct my dissertation, and thanks to Mrs. Hof and Mrs. Gill, for their help and support during my studies in the lab.

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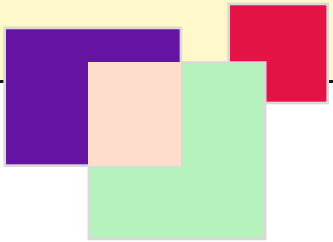
Cong Sui

Gießen, 2011



édition scientifique
VVB LAUFERSWEILER VERLAG

VVB LAUFERSWEILER VERLAG
STAUFENBERGRING 15
D-35396 GIESSEN

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

ISBN: 978-3-8359-5885-2