The Type III Transforming Growth Factor-β receptor (Betaglycan or BG) Modulates TGF-β Signaling and Wound Healing in Human Endometrial Cells and in Endometriosis

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by
Mwaura, Agnes Njoki
M.Sc (Biochemistry), UON
from Nairobi, Kenya

Giessen (2022)

From the Institute of Gynecology and Obstetrics UKGM
Director: Prof. Ivo Meinhold-Heerlein
Department of Gynecology Laboratory Manager: PD. Dr. Lutz Konrad of the Faculty of Medicine of the
Justus Liebig University Giessen

First Supervisor and Committee Member: PD. Dr. Lutz Konrad
Second Supervisor and Committee Member: Prof. Dr. Georgios Scheiner-Bobis
Committee Members: Prof. Dr. Christine Wrenzycki
Prof. Dr. Martin Götte

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ABBREVIATIONS

Act A	Activin A
ACVRI	Activin receptor type I
ACVR2	Activin receptor type II
ADAM	A disintegrin and metalloproteinase
ADAM12S	Short variant of a disintegrin and metalloproteinase12
ADAM12L	Long transmembrane variant of a disintegrin and metalloproteinase12
ALK	Activin receptor-like kinase
ANOVA	One-way analysis of variance
AREs	Activin-responsive elements
ATCC	American type culture collection
ATP	Adenosine triphosphate
AUC	Area under the curve
BAMBI	BMP and activin membrane-bound inhibitor
BCA	Bicinchoninic acid
bFGF2	Basic fibroblast growth factor-2
BG	Betaglycan
BMI	Body mass index
BMP	Bone morphogenetic protein
Bp	Base pair
BSA	Bovine serum albumin
°C	Degree Celsius
CCK-8	Cell counting kit-8
ccRCC	Clear-cell renal cell carcinoma
CD	Cluster of differentiation
cDNA	Complementary DNA
CO_2	Carbon dioxide
COV434	Ovarian granulosa cell line
CTGF	Connective tissue growth factor
Ctrl	Control
DAB	3,3′-Diaminobenzidine
DAPI	4′,6-diamidino-2-phenylindole
DIE	Deep infiltrating endometriosis
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
dNTP	2'-deoxynucleoside-5'-triphosphate

et al.	And others
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
ELF	Embryonic liver fodrin
ELISA	Enzyme-linked immunosorbent assay
EM	Endometriosis
EMT	Epithelial-mesenchymal-transition
Endo	Endometrium
ERK	Extracellular-signal-regulated kinases
EVT	Extravillous cytotrophoblast
FAK	Focal adhesion kinase
FCS	Fetal calf serum
Fig	Figure
FSH	Follicle-stimulating hormone
FST	Follistatin
G	Gravitational force
GAG	Glycosaminoglycan
GAIP	G alpha interacting protein
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GCT	Granulosa cell tumors
GDFs	Growth and differentiation factors
GIPC	GAIP-interacting protein C-terminus
GM6001	N-[(2R)-2-(hydroxamidocarbonylmethyl)-4-methylpentanoyl]-L-tryptophan methylamide
H	Hour
HB-EGF	Heparin-binding EGF-like growth factor
HEK	Human embryonic kidney cells
HRP	Horseradish peroxidase
HSCs	Hepatic stellate cells
IGFBP	Insulin-like growth factor binding protein
Inh A	Inhibin A
IGF	Insulin growth factor
IL-6	Interleukin 6
ITS	Insulin transferrin selenium
JNK	c-Jun N-terminal kinase
kDa	Kilodalton
KGN	Ovarian granulosa-like tumor cell line
LAP	Latency associated peptide

LTBP	Latent-TGF-β binding protein
LY294002	2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one
LY364947	4-[3-(2-pyridinyl)-1H-pyrazol-4-yl]-quinoline
M	Molar
MAPK/MEK	Mitogen activated protein kinase
mBG	Membrane-bound betaglycan
MEFs	Mice embryonic fibroblasts
MEM	Minimal essential medium
MH	Mad homology domain
MRI	Magnetic resonance imaging
Mg	Milligram
MT-MMPs	Membrane-type matrix metalloproteinases
Min	Minute
mL	Milliliter
MMP	Matrix metalloproteinase
mRNA	Messenger ribonucleic acid
MRKH	Mayer–Rokitansky–Küster–Hauser
MUC1	Mucin1
MuSK	Muscle-specific receptor tyrosine kinase
Myo	Myometrium
ng/mL	Nanograms per milliliter
Neg. siRNA	Control siRNA
NES	Nuclear export signal
NFκB	Nuclear factor kappa B
NLS	Nuclear localization signal
Nm	Nanometer
NSCLC	Non-small-cell lung carcinoma
OD	Optical density
OE	Ovarian endometriosis
PAI	Plasminogen activator inhibitor
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PE	Peritoneal endometriosis
Pen/strep	Penicillin/streptomycin
pg/mL	Picograms per milliliter
PI3K/akt	Phosphatidylinositol-3-Kinase/protein kinase B
PMS	Phenazinium methylsulfate
PMSF	Phenylmethylsulfonyl fluoride
RD	Rhabdomyosarcoma

ROC	Receiver operating characteristic curve
rhBG	Recombinant betaglycan
RGMs	Repulsive guidance molecules
rhTIMP	Recombinant tissue inhibitor of metalloproteinase
RNA	Ribonucleic acid
RNase	Ribonuclease
RT	Room temperature
RT-qPCR	Reverse transcription quantitative real-time PCR
SARA	SMAD anchor for receptor activation
SBE	SMAD-binding elements
sBG	Soluble betaglycan
SEM	Standard error of the mean
siRNA	Small interfering RNA
SIS3	Specific inhibitor of SMAD3
SM	Smooth muscles
SMAD	Small mothers against decapentaplegic
STAT3	Signal transducer and activator of transcription 3
TA	Trans-activation
TACE	Tumor necrosis factor-converting enzyme
TAE	Tris acetate EDTA
TAPI	TNF-α protease inhibitor
TBE	Transcription factor binding elements
TBRI	Type I TGF-β receptor
TBRII	Type II TGF-β receptor
TBRIII	Type III TGF-β receptor
TF	Transcription factors
TGF-β	Transforming growth factor-β
TGFBR3	Transforming growth factor beta receptor 3
THESC	hTERT human endometrial stromal cells
TIMP	Tissue inhibitor of metalloproteinase
TNF-α	Tumor necrosis factor alpha
Tris	Trisaminomethane
U	Unit
UO126	1,4-Diamino-2,3-dicyano-1,4-bis(o-aminophenylmercapto)butadiene
UV	Ultraviolet
V	Volt
w/o EM	Without endometriosis

WST-8	(2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium
µ	Micro
µg	Microgram
µL	Microliter
µM	Micromolar
ZPD	Zona pelucida domain

1 INTRODUCTION

1.1 Endometriosis

Approximately 0.7 - 8.6% of reproductive-age women suffer from endometriosis, a chronic and estrogen-dependent gynecological condition, typically associated with chronic pelvic pain, dyspareunia, dysmenorrhea, dyschezia, and infertility (Ballard et al., 2008; Vercellini et al., 2014; Zondervan et al., 2018; Ghiasi et al., 2020). Endometriosis is characterized by occurrence of endometrial glands and stroma outside the uterine cavity; primarily in the ovaries, pelvic peritoneum, bladder, bowel, and retro-vaginal septum (Fig. 1) or occasionally in the brain, cutaneous tissue, liver, bone, pleura, or pericardium (Vercellini et al., 2014; Zondervan et al., 2018).

Endometriosis is classified into three major phenotypes: ovarian endometriosis, superficial peritoneal endometriosis, and deep infiltrating endometriosis (Zondervan et al., 2018). Endometriosis involving the ovary is the most common form (17 - 44%) and affects up to 50% of women suffering infertility and abdominal pain (Busacca and Vignali, 2003). Endometriosis exhibits features similar to that of benign tumors including cell growth, cell migration and invasion, neo-vascularization and reduced apoptosis (Omwandho et al., 2010; Vercellini et al., 2014; Dutta et al., 2015; Zondervan et al., 2018).

1.1.1 Pathogenesis of endometriosis

Endometriosis was first described by Carl Von Rokitansky in 1860, but to date, more than 160 years later, the exact mechanisms responsible for development of endometriosis remain unclear and elusive (Vercellini et al., 2014). A number of hypotheses have been proposed to explain the development and survival of ectopic endometrial lesions.

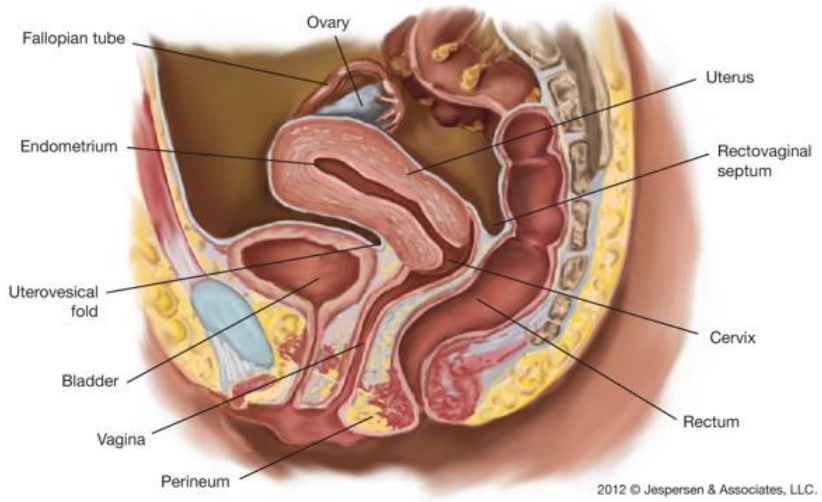


Figure 1. Anatomy of the female reproductive system showing common locations of endometriosis within the pelvic region (Smith et al., 2002).

Retrograde menstruation theory (Sampson, 1927) which postulates that endometriosis arises from reflux of endometrial fragments regurgitated via the fallopian tubes during menstruation with consequent implantation at ectopic locations is the most widely accepted theory. The theory, however, fails to explain why only about 0.7 - 8.6% of women develop endometriosis despite the fact that the majority (>70%) of women experience retrograde menstruation (Halme et al., 1984; Zondervan et al., 2018). Thus, although retrograde menstruation is regarded as an important origin of the endometrial fragments, other contributors may be involved. These include altered immune and inflammatory responses along with a favorable endocrine and metabolic environment in genetically susceptible women (Giudice and Kao, 2004; Rahmioglu et al., 2014; Dutta et al., 2015; 2018; Thézénas et al., 2020).

Other hypotheses of the origins of endometrial cells at ectopic sites include the coelomic metaplasia theory (supported for ovarian endometriosis) first described by Meyer (1924). Although this theory partly explains the occurrence of endometriosis at extra-uterine sites as well as the rare endometriosis in males, it lacks a molecular or cellular basis (Gazvani and Templeton, 2002). Additionally, recently we showed that Mayer–Rokitansky–Küster–Hauser (MRKH) patients, who sometimes lack a uterus or endometrium, only develop endometriosis if a uterus/endometrium is present, which underscores Sampson implantation

hypothesis (Konrad et al., 2019a). The vascular and lymphatic dissemination of endometrial cells has been postulated as a complimentary theory to explain presence of endometriosis outside the pelvic cavity (Javert, 1952). Moreover, other theories such as the endometrial stem cell implantation (Sasson and Taylor, 2008), Müllerian remnant abnormalities (supported for endometriosis infiltrating the cul-de-sac and uterosacral ligaments) and the neonatal uterine bleeding (Gargett et al., 2014) are still debated and require further investigation.

1.1.2 Diagnosis and treatment of endometriosis

The symptoms of endometriosis often overlap with other gynecological conditions like adenomyosis, pelvic inflammatory disease, ovarian cysts, pelvic adhesions, in addition to several other non-gynecological conditions like irritable bowel syndrome, inflammatory bowel syndrome, and intestinal cystitis (Carneiro et al., 2010; Dutta et al., 2018). Such diverse symptomatology, lack of awareness along with the dependence on laparoscopy as the gold standard for diagnosis causes delay in diagnosis of endometriosis which is estimated to be between 4-11 years (Agarwal et al., 2019).

Presently, diverse diagnostic methods for endometriosis such as transvaginal ultrasonography, recto-sigmoidoscopy, magnetic resonance imaging (MRI) and urinary apparatus imaging are available and may be useful in detection of deep nodular forms of endometriosis and ovarian endometriomas but not superficial peritoneal endometriosis (Vercellini et al., 2014). Conclusive diagnosis of endometriosis remains surgical assessment of the pelvic region by laparoscopy followed by histological confirmation (Giudice and Kao, 2004). These requirements underscore the need for identification of reliable molecular biomarkers, which are currently unavailable, for early diagnosis of the condition prior to investigative surgery. Thus, we investigated whether new biomarkers such as soluble betaglycan (sBG) and a disintegrin and metalloproteinase12 (ADAM12) concentrations in serum and/or endocervical mucus can be used as non-invasive diagnostic biomarkers for endometriosis.

Existing treatment options for endometriosis only transiently alleviate but do not cure the condition. Currently available therapy for endometriosis include surgery, hormone therapies

(gonadotrophin releasing hormone antagonists, selective progesterone receptor modulators, selective estrogen receptor modulators and aromatase inhibitors), and/or analgesics that may be contraceptive and associated with numerous undesirable side effects (Falcone and Lebovic, 2011; Vercellini et al., 2014; Parasar et al., 2017).

1.2 The transforming growth factor beta (TGF-β) superfamily

The TGF-β superfamily comprises structurally related proteins including the TGF-βs, activins, inhibins, bone morphogenetic proteins (BMPs), along with growth and differentiation factors (GDFs) (Derynck and Budi, 2019). These proteins are fundamental to normal cellular functions like cell proliferation, survival, differentiation, matrix production, motility, angiogenesis, apoptosis, as well as immune modulation (Omwandho et al., 2010; Finnson et al., 2013; Dela Cruz and Reis, 2015; Young et al., 2017). The proteolytic activity of matrix metalloproteinases is modulated by cytokines, growth factors, hormones and several other biologically active agents (Balkowiec et al., 2018). Therefore, in the current study, we investigated whether TGF-β, activin A and inhibin A influence secretion of MMP2 and MMP3 in endometrial cells.

1.2.1 Transforming growth factor beta

There are three mammalian TGF-β isoforms namely; TGF-β1, TGF-β2, and TGF-β3 and showing significant sequence homology (between 60 and 80%) (Penn et al., 2012). The three TGF-β isoforms are synthesized with large amino-terminal pro-domains (~ 250 residues), which are essential for proper folding and dimerization of the carboxy-terminal growth factor domains (~ 110 residues) (Lichtman et al., 2016). The isoforms are secreted in a dimerized, latent complex which also encompasses a latent TGF-β binding protein (LTBP). The large latent complex may associate with the extracellular matrix where TGF-β may be stored until activation by thrombin and other serum proteases. Activation involves cleavage of the LTBP to release mature active TGF-β (Tandon et al., 2010) (Fig. 2).

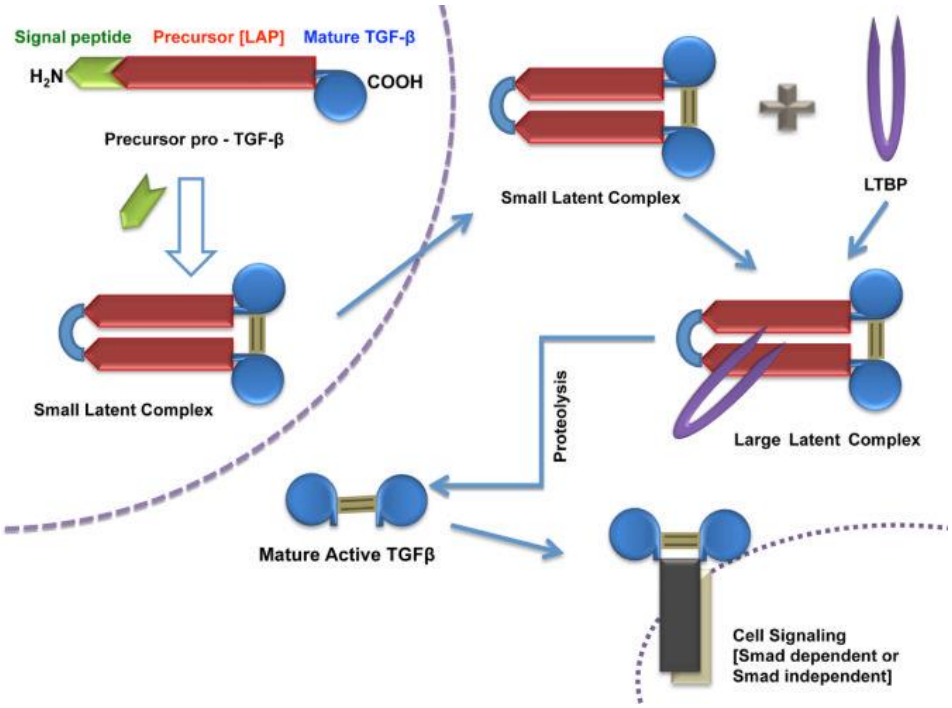


Figure 2. Schematic presentation of TGF-β maturation. The inactive TGF-β precursor undergoes enzymatic (endopeptidase) cleavage in the Golgi apparatus to release the small latent complex. The small complex then covalently binds with latent TGF-β binding protein (LTBP) and forms the large latent complex that is finally released into the extracellular matrix as mature TGF-β. After activation of bioactive TGF-β, a 25 kDa protein, signal transduction through various signaling pathways may ensue (Tandon et al., 2010).

1.2.2 Activins and inhibins

Activins and inhibins are among the 33 members of the TGF-β family and are implicated in a variety of biological processes including regulating erythropoiesis, development, nervous system, reproduction, body composition and energy metabolism and inflammation (Namwanje and Brown, 2016). They share common β subunits, with activins occurring as ββ homodimers, and inhibins as αβ heterodimers (Fig. 3). All activin and inhibin subunits are synthesized as pre-proproteins, which undergo proteolytic cleavage to form mature subunits (Jones et al., 2002). Dimerization of mature β subunits alone yields activin AB, activin A or activin B, whereas the mature α subunit (αC) can dimerize with mature βA or βB subunits to form inhibin A and inhibin B, respectively.

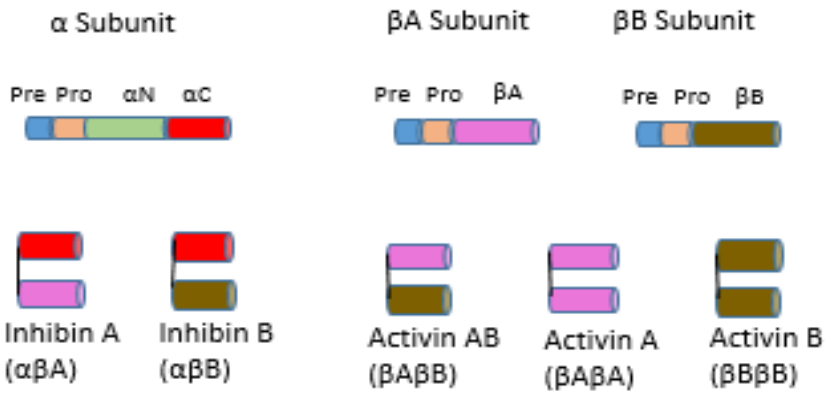


Figure 3. Structure of inhibins and activins. Inhibin and activin monomers are synthesized as pre-proproteins which are processed to form mature heterodimers (inhibins) and homodimers (activins) (Namwanje and Brown, 2016).

1.2.3 The mechanism of TGF-β signaling

Virtually all members of the TGF-β superfamily signal via pairs of serine/threonine kinase receptors, in the case of the TGF-βs, the type I and II TGF-β receptors (TBRI and TBRII), which assemble into heteromeric complexes on the cell surface (Massagué, 1998). TGF-β ligands bind to their respective cell surface receptors followed by a cascade of phosphorylation and activation events of specific downstream targets (Fig. 4).

In the canonical TGF-β signaling pathway, the TGF-β ligands activate members of the SMAD transcription factor family (Moustakas et al., 2001). TGF-βs and activins signal through SMAD2/3 while BMPs require SMAD1/5/8. Receptor-activated SMADs then bind to the common SMAD4 and translocate to the nucleus where they associate with SMAD-binding elements (SBE), transcription factors (TF) and transcription factor binding elements (TBE) resulting in transcriptional modification of target genes (Moustakas et al., 2001). In the non-canonical signaling pathway, the TGF-β ligands activate members of the mitogen activated protein (MAP) kinase signaling molecules, like p38, extracellular-signal-regulated kinases (ERKs), c-Jun N-terminal kinase (JNK) and the phosphatidylinositol-3-kinase/protein kinase B (PI3-K/Akt) pathways (Zhang, 2009). Accordingly, we investigated

which signaling pathways are utilized in TGF-β/activin A-mediated modulation of BG shedding in endometrial cells.

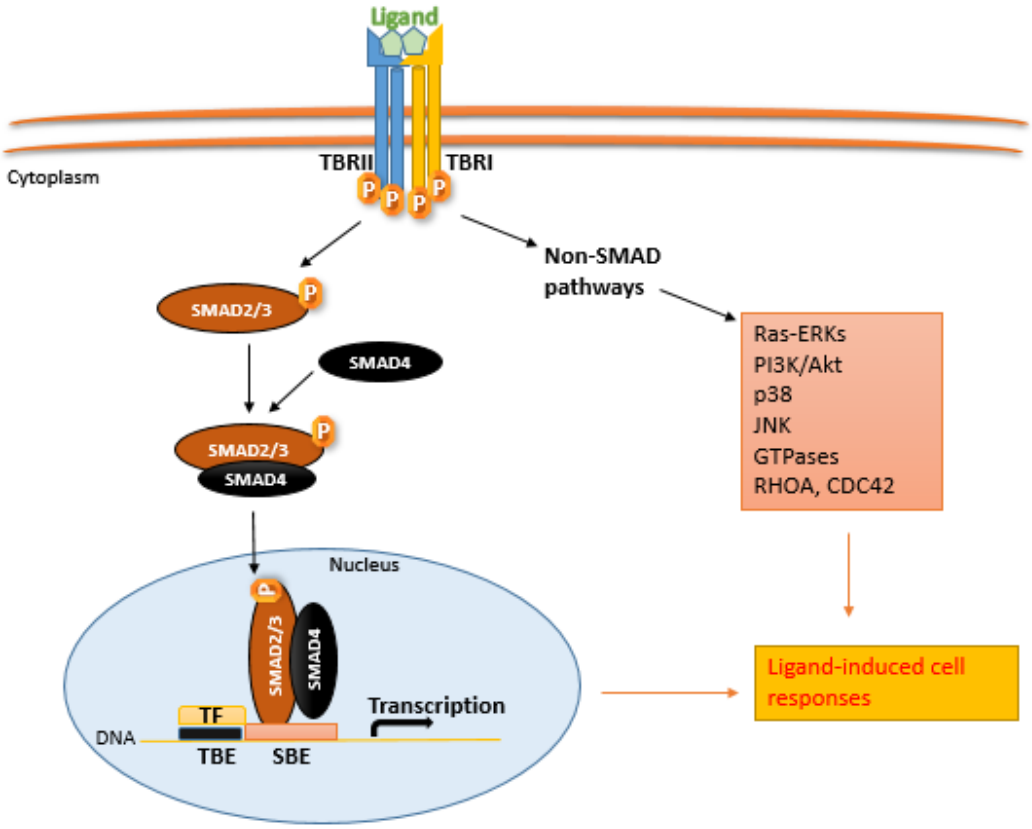


Figure 4. Schematic presentation of TGF-β signaling. Generally, signaling is initiated with ligand-induced oligomerization of signaling receptor kinases followed by phosphorylation of the cytoplasmic signaling transcription factors, SMAD2 and SMAD3. Carboxy-terminal phosphorylation of SMADs by activated receptors results in their partnering with the common signaling transducer, SMAD4, and translocation to the nucleus. Activated SMADs modulate diverse biological processes by partnering with transcription factors resulting in cell-specific regulation of transcription. In certain contexts, TGF-β signaling may also affect SMAD-independent pathways, including ERK, PI3K/Akt, JNK, and p38 MAPK pathways. SMAD-binding elements (SBE), transcription factors (TF) and transcription factor binding elements (TBE) (Moustakas et al., 2001; Zhang, 2009).

Activin signaling requires several steps which are initiated by binding of the activin dimer to two serine/threonine kinase type II activin receptors which are then phosphorylated and activated (Namwanje and Brown, 2016). This results in recruitment and association with two type I activin receptors, ACVR1B (ALK-4) or ACVR1C (ALK-7), that are subsequently phosphorylated and activated. The ligand-receptor complex then initiates SMAD signaling via phosphorylation of SMAD2 and/or SMAD3 close to their carboxyl termini, with subsequent association of the two phosphorylated SMADs with SMAD4. The complex then translocate to the nucleus where binding of the SMAD complex and transcription coactivators to activin-responsive elements lead to transcription of hundreds of target genes (Namwanje and Brown, 2016).

Inhibin antagonizes activin signaling via association of its β subunit with a single type II activin receptor as well as the association of its α subunit with betaglycan thus forming an inactive inhibin–receptor complex. This complex is incapable of signal transduction as it leads to sequestering of type II activin receptors thus inhibiting activin signaling (Lewis et al., 2000; Appiah Adu-Gyamfi et al., 2020).

1.2.4 The SMAD proteins

The TGF-β superfamily directly activates the SMAD signaling pathway, besides other SMAD-independent pathways (Moustakas et al., 2001). The SMAD family of proteins can be divided into three functional groups namely; the receptor-activated SMADs (R-SMADs; SMAD1/2/3/5/8), common mediator SMADs (Co-SMADs; SMAD4), and the inhibitory SMADs (I-SMADs; SMAD6/7) (Massagué, 1998). SMAD2 and SMAD3 are structurally similar and are considered to be equally important in mediating TGF-β signals (Moustakas et al., 2001). The SMAD signaling pathway is initiated by phosphorylation of SMAD2/3 on their C-termini by TBRI (Massagué, 1998). Typically, adaptor proteins like SMAD anchor for receptor activation (SARA) bind and present SMAD2 and SMAD3 to the TGF-β receptors. After activation, SMAD2 and SMAD3 oligomerize with SMAD4 and translocate to the nucleus where they interact with DNA, and recruit other transcription factors, co-activators, and/or co-repressors to regulate transcription of specific target genes (Massagué, 1998).

The SMAD proteins are characterized by two conserved regions identified as the N-terminal Mad homology domain-1 (MH1) and C-terminal Mad homology domain-2 (MH2), which are joined by a short, poorly conserved linker region (Moustakas et al., 2001). The R and Co-SMADs possess an N-terminal nuclear localization signal (NLS) and SMAD4 possesses a nuclear export signal (NES) in the MH1 domain (Brown et al., 2007). Activated type I receptors phosphorylate regulatory R-SMADs at the conserved Ser-Ser-X-Ser (SSXS) motifs located at their C-terminal. The SMAD2 and SMAD3 share 66% amino acid sequence identity between their MH1 domains and 96% amino acid sequence identity between their MH2 domains (Brown et al., 2007). The two domains interact with several proteins including ubiquitination adaptors and substrates, several transcription factors, and transcriptional co-activators and co-repressors (Moustakas et al., 2001). The MH1 domain is highly conserved among R-SMADs and Co-SMADs and functions in R- and Co-SMAD nuclear importation, cytoplasmic anchoring, DNA binding, as well as modulation of transcription of target genes. Contrary, the N-terminal parts of I-SMADs have only weak sequence similarity to MH1 domains (Moustakas et al., 2001). The MH2 domain is highly conserved among all SMADs and functions to regulate SMAD oligomerization, recognition by type I receptors, cytoplasmic anchoring, along with transcription (Brown et al., 2007) (Fig. 5).

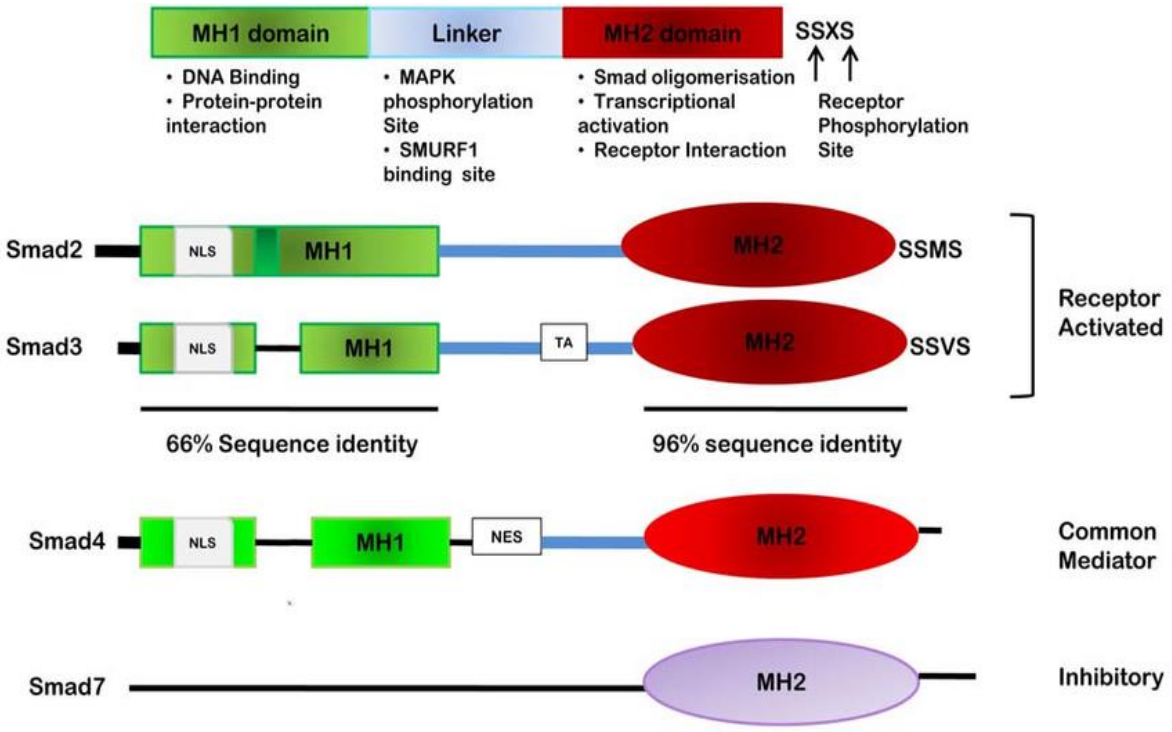


Figure 5. Schematic presentation of the structure of the three SMAD subfamilies. The MH1 domain is colored in green and the MH2 domain in red. MH1 domain of SMAD2 contains an additional 30 amino acids (dark green box). SMAD3 possesses a trans-activation (TA) region in its linker domain. SMAD4 contains a nucleus export signal (NES) in its linker region. SMADs2/3/4 contain a nucleus localization signal (NLS) in their MH1 domain. SMAD7 lacks MH1 domain. The SSXS motif of R-SMADs indicates the serine residues which are phosphorylated upon ligand binding (Samanta and Datta, 2012).

1.2.5 TGF-β superfamily co-receptors

Accessory receptors or co-receptors are cell surface receptors that control specificity, intensity, duration, and diversity of TGF-β superfamily signaling (Nickel et al., 2018). Co-receptors interact with TGF-β superfamily ligands and/or TBRI and TBRII receptors and are distinct from signaling TBRI and TBRII in that they do not possess a functional enzymatic motif (Nickel et al., 2018). Often, co-receptors exhibit lower affinities for TGF-β ligands, nonetheless, they are more abundant than the signaling receptors (Bilandzic and Stenvers, 2011). A ligand may initially associate with co-receptors which afterwards present it to (or sequester it from) the signaling receptors thus determining the signaling intensity and

duration (López-Casillas et al., 1993). Thus, co-receptors may determine whether a certain cell responds to a ligand or they can function as a decoy and thus antagonize signaling (Onichtchouk et al., 1999). TGF-β superfamily co-receptors include endoglin, betaglycan (BG), neuropilins, Cripto, BMP and activin membrane-bound inhibitor (BAMBI), repulsive guidance molecules (RGMs), and muscle-specific receptor tyrosine kinase (MuSK) (Nickel et al., 2018).

1.2.6 Betaglycan

1.2.6.1 Structure and function of betaglycan (BG)

BG is a ubiquitously expressed transmembrane co-receptor for some TGF-β superfamily ligands including TGF-βs, inhibins, GDFs, and BMPs (López-Casillas et al., 1991; 1993; Bilandzic and Stenvers, 2011; Derynck and Budi, 2019). It is encoded by the TGFBR3 gene, which is a single gene spanning a region 27,000 base pairs located on chromosome 1p32-33. The TGFBR3 gene comprises 16 exons and two promoters, a proximal and a distal one (Wang et al., 1991; Johnson et al., 1995). Structurally, BG is an 851 amino acid non-covalently linked heparan sulfate proteoglycan with a large extracellular domain, a single-pass hydrophobic transmembrane domain, and a short cytoplasmic domain lacking kinase activity (Cheifetz et al., 1988; López-Casillas et al., 1991; Bilandzic and Stenvers, 2011). A schematic presentation of the BG structure is shown in Figure 6.

The extracellular domain contains an N-terminal orphan domain with unknown homology, a zona pelucida domain (ZPD) which is thought to play a role in receptor oligomerization, as well as two independent TGF-β ligand binding domains. The two TGF-β ligand binding domains comprise of a distal binding domain located near the N-terminus of the extracellular domain which is separated from the proximal binding domain located at the C-terminus of the extracellular domain by an un-structured linker region (Wang et al., 1991; López-Casillas et al., 1991; 1994; Pepin et al., 1995; Mendoza et al., 2009).

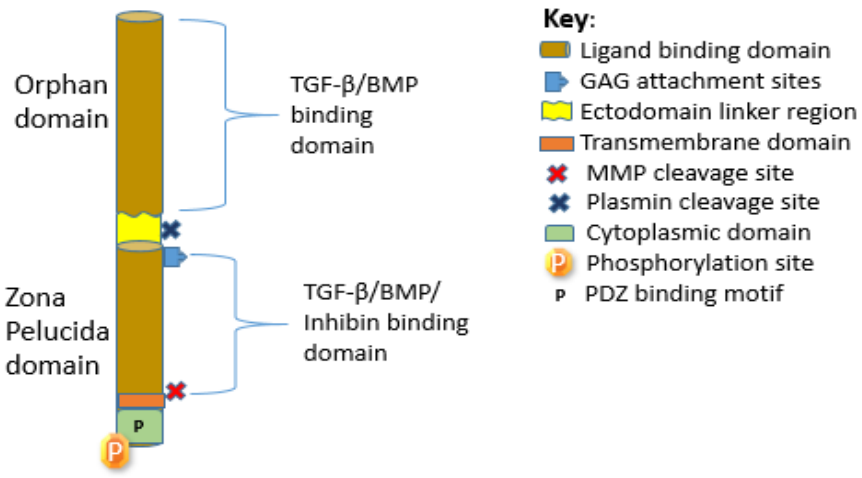


Figure 6. Structural presentation of betaglycan: BG is a type I membrane protein with a large extracellular domain that contains an N-terminal orphan domain with no known homology and a zona pelucida domain at the C-terminal. The orphan domain binds only TGF-β and BMP, whereas the zona pelucida domain can bind TGF-β1-3, BMPs, and inhibin. The structure depicts enzymatic (MMPs and plasmin) cleavage sites and sites for GAG attachment. The cytoplasmic domain is phosphorylated at Ser/Thr residues and contains consensus PDZ-binding motifs present at the carboxyl terminus (Bilandzic and Stenvers, 2011).

Structural studies revealed that the BG ligand-binding domains bind TGF-β1-3, BMP-2/4/7, and GDF-5, as well as inhibin A, whereas binding of basic fibroblast growth factor-2 (bFGF2) occurs through the glycosaminoglycan (GAG) side chain modifications (Gatza et al., 2010; Bilandzic and Stenvers, 2011). Unlike TGF-β1 and TGF-β3 which do not require BG to signal, TGF-β2 has only a weak intrinsic affinity for TβRII and thus requires BG for signaling (Bilandzic and Stenvers, 2011). Binding of BG to inhibin occurs exclusively within the ZP domain in the proximal region (Wiater et al., 2006). The extracellular domain of BG possesses two sites, at serine 535 and serine 546, of heparin and chondroitin sulfate GAG side chain modifications (Cheifetz et al., 1988; López-Casillas et al., 1994).

The short cytoplasmic domain of BG consists of a class I PDZ binding motif that lacks intrinsic enzymatic activity. The domain binds to the auto-phosphorylated cytoplasmic

domain of TβRII, and this enhances the assembly of an active TβRII-TβRI signaling complex prior to dissociation of BG from the complex (Blobe et al., 2001b). Furthermore, TβRII-mediated phosphorylation of the cytoplasmic domain of BG at threonine 841 promotes the interaction between BG and β-arrestin2 essential for co-internalization of the BG/TβRII/β-arrestin2 complex into endocytic vesicles and subsequent suppression of TGF-β signaling (Chen et al., 2003). Notably, the interaction between BG and β-arrestin2 determines the ability of BG to down-regulate nuclear factor kappa B (NFκB) signaling in breast cancer (You et al., 2009). Similarly, BG/β-arrestin2 interaction regulates the capability of BG to modulate cell migration (Mythreye and Blobe, 2009). Besides β-arrestin2, the class I PDZ binding domain of the cytoplasmic domain of BG also interacts with GIPC (GAIP-interacting protein C-terminus), a PDZ domain-containing protein, to stabilize BG cell surface expression (Blobe et al., 2001a).

1.2.6.2 The shedding of betaglycan

The core BG protein has a predicted molecular weight of 100 kDa, although the fully processed BG protein has an apparent molecular weight of 180-300 kDa as a result of glycosaminoglycan post-translational modifications (Wang et al., 1991; López-Casillas et al., 1994). Membrane-bound BG (mBG) undergoes ligand-independent proteolytic ectodomain cleavage, a process termed shedding, releasing a soluble domain (sBG) that can be detected in the extracellular matrix and body fluids such as milk, serum and plasma (Andres et al., 1989; Zhang et al., 2001; Velasco-Loyden et al., 2004; Grgurevic et al., 2020). The concentration of sBG was shown to correlate with the cell surface expression of BG although little is known about the specific cleavage site and regulation of soluble BG production (Andres et al., 1989). Some studies proposed that shedding of BG may be modulated by pervanadate and may partly be mediated by the membrane type matrix metalloproteases (MT-MMPs) as well as by plasmin (Lamarre et al., 1994; Velasco-Loyden et al., 2004). In the current study, we investigated whether matrix metalloproteinases are involved in shedding of BG in endometrial cells. The soluble fragment is an inhibitor of TGF-β signaling and is reported to have therapeutic value in certain cancers and fibrotic diseases (Vilchis-

Landeros et al., 2001; Bandyopadhyay et al., 2002a; 2002b; 2005; Liu et al., 2002; Juárez et al., 2007; Naumann et al., 2008).

1.2.6.3 The role of BG in diseases

BG function is to establish the potency of its ligands, chiefly TGF-β2 and inhibin A, on their target cells (Bilandzic and Stenvers, 2011) but has additional ligand-dependent and -independent roles like regulation of reproduction and tumor suppression (Gatza et al., 2010; Lee et al., 2010; Nishida et al., 2018; Grgurevic et al., 2020). BG null embryos are not viable and die between E13.5 and birth, due to cardiovascular and hepatic defects (Stenvers et al., 2003; Compton et al., 2007). Differential expression of BG within the testis and ovary was reported by Sarraj et al. (2007) who detected BG expression within the fetal testis interstitium primarily by Leydig cells, although this expression shifted inside the seminiferous cords at birth. On the other hand, BG was observed in both the somatic and germ cell lineages in both the fetal and neonatal ovary. In the uterus, BG was found to be expressed in endometrial glands and endothelial cells of both the endometrium and the myometrium (Florio et al., 2005). Elsewhere, Jones et al. (2002) reported BG localization primarily in stromal cells and in a subset of epithelial cells. In the same study, strong staining of BG was noted in decidualized cells during pregnancy, whereas in the placenta intense staining was observed on the apical surface of the syncytiotrophoblast layer.

Since BG can bind numerous classes of TGF superfamily ligands, it is not surprising that the functional impact of BG is both cell type- and context-dependent. In fact, increasing evidence suggest a role of BG in human cancers and other diseases (Gatza et al., 2010). Consequently, our current study investigated the role of BG in the pathophysiology of endometriosis. Indeed, dysregulation of BG expression has been observed in multiple cancers, with loss of expression correlated to disease progression or metastasis and/or a poorer prognosis in patients (Gatza et al., 2010; Lee et al., 2010; Nishida et al., 2018; Grgurevic et al., 2020). Loss of BG expression in endometrial adenocarcinoma is suggested to involve BG regulation of inhibin action, since the α-subunit of inhibin is also disrupted in endometrial adenocarcinomas (Florio et al., 2005). The loss of BG expression in early stage disease of several cancer types suggest that BG may inhibit tumor progression (Gatza et al., 2010).

Indisputably, BG has direct functions in suppressing cell migration, invasion, angiogenesis, as well as metastasis in *in vivo* models suggesting an emerging role for BG as a suppressor of tumor progression and/or metastasis (Naumann et al., 2008; Gatza et al., 2010; Lee et al., 2010; Nishida et al., 2018; Grgurevic et al., 2020).

It has been reported that restoring or increasing BG expression in numerous cancer models decreases cancer cell motility and invasion *in vitro* and invasion, angiogenesis and metastasis *in vivo* (Bandyopadhyay et al., 1999; 2002a; Criswell et al., 2008; Naumann et al., 2008; Nishida et al., 2018). Several studies reported that BG reduces cell migration and invasion of breast, ovarian, pancreatic, prostate, non-small cell lung, and clear-cell renal cell carcinoma (ccRCC) cancer models (Turley et al., 2007; Finger et al., 2008; Gordon et al., 2008; Mythreye and Blobe, 2009; Lee et al., 2010; Nishida et al., 2018).

1.3 ADAM12 structure and function

A disintegrin and metalloproteinase-12 (ADAM12) is a member of the ADAM family of enzymes. The ADAMs, also known as Zinc-dependent proteases, are a Zn^{2+}-mediated, largely membrane-bound (soluble forms have also been described) family of multi-domain, multifunctional proteases which belong to the zinc protease superfamily (Seals and Courtneidge, 2003). The ADAMs consists of 8 domains namely; a prodomain, a signal domain, a cysteine-rich domain, an intracellular C-terminal domain, a metalloproteinase domain, a trans-membrane domain, a disintegrin or integrin binding domain and an epidermal growth factor-like domain (Seals and Courtneidge, 2003; Edwards et al., 2008). They are synthesized as inactive protein precursors and are activated via release of the zinc ion from the cysteine residue at the catalytic site via autocatalysis or furin-like convertase activity (Seals and Courtneidge, 2003). Downstream the MMP domain of ADAMs is the disintegrin domain that binds integrin proteins involved in adhesion, migration as well as cell signaling (Jacobsen and Wewer, 2009; Nyren-Erickson et al., 2013). There are presently 40 gene members known for this family; nonetheless only 21 of these are believed to function in humans (Edwards et al., 2008). Of the 21, only 13 exhibit protease activity and these include ADAM9, 10, 12, 15, 17, 28 and 33 (Giebeler and Zigrino, 2016).

At the molecular level, human ADAM12 exists as two alternating splicing forms, i.e.; the short secreted form (ADAM12S) and the long transmembrane form (ADAM12L; Roy et al., 2011). The two isoforms share a high overall sequence homology, differing only in the transmembrane domain (absent in ADAM12S) and a C-terminus that is distinct in each isoform (Jacobsen and Wewer, 2009). ADAM12L sheds several membrane-bound ligands, including epidermal growth factor (EGF), heparin-binding EGF-like growth factor binding protein (HB-EGF), betacellulin, Notch ligand delta-like 1, and placental leucine aminopeptidase. On the other hand, ADAM12S can cleave insulin-like growth factor binding protein (IGFBP)-3 and -5 and degrade extracellular matrix substrates (Seals and Courtneidge, 2003; Roy et al., 2004; Edwards et al., 2008; Nyren-Erickson et al., 2013). ADAM12L interacts with a number of proteins involved in signaling pathways forming a tightly connected protein network. For instance, the protein is implicated in TGF-β signaling pathways which is critical in cell proliferation, epithelial-mesenchymal-transition (EMT) as well as metastasis (Joshi and Cao, 2010; Ruff et al., 2015).

ADAM12 has been implicated in pathogenesis of countless cancers, liver fibrogenesis, osteoarthritis, cardiac hypertrophy, and numerous pregnancy complications (Jacobsen and Wewer, 2009; Nyren-Erickson et al., 2013). ADAM12 is markedly elevated, both at the mRNA and protein level, in numerous cancers and its two isoforms are associated with adhesion, migration as well as invasion of cancer cells (Rocks et al., 2006; Roy et al., 2011). Notably, ADAM12L is involved in earlier stages of breast cancer while ADAM12S is involved in the later stages which include migration and invasion of cancer cells (Roy et al., 2004, 2011). Ergo, we investigated whether the expression of ADAM12 is different in eutopic and ectopic endometrium of endometriosis patients.

ADAM12 can be detected in urine of breast cancer patients, and its levels have been shown to correlate with disease status, stage, as well as cancer risk (Roy et al., 2004). A study by Laigaard et al. (2005b) reported significantly lower maternal serum ADAM12 levels during the first trimester in patients who later developed preeclampsia during pregnancy relative to levels in women with normal pregnancies, suggesting that ADAM12 may be a valuable early marker for preeclampsia. Furthermore, ADAM12 levels in maternal serum was markedly lower in trisomy 18 pregnancies than in normal pregnancies suggesting that maternal serum

ADAM12 is a promising biomarker for fetal trisomy 18 (Laigaard et al., 2005a). Interestingly, González-Foruria et al. (2017) reported a significant correlation between increased oxidative stress and hyper-activation of the ADAM17/Notch signaling in women with endometriosis relative to controls.

1.4 TGF-β family and endometriosis

1.4.1 The role of TGF-β in pathophysiology of endometriosis

The roles of specific TGF-β family members vary and can even be opposing in various cells and at various developmental stages (Massagué, 2012). An example of the context-dependent functions is the dichotomy of TGF-β's roles in cancer. In tumorigenesis, TGF-β superfamily ligands and their signaling pathways play both a tumor suppressive and tumor promoting role (Bierie and Moses, 2006; Gordon and Blobe, 2008). In early-stage cancer, the ligands enhance tumor suppression mainly via their capacity to maintain tissue architecture, inhibit both cell proliferation and genomic instability as well as promote senescence and apoptosis. Contrary, perturbations in TGF-β family components in many late-stage cancers cause tumor promoting effects on both the cancer cells themselves and the stromal compartment, for instance, through suppressed immune surveillance, increased angiogenesis, enhanced EMT, as well as enhanced migration and invasion (Bierie and Moses, 2006; Pardali and Moustakas, 2007; Gordon and Blobe, 2008).

TGF-βs, their receptors and signal transducers are amply and differentially expressed in the endometrium under hormonal control, signifying a role of the TGF-βs in the normal function of the human endometrium and in the pathogenesis of endometriosis (Omwandho et al., 2010; Dela Cruz and Reis, 2015; Young et al., 2017). Beginning with menstruation, endometriosis is categorized into six stages namely; cell shedding into peritoneal cavity, cell survival, escape from immune surveillance, cell adhesion and invasion at ectopic sites, neo-angiogenesis and growth of the implants and bleeding (Omwandho et al., 2010). In most of these processes, TGF-βs and their receptors are directly or indirectly involved (Omwandho et al., 2010; Dela Cruz and Reis, 2015).

Since mRNA and protein expression of the three TGF-β isoforms is differentially regulated around menstruation, it is suggested that TGF-βs might be involved in initiation of

menstruation as well as in scarless postmenstrual regeneration (influenced by the TGF-β3: TGF-β1/2 ratio) of the endometrium (Omwandho et al., 2010). TGF-βs exert their effects on wound healing particularly by modulating cell differentiation, proliferation, migration, as well as extracellular matrix production, and immune modulation (Penn et al., 2012; Lichtman et al., 2016)

TGF-β1 was reported to be significantly elevated in serum, peritoneal fluid, ectopic endometrium and peritoneum of women with peritoneal endometriosis compared to healthy controls (Young et al., 2017). The review reported that increasing levels of TGF-β ligands are correlated with reduced immune cell activity within the peritoneum, as well as enhanced ectopic endometrial cell survival, proliferation, attachment, and invasion during development of endometriotic lesions *in vivo*. A study by Soni et al. (2019) reported augmented development of endometriosis via increased cell migration, adhesiveness, colonization, and invasiveness upon administration of high concentrations of TGF-β1 to a mouse model. Recently, TGF-β1 levels were found to be significantly elevated in serum of women with endometriosis than in controls (Nanda et al., 2020). TGF-β1 is linked to the initiation of neo-angiogenesis together with alterations in ectopic endometrial and peritoneal cell metabolism further promoting endometriosis lesion development (Young et al., 2017).

1.4.2 The role of activin and inhibin in pathophysiology of endometriosis

Activins and inhibins are key players in physiological processes like germ cell development, oocyte maturation and follicular development, ovulation, decidualization, endometrial receptivity and embryo implantation, placentation, as well as endometrial repair after menstruation (Kaitu'u-Lino et al., 2009; Namwanje and Brown, 2016; Appiah Adu-Gyamfi et al., 2020). Dysregulation of these glycoproteins promote pathogenesis and pathophysiology of numerous diseases including pregnancy complications like incomplete and complete miscarriages, recurrent abortion, ectopic pregnancy as well as in anovulatory bleeding, processes which collectively disrupt reproduction (Florio et al., 2010; Namwanje and Brown, 2016).

Aberrant expression of activins and inhibins has also been reported in women with endometriosis (Dela Cruz and Reis, 2015). Activin A, ALK-4, ALK-7, ACVR2A, ACVR2B,

SMAD2/3/4 but not inhibins, are expressed in ovarian endometriotic lesions (Mabuchi et al., 2010). Moreover, mRNAs of the α, βA, βB, ACVR2A and ACVR2B subunits are highly expressed in peritoneal endometriotic cells (Florio et al., 1998). Alpha and βA-subunits of activin and inhibin were abundantly expressed in eutopic glandular cells from patients with minimal and mild endometriosis relative to controls (Rombauts et al., 2006). In the same study, activin A secretion by glandular and stromal endometrial cells was 7-fold and 3-fold higher, respectively, in women with endometriosis relative to women without the disease.

Reis et al. (2001) reported significantly elevated concentrations of dimeric activin A and inhibin A in cystic fluid of ovarian endometriomas suggesting a role of these glycoproteins in local modulation of endometriosis cell growth and differentiation. The concentrations were slightly higher in cystic fluid than in peritoneal fluid and significantly higher than in peripheral blood (Reis et al., 2001). Moreover, activin A was reported to significantly promote invasion of endometrial stromal and epithelial cells through a modeled peritoneal mesothelial monolayer *in vitro*, partly via down-regulation of E-cadherin, an effect which was partially reversed by inhibin A and follistatin (Ferreira et al., 2008). Activin A induced secretion of estradiol by modulating expression of cytochrome P450 through the ALK4-SMAD pathway in endometrial lesions suggesting that activin A promotes ectopic lesion development and survival (Zheng et al., 2016). Activin A was also reported to promote proliferation of endometriotic lesions via augmented expression of interleukin 6 (IL-6) and tumor necrosis factor alpha (TNF-α) in a mouse model of endometriosis (Kana et al., 2019). Activin A also enhanced myofibroblast differentiation of endometrial mesenchymal stem cells through signal transducer and activator of transcription 3 (STAT3)-dependent SMAD/connective tissue growth factor (CTGF) pathway promoting fibrosis during endometriosis *in vitro* and *in vivo* (Zhang et al., 2019).

Thus, dysregulation of members of the TGF-β superfamily components promote pathogenesis and pathophysiology of numerous diseases including endometriosis. Hence, we investigated whether BG is dysregulated in serum/endocervical mucus of endometriosis patients. Since sBG is an antagonist of TGF-β and mBG is required in inhibin-mediated inhibition of activin, we investigated whether TGF-β, activin A and inhibin A modulate production of the soluble form of BG and the exact molecular mechanisms involved and

whether or not sBG influences TGF-β secretion, cell viability and wound healing of endometriotic cells.

1.5 Aims and objectives

TGF-β superfamily signaling pathways are ubiquitous and indispensable modulators of cellular processes like cell differentiation, proliferation, migration, invasion, and survival, along with physiological processes such as angiogenesis, embryonic development, and wound healing. Dysregulation of these pathways, through either mutations or alterations in the expression of the components of these signaling pathways frequently give rise to human diseases. Accordingly, apt regulation of these pathways is obligatory at all levels, chiefly at the ligand level, with either overexpression or downregulation of specific TGF-β superfamily ligands leading to diseases like endometriosis and numerous cancers.

One protein involved in the regulation of several pathways of TGF-β superfamily members is BG. It has been shown to modulate TGF-βs, activin, inhibin, GDFs and BMP signaling in various cells. Although BG has been demonstrated to participate in multiple diseases including endometrial cancer, its role in pathophysiology of endometriosis remains unknown. Consequently, it is of great significance to elucidate how TGF-βs modulate BG and *vice versa* in the context of endometriosis. In the current investigation, the regulation of BG shedding by TGF-β, activin A and inhibin A and the molecular mechanisms involved using endometrial *in vitro* models are addressed. We further analyzed the influence of TGF-βs and BG on wound healing in endometriotic cells.

Conclusive diagnosis of endometriosis remains surgical assessment of the pelvic region by laparoscopy followed by histological validation. These requirements underscore the need for identification of reliable molecular biomarkers for early diagnosis of the condition prior to investigative surgery. Thus, our study investigated the utility of serum/endocervical mucus sBG and ADAM12 levels as potential non-invasive diagnostic biomarkers for endometriosis. Analysis of ADAM12 expression in eutopic and ectopic endometrium was also performed using immunohistochemistry.

1.5.1 Aims of the study

The following topics were addressed:

- The effects of TGF-β on BG shedding in different endometrial cell types
- Signaling mechanisms involved in TGF-β-mediated reduction in BG shedding
- Influence of activin A and inhibin A on BG shedding in different endometrial cell types
- The signaling mechanisms involved in activin A-mediated reduction in BG shedding
- Effects of recombinant BG on cell viability and TGF-β1/β2 secretion
- Modulation of BG shedding by matrix metalloproteinases
- Influence of TGF-β, activin A, and inhibin A on MMP2, MMP3 and ADAM12 secretion
- Influence of TGF-β1/β2 and BG on wound healing in endometriotic cells
- Evaluation of sBG in serum and endocervical mucus samples as a biomarker for endometriosis
- Analysis of the pattern of localization and staining intensity of ADAM12 in eutopic and ectopic endometrium
- Evaluation of whether ADAM12 levels in serum/endocervical mucus are altered in women with endometriosis

2 MATERIALS AND METHODS

2.1 Materials and chemicals

2.1.1. Chemicals

Table 1. Chemicals

Name	Manufacturer	City
Agarose	Roth	Karlsruhe
Amphotericin B	Sigma-Aldrich	Steinheim am Albuch
BSA	Sigma-Aldrich	Steinheim am Albuch
Cell lysis buffer	Cell signaling Tech.	Frankfurt am Main
Citrate buffer	DAKO	Hamburg
DAB+ substrate chromogen system	DAKO	Hamburg
DAPI	Invitrogen	Karlsruhe
Dimethyl sulfoxide (DMSO)	Roth	Karlsruhe
Ethanol	Sigma-Aldrich	Steinheim am Albuch
Ethidium bromide	Roth	Karlsruhe
EnVision Plus System	DAKO	Hamburg
Eukitt® mounting medium	Fluka Analytical/Sigma	Schwerte
Halt™ protease inhibitor cocktail	ThermoScientific	Brunswick
Hematoxylin Mayer	Waldeck GmbH	Münster
Hydrogen peroxide	Roth	Karlsruhe
1x protease inhibitor cocktail	Sigma	Taufkirchen
Methanol	Sigma-Aldrich	Steinheim am Albuch
Neo-Clear	Merck/Sigma	Taufkirchen
Phenylmethylsulfonyl fluoride	Sigma-Aldrich	Steinheim am Albuch
Quick-Load® 100 bp DNA Ladder	BioLabs inc.	Amsterdam
Reagent diluent	R & D Systems	Wiesbaden
Stop solution	R & D Systems	Wiesbaden
Trypan Blue Dye, 0.4%	Biorad	München
Tween 20	Sigma	Taufkirchen

Xylene	Roth	Karlsruhe
β-Mercaptoethanol	AppliChem	Darmstadt

2.1.2 Cell culture reagents

Table 2. Cell culture reagents

Name	Manufacturer	City
Accutase	Gibco	Schwerte
DMEM 4.5 g/L high glucose	Gibco	Schwerte
DMEM/F12	Gibco	Schwerte
PBS (1x) without Ca^{2+} & Mg^{2+}	Gibco	Schwerte
FCS	Bio&Sell	Feucht
Glutamine	Life technologies co.	Renfrew
ITS (insulin transferrin selenium)	Gibco	Schwerte
Non-essential amino acids	Sigma-Aldrich	Steinheim am Albuch
Penicillin/Streptomycin (100x)	Life technologies co.	München

2.1.3 Antibodies

Table 3. Antibodies used for immunofluorescence and immunohistochemistry

Name	Catalog No.	Manufacturer	City
ADAM12	A7940	ABclonal	Woburn
Alexa Fluor 488	A21202	Invitrogen	Karlsruhe
Alexa Fluor 555	A-31570	Invitrogen	Karlsruhe
CD10	M7308	DAKO/Agilent	Waldbronn
CD248	HPA051856	Sigma	Steinheim am Albuch
MUC1	M061301-2	DAKO/Agilent	Waldbronn
Secondary antibody	K4003	DAKO	Santa Clara

2.1.4 Cytokines

Table 4. TGF-β family Ligands

Name	Catalog No.	Manufacturer
Activin A, recombinant human	C-60057	Promokine
Inhibin A, recombinant human	8506-AB	Novus Biologicals
TBRIII, recombinant human	242-R3	R & D systems
TGF-β1, recombinant human	C-63503	Promokine
TGF-β2, recombinant human	C-63498	Promokine
TGF-β3, recombinant human	C-63508	Promokine

2.1.5 ELISA kits

Table 5. ELISA kits

Name	Species	Catalog No.	Sensitivity Range	Manufacturer
ADAM12 Duoset ELISA kit	Human	DY4416	125-8,000 pg/mL	R & D systems
MMP2 Duoset ELISA kit	Human	DY902	0.6-20 ng/mL	R & D systems
MMP3 Duoset ELISA kit	Human	DY513	31.2-2,000 pg/mL	R & D systems
TGF-β RIII Duoset ELISA kit	Human	DY242	156-10,000 pg/mL	R & D systems
TGF-β1 Duoset ELISA kit	Human	DY240	31.2-2,000 pg/mL	R & D systems
TGF-β2 Duoset ELISA kit	Human	DY302	31.2-2,000 pg/mL	R & D systems

Duoset ELISA kit contains:

- Capture Antibody - Reconstituted with PBS
- Detection Antibody - Reconstituted with reagent diluent
- Standard - Reconstituted with reagent diluent

- Streptavidin-HRP - Streptavidin conjugated to horseradish-peroxidase

2.1.6 Inhibitors

Table 6. Inhibitors

Name	Inhibition of	Catalog No.	Manufacturer
Follistatin	Activin	C-60040	Promokine
GM6001	Broad MMPs inhibitor	G8237	US biological
LY364947	TGF-β Type I receptor kinase	L6293	Sigma-Aldrich
LY294002	PI3-Kinase	19-142	Sigma-Aldrich
SIS3	SMAD3	566405	Merck
rhTIMP1	MMPs	10934-HNAH	Sino Biologicals
rhTIMP2	MMPs	10396-HNAH	Sino Biologicals
rhTIMP3	MMPs, TACE	973-TM	R & D Systems
UO126	MEK1/2	19-147	Sigma-Aldrich

2.1.7 RNAi transfection reagents

Table 7. Transfection reagents and siRNAs

Name	Identification No.	Manufacturer	City
Lipofectamine™ RNAiMAX	13778030	Invitrogen	Karlsruhe
Negative control No. 2 siRNA	4390847	Invitrogen	Karlsruhe
Opti-MEM™	31985062	ThermoFisher Scientific	Brunswick
SMAD2 siRNA	107873	Invitrogen	Karlsruhe

SMAD3 siRNA	s535081	Invitrogen	Karlsruhe

Table 8. siRNA sequences

siRNA oligo (Species)	Sequence (5'→3')	Length (bp)
SMAD2 (Human)	Sense: GGAGUGCGCUUAUACUACAtt	21
	Antisense: UGUAGUAUAAGCGCACUCCtc	21
SMAD3 (Human)	Sense: GGUGCUCCAUCUCCUACUAtt	21
	Antisense: UAGUAGGAGAUGGAGCACCag	21

2.1.8 PCR reagents

Table 9. PCR reagents

Name	Manufacturer	City
iTaq Universal SYBR Green Supermix	Bio-Rad	München
Nuclease-free water	Qiagen	Hilden

TAE buffer 50x stock recipe

- 242 g Tris base in double-distilled H_2O
- 57.1 mL glacial acetic acid
- 100 mL 0.5 M EDTA solution (pH 8.0)
- Adjust volume to 1 L.

2.1.9 Primers

All primers were designed using the primer blast software available at http://www.ncbi.nlm.nih.gov/tools/primer-blast and were exon-exon junction spanned. Forward and reverse sequence, annealing temperature, size and accession number of each gene is described in Table 10.

Table 10. PCR primers

Gene (Species)	Sequence (5'→3')	AT	Acc. No.	Size (bp)
GAPDH (Hu)	GACCCCTTCATTGACCTCAAC fwd GATGACCTTGCCCACAGCCTT rev	59°C	NM_001256799	561
SMAD2 (Hu)	ATCCTAACAGAACTTCCGCC fwd CTCAGCAAAAACTTCCCCAC rev	59°C	NM_001003652	481
SMAD3 (Hu)	GTCTGCAAGATCCCACCAGG fwd CTTGTCAAGCCACTGCAAAG rev	59°C	NM_001145102	237

Hu, human; AT, annealing temperature; Acc. No., accession number; fwd, forward; rev, reverse; bp, base pairs; GAPDH, Glyceraldehyde 3-phosphate dehydrogenase.

2.2 Equipment

Table 11. Equipment

Name	Manufacturer	City
Cell culture CO2 incubator	Memmert	Schwabach
Desktop centrifuge Biofuge Fresco	Hettich	Tuttlingen
DNA electrophoresis system	BioRad	München
Electronic balance SPB50	Sartorius	Göttingen
Heidolph polymax 1040 orbital shaker	Heidolph	Schwabach
Infinite® 200 microplate reader	Tecan	Männedorf
Inverse microscope FSX100	Olympus	Hamburg
Leica DM IL microscope	Leica	Wetzlar
Megafuge 1.0R centrifuge	Thermo Electron Co.	Karlsruhe
Microwave	Samsung	Schwalbach
Mini centrifuge Galaxy	Heathrow Scientific	Vernon Hills
MiniOpticon™ Real-Time PCR System	Bio-Rad	Dusseldorf

Multi Gourmet steamer	Braun	Kronberg
P300 NanoPhotometer®	Implen	München
PowerPac Power Supplies	BioRad	München
TC 10 Automatic cell counter	BioRad	München
Thermo Scientific™ Herasafe™ KS biological safety cabinet	Thermo Electron Co.	Karlsruhe
Thermo-Shaker (PSC18)	Grant Instruments Ltd.	Amsterdam
Ultrasonic homogenizer Bandelin Sonopuls	Bandelin electronic	Berlin
ChemiDoc™ XRS gel imaging system	BioRad	München

2.3 Miscellaneous

Table 12. Miscellaneous reagents and kits

Name	Manufacturer	City
Ancillary Reagent Duoset Kit 2	R & D Systems	Wiesbaden
Bicinchoninic acid (BCA) assay kit	Pierce, ThermoScientific	Brunswick
Cell counting kit-8	Dojindo EU GmbH/Sigma	München
Cell strainers	Corning	Kaiserslautern
***Clostridium histolyticum* collagenase**	Sigma-Aldrich	Steinheim am Albuch
RNeasy Micro Kit	Qiagen	Hilden
RevertAid First Strand cDNA Synthesis Kit	ThermoScientific	Brunswick
Sample activation kit	R & D System	Wiesbaden

Ancillary reagent Duoset kit 2 contains:

- 96 well microplates
- Plates sealers

- Reagent Diluent concentrate 2 - 1% BSA in PBS, pH = 7.2 - 7.4, 0.2 μm filtered
- Phosphate buffered saline (PBS) - 137 mM NaCl, 1.5 mM KH_2PO_4, 8.1 mM $Na_2HPO_4 \cdot 2H_2O$, 2.7 mM KCl, pH = 7.2 - 7.4, 0.2 μm filtered
- Wash Buffer - 0.05% Tween 20 in PBS, pH = 7.2 - 7.4
- Block Buffer - 5% Tween 20 in PBS
- Substrate solution - 1:1 mixture of color reagent A (H_2O_2) and color reagent B (Tetramethylbenzidine)
- Stop Solution - 2 N H_2SO_4

2.4 Cell lines and cell culture

Epithelial endometriotic 12Z and endometrial stromal THESC cell lines were selected as they represent the glandular epithelial and stromal components of the endometrium which are essential in pathogenesis of endometriosis.

2.4.1 Endometriotic 12Z cells

The immortalized and well-characterized 12Z cell line (Zeitvogel et al., 2001; Banu et al., 2008) was kindly provided by Prof. Anna Starzinski-Powitz (Department of Biology, University of Frankfurt, Frankfurt, Germany). The 12Z cells were originally generated via the in-situ electroporation of primary peritoneal epithelial endometriotic cells with SV40 T-antigen (Zeitvogel et al., 2001). The cells are epithelial-like (Fig. 7A) and express cytokeratins, E-Cadherin and vimentin typically present in endometrial epithelial tissue (Zeitvogel et al., 2001). They also express estrogen receptor α and β, progesterone receptor and steroidal stimulating factor-1 (Banu et al., 2008). The cells show features of the active/progressive phase of endometriosis and hence are suitable for studying cellular action of endometriosis (Zeitvogel et al., 2001; Dietze et al., 2018; Romano et al., 2020; Fan, 2020).

2.4.2 Endometrial stromal THESC cells

The immortalized stromal THESC cells are fibroblast-like (Fig. 7B), show typical endometrial characteristics (Krikun et al., 2004) and were purchased from ATCC (Cat. No. CRL-4003). The cells are telomerase-immortalized endometrial stromal cells and show

extensive and constitutive expression of progesterone receptor mRNA, estrogen receptor beta and P-450 aromatase (Krikun et al., 2004; Banu et al., 2008).

2.4.3 Endometrial epithelial Ishikawa cells

The Ishikawa cell line was originally established from an endometrial adenocarcinoma and was purchased from Sigma Aldrich (Cat. No. 99040201). The Ishikawa cells, although they do not represent healthy endometrial or endometriotic tissue, possess characteristics of endometrial glandular epithelium (Fig. 7C) and hence are applied in numerous endometriosis studies as a representative cell line for the normal human endometrium (Correa et al., 2016; Dietze et al., 2018).

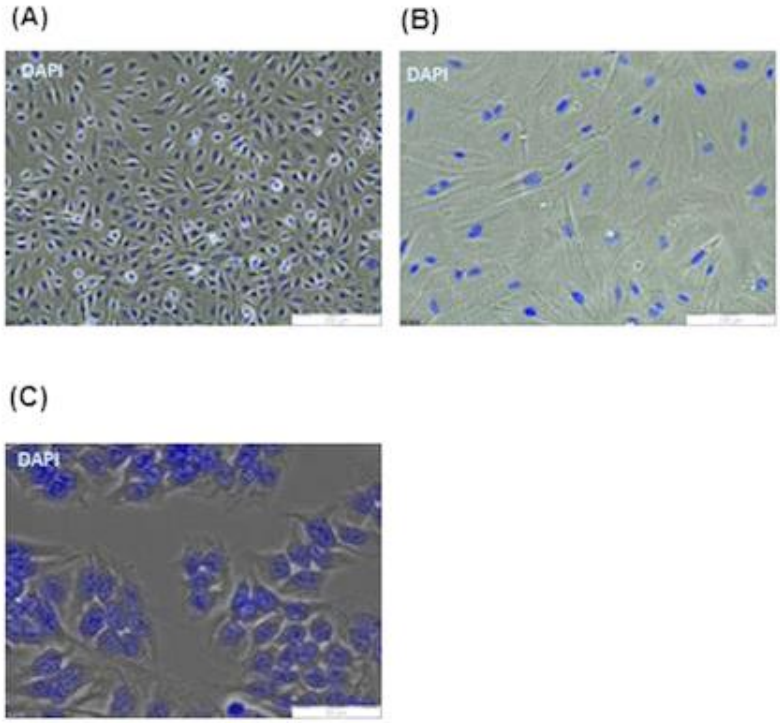


Figure 7. Endometrial epithelial and stromal cells. Endometriotic epithelial 12Z cells (scale; 200 µm) **(A)**, endometrial stromal THESC cells (scale; 200 µm) **(B)**, and epithelial Ishikawa cells (scale; 50 µm) **(C)**. Blue DAPI nuclear staining.

2.4.4 Primary human endometrial stromal cells

This study was approved by the Ethics Committee of the Medical Faculty of the Justus-Liebig-University, Giessen, Germany (registry number 95/09) and experiments performed in accordance with relevant guidelines and regulations. Primary endometrial stromal cells were obtained from eutopic endometrial tissue of a 45-year-old woman who underwent surgery (abdominal total laparoscopic hysterectomy) due to endometriosis and uterine myoma. The patient had not received hormonal therapy before surgery. Preoperative informed consent form was obtained from the patient.

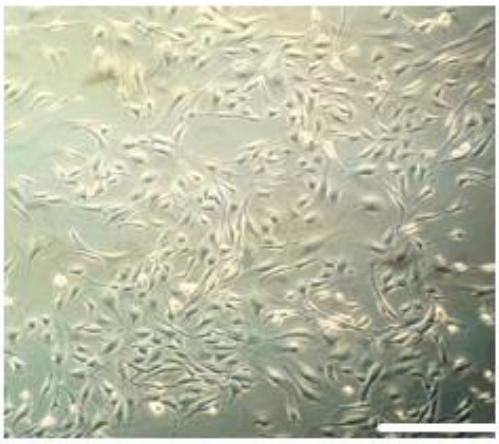


Figure 8. Phase-contrast microscopy of cultured primary human endometrial stromal cells (scale; 200 µm).

The cells were isolated and cultured by Dr. Muhammad A. Riaz following the procedures described by Swangchan-Uthai et al. (2012) and Raheem and Fouladi-Nashta (2014) with some modifications. For transport to the laboratory, tissue specimens were placed in ice-cold Dulbecco's modified Eagle's medium (DMEM)/F12 supplemented with 100 IU/ml penicillin, 100 mg/ml penicillin/streptomycin (pen-strep) solution and 2.5 mg/ml amphotericin B. After washing with PBS, endometrial tissue was minced into small pieces (1-3 mm) and digested in DMEM/F12 containing 1 mg/mL *clostridium histolyticum* collagenase at 37°C for 2 h in a shaking water bath and the suspension passed through 70 µm cell strainers. The filtrate containing both endometrial stromal and epithelial cells was centrifuged at 500 x g for 5 min and the cell pellet re-suspended in DMEM/F12 medium

supplemented with 10% FCS, 1% pen-strep and 1% insulin, transferrin, and selenium (ITS) solution. A differential method was used to separate the stromal cells from epithelial cells since stromal cells unlike epithelial cells adhere firmly to cell culture plates after 18 h (Fortier et al., 1988). After pre-plating for 18 h, the supernatant containing floating epithelial cells and other types of cells was removed and the remaining stromal cells (Fig. 8) maintained in a humidified incubator at 37°C and 5% CO_2. Culture medium was changed every 2 days for 7-8 days to attain confluence. Purity of the stromal cells was assessed by morphological examination as well as CD10 and CD248 (both stain positive for stromal cells) and mucin 1 (MUC1) (positive for epithelial but negative for stromal cells) staining. Cultured primary stromal cells at 4-5 passages were used for further analysis.

2.4.5 Characterization of primary cells by immunofluorescence

Primary stromal cells were grown in 4- or 8-well chamber slides and incubated at 37°C and 5% CO_2 for 24 h. The cells were rinsed with 1x PBS and fixed with 100% ice-cold methanol on ice for 10 min, washed thrice with PBS for 5 min and blocking solution (PBS with 3% BSA and 0.1% Tween 20) added for 1 h at room temperature (RT) on an orbital shaker. The blocking solution was removed and replaced with fresh blocking solution containing the primary antibodies at a 1:300 dilution for detection of CD10, CD248, and MUC 1, respectively, and incubated overnight at 4 °C. The cells were washed thrice with PBS for 3 min each at RT on an orbital shaker and fresh blocking solution with the appropriate secondary antibody (Alexa Fluor 488-conjugated donkey anti-rabbit, donkey anti-mouse, Alexa Fluor 555-conjugated donkey anti-mouse) diluted 1:500 added and incubated for 1 h at RT. After washing 3 times with PBS, cell nuclei were stained with 0.5 µg/µL 4′-6-diamidino-2-phenylindole (DAPI) solution diluted in PBS. MUC 1 was used as a negative control. The cells were washed twice with PBS and images were obtained using an inverted Olympus IX81 microscope equipped with a fluorescence system.

2.4.6 Cell culture

Cells were maintained in medium as follows: 12Z cells in DMEM 4.5 g/L glucose supplemented with 10% FCS, 2 mM glutamine, and 1% pen-strep; THESC and primary

human endometrial stromal cells in DMEM/F12 with 2 mM glutamine supplemented with 10% FCS, 1% pen-strep, and 1% ITS solution; Ishikawa cells in MEM supplemented with 5% FCS, 1% non-essential amino acids, 2 mM glutamine, and 1% pen-strep. All cell types were cultured in a humidified incubator at 37 °C and 5% CO_2 and medium was routinely renewed every 3-4 days. Cells were washed once with 1x PBS without Ca^{2+} and Mg^{2+} before detachment with 0.25% accutase and passaged at about 80% confluence.

2.5 Treatment and collection of supernatants for ELISAs

2.5.1 Treatment of cells with various agents

Cells (2 x 10^5 cells) were cultured in 6-well plates and serum-starved (1% FCS) for 24 h and treated in duplicates with TGF-β1 (1 - 15 ng/mL), TGF-β2 (1 - 15 ng/mL), TGF-β3 (1 - 15 ng/mL), activin A (5 - 50 ng/mL), follistatin (200 ng/mL), inhibin A (5 - 50 ng/ml), recombinant BG (10 - 100 ng/mL), recombinant TIMP3 (2.5 - 10 nM), recombinant TIMP1/2 (10 – 200 ng/mL) or GM6001 (10 µM) for 24 - 72 h. For inhibition studies, cells were pre-treated with the respective inhibitors for 2 h (10 µM LY364947, 50 µM UO126, 5 µM LY294002 or 5 µM SIS3 diluted in 0.01% DMSO; Cui et al., 2019) prior to stimulation with TGF-β1 or TGF-β2 (each 10 ng/mL) or activin A (25 ng/mL). Negative controls consisted of untreated samples without the recombinant proteins or containing the vehicle (0.01% DMSO).

2.5.2 Collection of supernatants for ELISA

Cell culture supernatants were collected in Eppendorf tubes and stored at -20°C until use. For normalization of ELISA concentrations, cells were washed once with 1× PBS and detached with 500 µL/well accutase for 3 min at 37°C before re-suspending in medium. The single cell suspension (10 µL) was afterwards stained with trypan blue (10 µL) and the number of viable cells assessed with the TC10™ automated cell counter system. For preparation of cell lysates, supernatants were collected and cell lysis performed as described in the manufacturer's protocol (Cell signaling, Frankfurt). Briefly, cells were washed with 2 mL ice-cold PBS before lysis with 300 µl lysis buffer containing 1x Halt™ protease inhibitor cocktail. The lysates were collected with a cell scraper and sonicated twice on ice at 60 kHz

for 5 sec with 5 sec intervals before centrifugation at 13,000 x g for 15 min at 4°C. Supernatants were collected and protein concentration determined using the BCA protein assay kit (Pierce) following manufacturer's instructions. Protein concentrations were used for normalization of ELISA results.

2.6 TGF-beta RIII DuoSet ELISA

Samples consisting of cell culture supernatants, patient serum or endocervical mucus were analyzed with the human TGF-beta RIII DuoSet ELISA kit according to manufacturer's instructions. Supernatants were collected after treatment as described in 2.5.2. Briefly, 96-wells ELISA plate was coated overnight with 4 µg/mL capture antibody diluted in 1x PBS pH 7.2 at 4°C. Wells were blocked by adding 300 µL of reagent diluent to each well and incubated at RT with shaking for 1 h. Subsequently, 100 µL sample and TBRIII standards in reagent diluent were added and incubated for 2 h at RT. After an additional 2 h incubation step with 400 ng/mL detection antibody, wells were washed and streptavidin-HRP solution (1:200) added to the wells and plates incubated for 20 min, washed and substrate solution (100 µL) added and plates incubated for 20 min at RT and the reaction stopped with 50 µL 2N H_2SO_4. Optical densities (OD) were read using an infinite® 200 microplate reader set at a wavelength of 450/540 nm. Soluble BG concentrations were standardized against the number of viable cells or the total protein concentrations of the corresponding lysates.

2.7 SiRNA transfection

Epithelial 12Z cells (1 x 10^5cells/mL) were grown to 60% confluency in 12-well plates and transfected with either transfection reagent alone, silencer™ select negative control No. 2 siRNA, SMAD2 siRNA or SMAD3 siRNA using Lipofectamine™ RNAiMAX transfection reagent in medium without antibiotics following the manufacturer's guidelines. Briefly, siRNA and transfection reagent were diluted 1:3 (siRNA: Lipofectamine) each in 150 µL Opti-MEM medium. Equal parts of diluted siRNA and diluted Lipofectamine were mixed and incubated for 5 min at RT. The mixture (300 µL) was added to the cells (700 µL antibiotic-free medium) to achieve a final siRNA concentration of 100 nM (SMAD2 siRNA) or 150 nM (SMAD3 siRNA). The cells were cultured in a humidified incubator at 37°C and

5% CO_2 and medium was replaced with complete growth medium after 24 h and the cells incubated for 48 h. Efficiency of the siRNA gene knockdown was determined after every transfection procedure by RT-qPCR. Cells were treated with either TGF-β1 (10 ng/mL), TGF-β2 (10 ng/mL), or activin A (25 ng/mL) as previously described in section 2.5.1.

2.8 RNA isolation

Total RNA was extracted from transfected 12Z cells using the RNeasy Kit in accordance to the manufacturer's protocol. Briefly, cells were washed twice with ice-cold PBS, lysed using 350 µl RLT lysis buffer containing β-mercaptoethanol (diluted 1:100 with RLT buffer) and transferred to 1.5 mL Eppendorf tubes. The lysate was subsequently passed at least 5 times through a blunt 27-gauge needle fitted to a 1 mL syringe. An equal volume of 70% ethanol was added to the homogenized lysate before transferring to an RNeasy spin column placed in a 2 mL collection tube and centrifuged at 15000 x g for 30 sec (all centrifugation steps were performed at RT). The flow-through was discarded and 700 µl of RW1 wash buffer added to the column prior to centrifugation 15000 x g for 30 sec. RPE buffer (500 µl) was added to the column and centrifuged at 15000 x g for 30 sec to wash the spin column membrane. This step was repeated with centrifugation for 2 min before transferring the column to fresh 1.5 mL microfuge tubes. To elute the total RNA, 30 µL of RNase-free water was added to the column and centrifuged at 15000 x g for 1 min. RNA quantity and quality were determined spectrometrically using a P300 NanoPhotometer®.

2.9 Complementary DNA synthesis

A First Strand cDNA Synthesis kit was used to reverse-transcribe the total RNA following the manufacturer's instructions. Briefly, total RNA template was mixed with oligo-dTs and the volume made up to 12 µL with nuclease-free water. The reaction mixture was incubated in a PCS18 thermo-shaker set at 65°C for 5 min and rapidly chilled on ice.

Table 13. cDNA synthesis reagents

Component	Amount
RNA template	1 µg
Oligo $(dT)_{18}$ primer	1 µL
Nuclease free water	To 12 µL
5 x Reaction buffer	4 µL
PeqGOLD RNase inhibitor (20 u/µL)	1 µL
10 mM dNTPmix	2 µL
PeqGOLD M-MuLV H Minus reverse transcriptase	1 µL

After the annealing step, the rest of the reagents were added (Table 13) and the mixture spun briefly before incubation at 42°C for 1 h. The reaction was afterwards terminated by heating at 70°C for 5 min and reaction products stored at -80°C for further analysis.

2.10 Real time-qPCR and gel electrophoresis

2.10.1 Real time-qPCR

PCR primers (Table 10) were designed using the NCBI Primer-Blast algorithm available at http://www.ncbi.nlm.nih.gov/tools/primer-blast accessed on March 2021. RT-qPCR was conducted with 0.5 µg cDNA in a 20 µl PCR reaction using iTaq™ Universal SYBR® Green Supermix on a MiniOpticon™ Real-Time PCR System. The PCR reaction components were added as shown in Table 14.

Table 14. PCR reagent mix

Component	Volume/20 µL reaction
cDNA template	1 µL
Nuclease free water	8 µL
iTaq™ Universal SYBR® Green Supermix	10 µL
Forward primer	0.5 µL
Reverse primer	0.5 µL

The reaction was performed in duplicate. The cycling program was initiated by a first denaturation at 95°C for 5 min, followed by 35 cycles of denaturation at 95°C for 30 sec, annealing at 59°C for 20 sec and with a final elongation step at 72°C for 30 sec except for the final extension which lasted 10 min. GAPDH was used for normalization. Results were validated by agarose gel electrophoresis.

2.10.2 Agarose gel electrophoresis

Agarose powder (1.7 g) was placed in a volumetric flask to prepare a 1.7% gel. A 1x TAE buffer (100 mL) was added and the mixture microwaved for 3-5 min until the agarose was completely dissolved. Next, 2 µL ethidium bromide was added and the solution poured into a gel tray with the well comb fixed in place and the gel allowed to polymerize. Once solidified, the gel was placed into an electrophoresis chamber containing 1x TAE buffer. Molecular weight ladder (6 µL) and DNA samples (15 µL) diluted in loading buffer (1:3) were loaded into the wells and the gel run at 125V for 45 min. After the run, the gel was removed and the bands visualized using a ChemiDoc gel imaging system.

2.11 CCK-8 cell viability assay

Cell viability assay was performed using the cell counting kit-8 (CCK-8) following the manufacturer's instructions. Briefly, endometriotic 12Z cells were first counted, and approximately 4000 cells seeded in a 96-well cell culture plate. The cells were incubated in a humidified atmosphere at 37°C and 5% CO_2 and allowed to attach for 24 h. The complete medium was replaced with starvation medium for another 12 h. The culture medium was replaced by a series of concentrations of human recombinant BG (10 ng/mL – 200 ng/mL) diluted in starvation medium. Cells were cultured for 21 h and 10 µL of the CCK-8 reagent added into each well and incubated for an additional 3 h. CCK-8 uses WST-8 (2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium monosodium salt) as a substrate which produces a water-soluble formazan dye following bio-reduction in the presence of 1-Methoxy phenazinium methylsulfate (PMS) which acts as an electron carrier (Fig. 9). WST-8 is reduced by cellular dehydrogenases to the orange-colored product

formazan, which is soluble in culture medium. The amount of formazan produced is directly proportional to the number of living cells. Cell viability was determined by reading optical density (OD) values using an infinite® 200 microplate reader set at a wavelength of 450 nm. Cell viability was presented as the percentage of each concentration relative to the control.

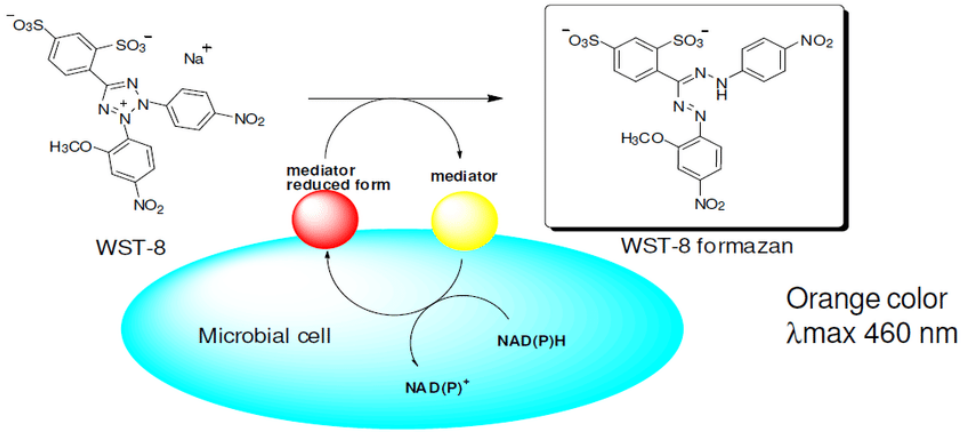


Figure 9. Colorimetric formation of WST-8 formazan from WST-8 in the presence of 1-Methoxy PMS. WST-8 is reduced to water-soluble and orange-colored WST-8 formazan. The figure was adopted from the technical manual by Dojindo Molecular Technologies, Inc. (Rockville, USA).

2.12 Effects of recombinant BG on secretion of TGF-β

The levels of TGF-β1 and TGF-β2 in the supernatants of recombinant BG-treated 12Z cells were measured using ELISAs according to the manufacturer's protocol. Briefly, samples were activated by adding 25 µL 1N HCl to 125 µL sample and incubated at RT for 10 min and neutralized with 25 µL 1.2N NaOH/0.5M HEPES before diluting with 175 µL reagent diluent. The samples or standards (100 µL/well) were added to a 96-well microplate previously pre-coated with 100 µL capture antibody overnight and blocked with 300 µL reagent diluent (all incubations were performed at RT and with shaking). The wells were washed thrice before incubation with 100 µL detection antibody for 2 h. Washing was repeated and 100 µL Streptavidin-HRP solution (diluted 1:200) added to the wells and

incubated for 20 min. The wells were washed and substrate solution (100 µL) subsequently added and incubated for 20 min in the dark and the reaction stopped using 50 µL stop solution. OD values were immediately determined at a wavelength of 450/540 nm using an infinite® 200 microplate reader and data standardized against 1 x 10^6 viable cells.

2.13 MMP2, MMP3 and ADAM12 ELISAs

Epithelial 12Z or stromal THESC cells were treated with TGF-β1, TGF-β2, activin A or inhibin A and supernatants collected as previously described in sections 2.5.1 and 2.5.2. MMP2, MMP3 and ADAM12 ELISAs were performed according to the manufacturer's guidelines. Briefly, 96-wells ELISA plates were coated overnight with 100 µL capture antibody diluted in 1x PBS pH 7.2 at 4°C. This was followed by blocking using 300 µL reagent diluent for a minimum of 1 h. All incubations were carried out at RT and with shaking. After washing, 100 µL samples or standards in reagent diluent were added and incubated for 2 h prior to washing and addition of 100 µL detection antibody for an additional 2 h. Streptavidin-HRP solution was added to the wells for 20 min. Next, the wells were washed and 100 µL substrate solution added for 20 min and the reaction stopped with 50 µL stop solution. OD values were obtained using an infinite® 200 microplate reader set at a wavelength of 450/540 nm. MMP2, MMP3 and ADAM12 concentrations were standardized against the number of viable cells.

2.14 Scratch assay

The wound healing assay according to Liang et al. (2007) was used with some modifications. In brief, 1 x 10^6 12Z cells were cultured in 6-well plates in a humidified incubator at 37°C and 5% CO_2 and allowed to grow until a cell monolayer was formed. The confluent cell monolayer was then disrupted by scratching with a sterile 200 µl yellow pipette tip. Four markings were created along the scratch to serve as reference points. Culture medium was immediately removed and after washing with 1x PBS, cells were grown in serum-free medium with or without 10 ng/mL TGF-β1 or TGF-β2 or 25 ng/mL human recombinant BG. Migration of cells into the cell-free areas was monitored at 0, 9, 24, and 48 h by capturing images under a Leica DM IL microscope fitted with a Canon EOS 450D camera. Four fields

were quantitatively analyzed per well using ImageJ software version 1.53h accessed on March 2021 at http://rsbweb.nih.gov/ij/ and the percentage area of each treatment calculated relative to the controls.

2.15 Patient recruitment and sample analysis

2.15.1 Patients

The current study was approved by the Ethics Committee of the Medical Faculty of the Justus-Liebig-University, Giessen, Germany (registry number 95/09). Informed written consent was obtained from all participants prior to sample collection in accordance with the approved protocol. The study group for serum sBG consisted of 166 women with and 72 women without endometriosis while that of endocervical mucus sBG consisted of 100 women with and 82 women without endometriosis (Table 15). The study for serum ADAM12 consisted of 179 women with and 114 women without endometriosis while that of ADAM12 in endocervical mucus consisted of 35 women with and 44 women without endometriosis (Table 23). Patients on hormone therapy consisted of women taking dienogest- or dienogest plus ethinylestradiol- or progestin-based medications. Diagnosis of endometriosis was performed by laparoscopic surgery followed by histological confirmation. Healthy subjects were examined by anamnesis, palpation and transvaginal ultrasound and in cases of DIE with MRI to rule out endometriosis.

Table 15. Patient demographics and clinical characteristics

	Serum Samples		**Endocervical mucus sample**	
	EM	**w/o EM**	**EM**	**w/o EM**
N	166	72	100	82
Mean age (SEM)	33.6 (0.6)	26.7 (0.9)	32.7 (0.7)	28.8 (0.8)
BMI (kg/m^2)	23.7	22.3	22.9	23.1
Smoking (%)	24	19	28	23
Allergy (%)	59	64	51	48
Menstrual phase (n)				
Proliferative	28	19	27	19
Secretory	41	31	43	36
Menstruation	17	9	-	-
Unknown	80	13	30	27
Contraception use (n)				

Yes	68	43	30	31
No	98	29	70	51
Fertility (n)				
Yes	43	6	37	10
No	56	7	24	14
Unknown	67	59	39	58
Pain (n)				
Dysmenorrhea				
Yes	112	58	89	70
No	38	13	11	11
Unknown	16	1	-	1
Dyspareunia				
Yes	92	30	58	35
No	54	38	42	46
Unknown	20	4	-	1
Dyschezia				
Yes	60	8	45	29
No	95	60	55	53
Unknown	11	4	-	-
Dysuria				
Yes	33	7	29	15
No	125	63	71	66
Unknown	8	2	-	1
sBG detected (%)	166 (100)	72 (100)	96 (96)	81 (99)

EM, endometriosis; w/o EM, without endometriosis; SEM, standard error of the mean; BMI, body mass index; sBG, soluble betaglycan; Pain yes denotes mild to strong pain (scale 2 – 10); unknown means data not available.

2.15.2 Sample collection and analysis

Two to three milliliters of anticoagulant-free venous blood was obtained from each subject during the clinical examination. Samples were centrifuged at 3000 x g for 15 min at 4°C and stored immediately as aliquots at -80°C until further use. Endocervical mucus samples from patients were obtained in the afternoon with a cotton swab and immediately placed in ice-cold 1x PBS containing 1x protease inhibitor cocktail. Samples were centrifuged at 3000 x g for 15 min at 4°C and supernatants collected, weighed and stored as aliquots at -80°C until further analysis. In all cases, the use of contraception and menstrual cycle phases were monitored by anamnesis and a questionnaire (Hackethal et al., 2011). Levels of sBG in serum and endocervical mucus were detected using the human TGF-beta RIII DuoSet ELISA kit while that of serum and endocervical mucus ADAM12 were detected using the human

ADAM12 Duoset ELISA kit following the manufacturer's guidelines (section 2.6 and 2.21). Samples were diluted using reagent diluent (serum sBG, 1:20; endocervical mucus sBG, 1:2; serum ADAM12, 1:2).

2.15.3 Immunohistochemical analysis and quantification

Immunohistochemistry was performed on 5 µm thick consecutive sections (Table 16) of Bouin-fixed specimens as published previously (Konrad et al., 2019b). The EnVision Plus System was used according to the manufacturer`s instructions. Briefly, 5 µm thick tissue sections were deparaffinized in Neo-clear and rehydrated in an ethanol gradient (100%, 96% and 70%). Antigen retrieval was performed in citrate buffer (pH 6) and the slides heated using a Braun Multi-Gourmet steamer at 100°C for 20 min followed by cooling at RT for 20 min. Blocking of endogenous peroxidase was carried out using 3% H_2O_2 in methanol for 30 min. The slides were then incubated with a rabbit anti-ADAM12 polyclonal antibody (diluted 1:100, Table 3) overnight at 4 °C in a humidified chamber and afterwards with a horseradish peroxidase (HRP)-conjugated secondary antibody at room temperature for 30 min. The slides were incubated with diaminobenzidine (liquid DAB+ substrate chromogen system) for 1 min and washed with distilled water before counterstaining with hematoxylin. The slides were washed with distilled water and placed in running tap water for at least 10 min and dehydrated in an ethanol gradient (70%, 96% and 100%) and xylene (100%) for 2 min each. The slides were mounted with Eukitt and images obtained using an inverted Olympus microscope FSX100 fitted with Olympus FSX-BSW software and Leica DM 2000/Leica MC170/Leica application suite LAS 4.9.0. Immunohistochemistry quantification was done using the HSCORE (0, no staining; 1+, weak, but detectable; 2+, moderate or distinct; 3+, intense) which was calculated for each tissue by summing up the percentages of cells grouped in one intensity category and multiplying this number with the intensity of the staining. Glands, stromal cells as well as smooth muscles staining were used for evaluation of the HSCORE.

Table 16. Overview of the tissue samples for detection of ADAM12

Tissue	Endometrium	Ovarian endometriosis	Deep infiltrating endometriosis	Peritoneal endometriosis
All samples	n = 73	n =18	n = 24	n = 21
(Median age)	(43)	(42.1)	(45)	(44.5)
Proliferative	n = 37			
(median age)	(41.7)			
Secretory	n = 26			
(median age)	(43.8)			
Adenomyosis	n = 23			
(median age)	(45)			
Leiyomyoma	n = 20			
Bladder			4	6
Uterosacral ligament			5	1
Ovarian fossa			1	4
Pouch of Douglas			3	4
Peritoneum				3
Infundibulum of fallopian tube			2	1
Pelvic wall				1
Rectum			4	
Rectosigmoid			1	
Rectovaginal septum			2	
Paraurethral			1	
Sigmoid colon			1	
Round ligament of uterus				1

2.16 Statistical analyses

All statistical analyses were performed with the GraphPad Prism software (Version 8.0, GraphPad Inc. La Jolla, CV, USA). Each *in vitro* experiment was performed at least three times in duplicates. Statistical comparisons of the means between two groups were done using t-test and among multiple groups by one-way analysis of variance (ANOVA) followed by Dunnett's or Tukey's post hoc tests. Analyses of serum and endocervical sBG and ADAM12 levels were performed with the Mann-Whitney U-test (for two group comparisons)

or the Kruskal-Wallis test (for more than two groups). A receiver operating characteristic (ROC) curve with a 95% confidence interval was calculated to define the optimal cut-off for endocervical mucus sBG levels. Differences were considered statistically significant at $P \leq 0.05$. All data are reported as means ± SEMs (SEM = Standard error of the mean).

3 RESULTS

3.1 Characterization of primary human endometrial stromal cells

Primary endometrial stromal cells were isolated from tissue of a woman who underwent abdominal laparoscopic hysterectomy due to endometriosis and uterine myoma.

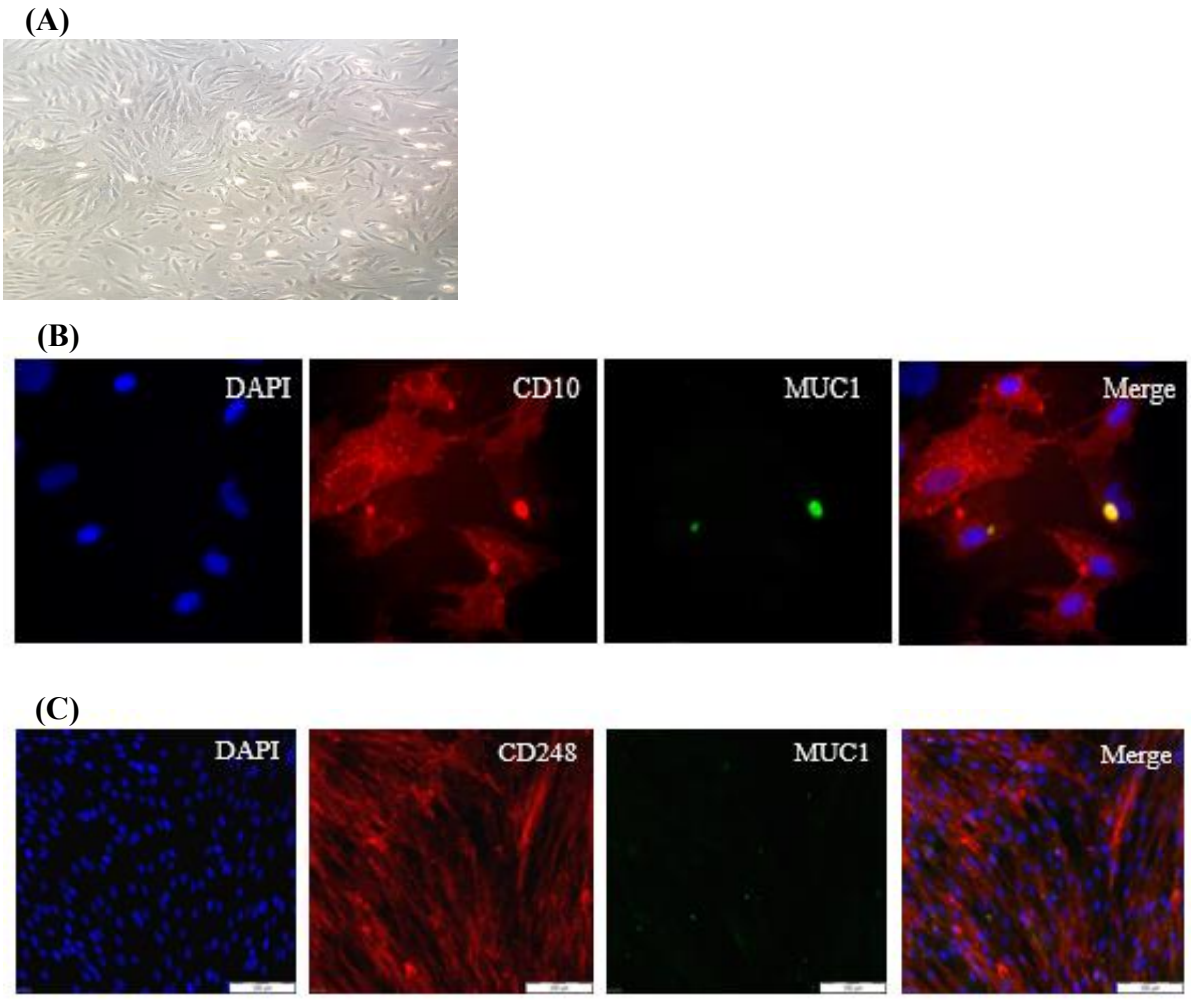


Figure 10. Characterization of primary human endometrial stromal cells by morphology and immunofluorescence. Phase contrast microscopy of isolated stromal cells showing morphology **(A)**. Fluorescence microscopy of isolated stromal cells showing expression of CD10 (magnification; 50x) and CD248 (scale; 200 µm) staining (both red) **(B, C)**. Staining with MUC1 showed only bluish nuclear counterstain with DAPI indicating no epithelial endometrial cells. Isolation and staining procedures were conducted by Dr. Muhammad A. Riaz.

Purity of the cells was assessed by morphological examination (Fig. 10A) as well as immunofluorescence using CD10, CD248 and MUC1 staining (Fig. 10B, C). The primary stromal cells showed a fibroblastic morphology with large, flat, spindle-shaped cells having processes extending from the ends of the cell body (Fig. 10A).

CD10, a cell surface ectoenzyme, is a valuable marker of endometrial stromal cells that is also useful in diagnosing endometriosis (Sumathi and McCluggage, 2002). CD248, also known as endosialin, is a transmembrane glycoprotein dynamically expressed on pericytes, fibroblasts and some stromal cells in numerous tumors (Teicher, 2019). MUC1 is a large glycoprotein located on the apical surface of most simple secretory epithelia and is highly expressed in the female reproductive tract like the uterus (Konrad et al., 2019b) as well as mammary glands (Brayman et al., 2004). In our study, the primary endometrial stromal cells stained positive for both CD10 and CD248, but negative for MUC1 validating their purity (Fig. 10B, C).

3.2 Effects of TGF-β on BG shedding

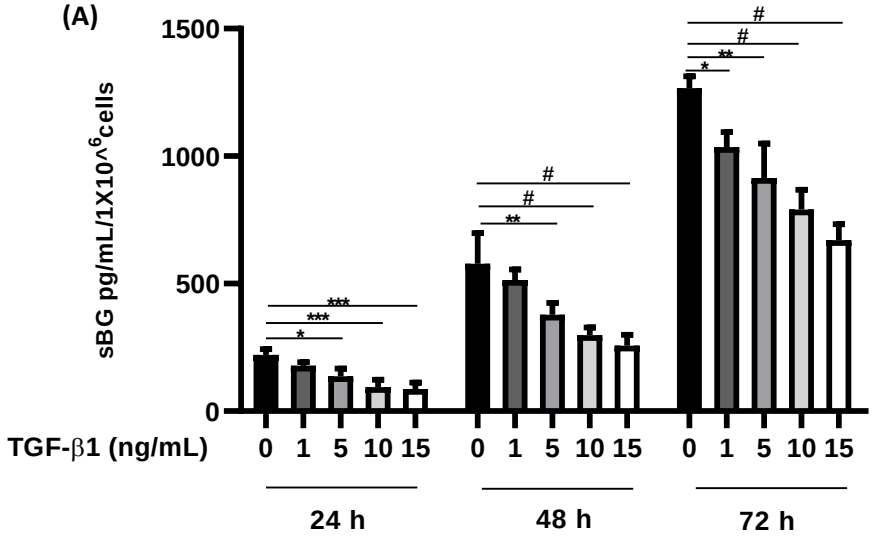
3.2.1 Effects of TGF-β1/2/3 on BG shedding in endometriotic cells

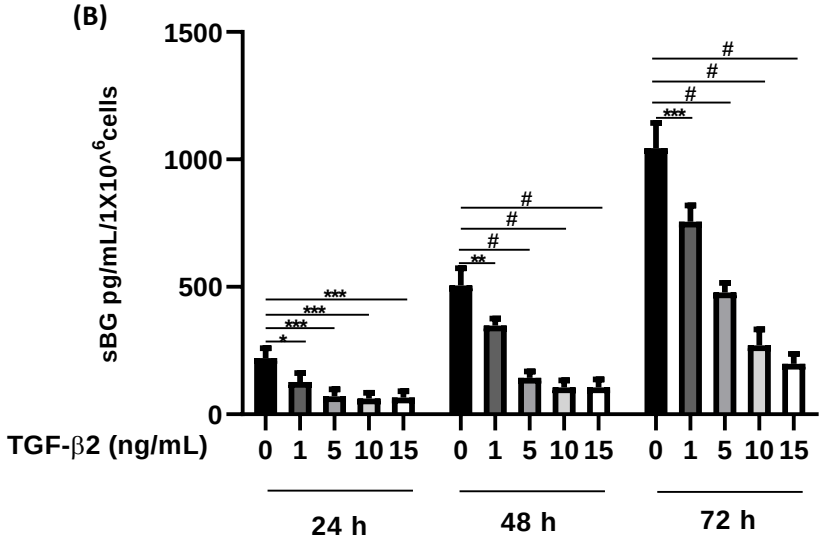
Previous studies on pathophysiology of endometriosis revealed disrupted TGF-β expression and signaling that in turn facilitated implantation and maintenance of ectopic endometrium (Omwandho et al., 2010; Young et al., 2017; Soni et al., 2019). BG is an important co-receptor and modulator of TGF-βs, especially of TGF-β2. It undergoes proteolytic ectodomain cleavage releasing a soluble domain (López-Casillas et al., 1993).

Given the significance of BG in modulating TGF-β signaling, we investigated whether TGF-βs regulate BG shedding in endometriotic cells. To begin with, we evaluated time and concentration dependency in BG shedding following TGF-β1, TGF-β2, and TGF-β3 treatment. Endometriotic epithelial 12Z cells were stimulated with increasing concentrations of TGF-β1/2/3 and supernatants analyzed by sBG ELISAs. We observed a significant time- and concentration-dependent decrease in BG shedding after stimulation with the TGF-βs (Fig. 11).

Approximately 20% - 60% decrease with 1 – 15 ng/mL TGF-β1 after 24, 48 and 72 h was observed (Fig. 11A). Additionally, TGF-β2 and TGF-β3 led to a decrease in BG shedding of

30% to 80% in epithelial cells after the three time points (Fig. 11B, C). Notably, a plateau effect was exhibited beyond the concentration of 5 ng/mL after 24 and 48 h for TGF-β2 and after 24, 48 and 72 h for TGF-β3 (Fig. 11B, C). Since the strongest effects were observed with 10 ng/mL TGF-β1/2/3, subsequent experiments were conducted with this concentration. In brief, these results show that TGF-β1, TGF-β2 and TGF-β3 reduce BG shedding in a time- and concentration-dependent manner in endometriotic epithelial cells.





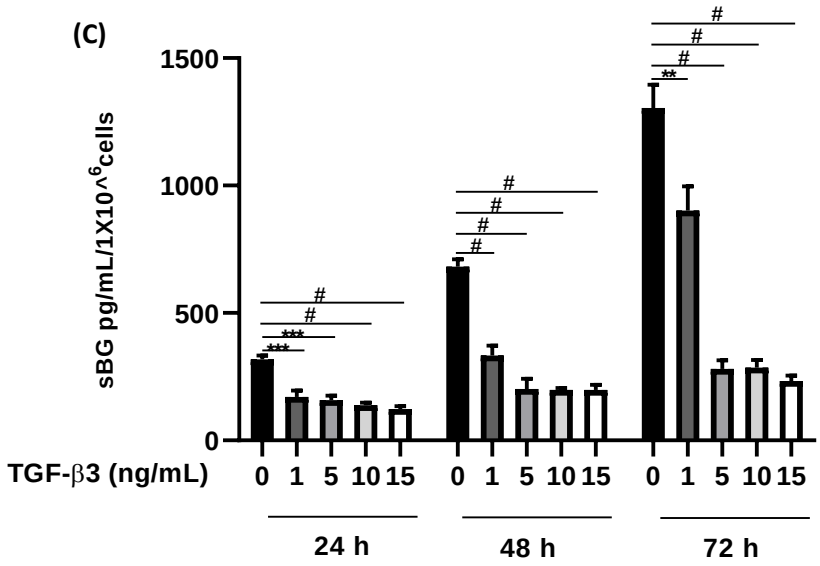


Figure 11. Time- and concentration-dependent effects of TGF-β1/2/3 on BG shedding. Epithelial 12Z cells were stimulated with increasing concentrations (1 - 15 ng/mL) of TGF-β1 **(A)**, TGF-β2 **(B)** and TGF-β3 **(C)** for 24, 48 and 72 h and supernatants analyzed by sBG ELISAs. TGF-β1, -β2 and -β3 treatment significantly reduced BG shedding in a time- and concentration-dependent manner. Untreated cells were taken as controls. Each bar represents the mean ± SEM of 3 independent experiments performed in duplicate. Dunnett's test was used for statistical analysis; $*p \leq 0.05$; $**p < 0.01$; $***p < 0.001$; $^{\#}p < 0.0001$; sBG, soluble betaglycan.

3.2.2 Effects of TGF-β1/2/3 on BG shedding in different cell types

Besides the epithelial endometriotic 12Z cells, we also examined BG shedding in epithelial Ishikawa and endometrial stromal THESC cell lines as well as in primary human endometrial stromal cells. Cells were treated with TGF-β1/2/3 and supernatants analyzed by sBG ELISAs. Soluble BG was detected in all four cell types; 12Z cells (~ 670 pg/mL/1x10^6 cells), Ishikawa cells (~ 550 pg/mL/1x10^6 cells), primary stromal cells (~ 350 pg/mL/1x10^6 cells) and THESC cells (~ 800 pg/mL/1x10^6 cells) after 48 h (Fig. 12A-D).

Our findings revealed that compared to controls, TGF-β1, -β2 and -β3 significantly reduced BG shedding in epithelial 12Z cells (~35%, 80% and 70%, respectively; Fig. 12A), Ishikawa cells (~45%, 70% and 75%, respectively; Fig. 12B), primary human endometrial stromal

cells (~25%, 40% and 35%, respectively; Fig. 12C) and stromal THESC cells (~40%, 75% and 55%, respectively; Fig. 12D). A stronger reduction in BG shedding was observed with TGF-β2 and TGF-β3 relative to TGF-β1. Equally, a stronger effect in the TGF-β1/2/3-mediated reduction in BG shedding was observed in the epithelial cells compared to the stromal cells (Fig. 12A-D). Briefly, these results imply that TGF-β significantly reduces BG shedding in both endometrial epithelial and stromal cells.

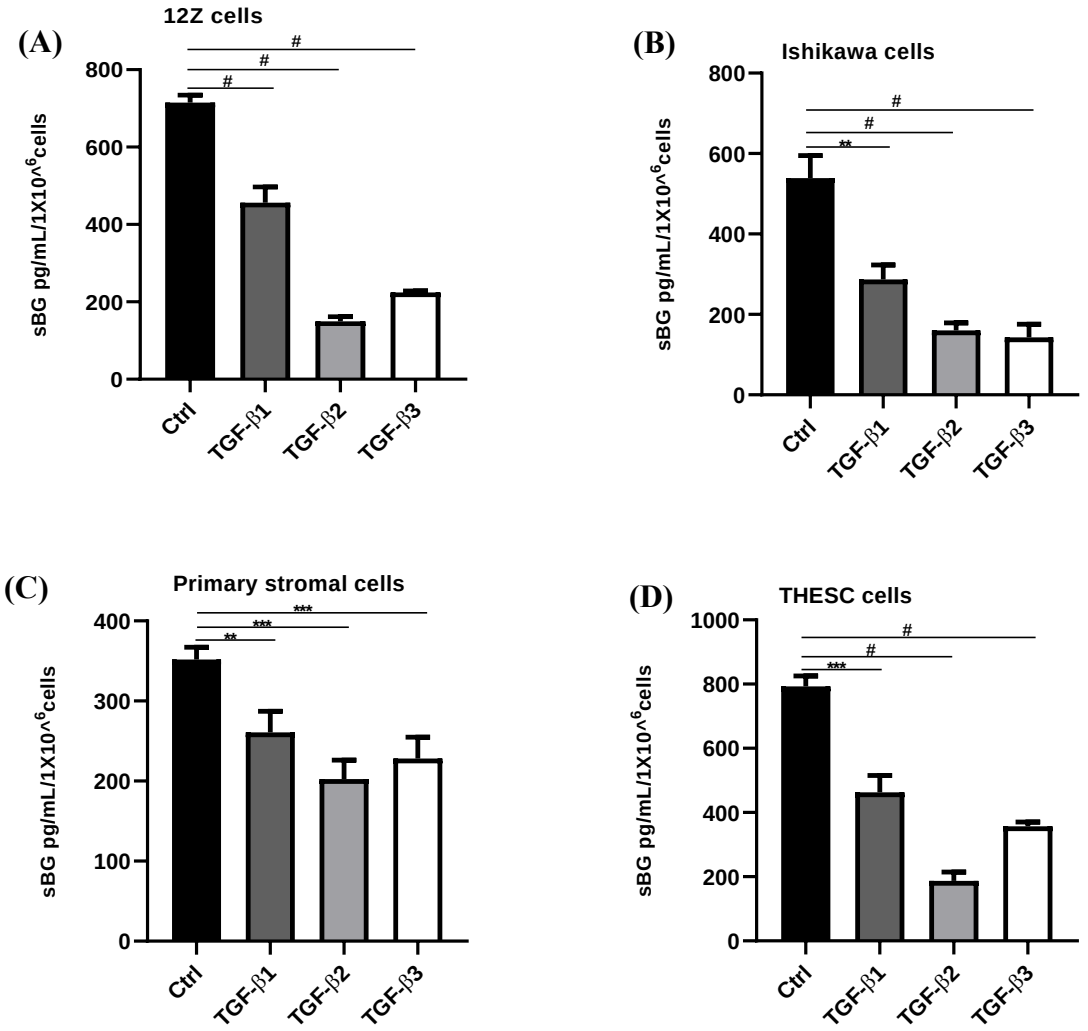
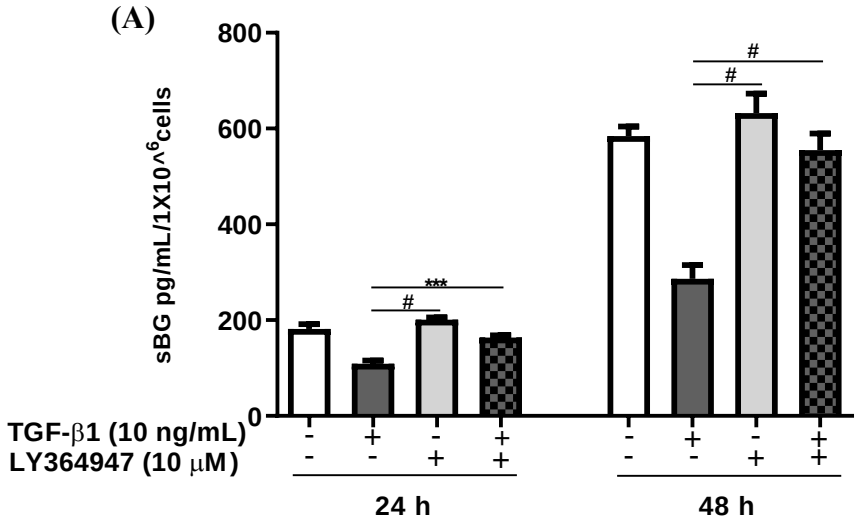


Figure 12. TGF-β1, -β2 and -β3 decrease shedding of BG in epithelial and stromal cells. Endometriotic epithelial 12Z **(A)** and endometrial epithelial Ishikawa **(B)** cells as well as primary human endometrial stromal **(C)** and endometrial stromal THESC **(D)** cells were treated with TGF-β1/2/3 (10 ng/mL) for 48 h and supernatants analyzed by sBG ELISAs. TGF-β1, -β2 and -β3 significantly attenuated BG shedding in all four cell types. Untreated cells were used as controls. Each bar represents the mean ± SEM of 3 independent experiments performed in duplicate. Dunnett's test was used for statistical analysis; **p < 0.01; ***p < 0.001; #p < 0.0001; Ctrl, control; sBG, soluble betaglycan.

3.2.3 Involvement of TGF-β type I receptor kinase (ALK5) in BG shedding

Subsequently, we investigated the signaling mechanisms involved in TGF-β-mediated reduction in BG shedding using endometriotic epithelial cells. Nearly all members of the TGF-β superfamily signal via pairs of serine/threonine kinase receptors, the type I and II receptors (Moustakas et al., 2001). TGF-β type I receptor (ALK-5) forms a heteromeric complex with TGF-β type II receptor which binds TGF-β with high affinity (Ten Dijke et al., 1994). Consequently, we investigated the involvement of ALK-5 in the TGF-β-mediated reduction in shedding of BG (Fig. 13A, B). Endometriotic 12Z cells were pretreated with LY364947, a selective and potent ATP-competitive inhibitor of ALK-4/5 (Cui et al., 2019), prior to stimulation with either TGF-β1 or TGF-β2.

Our results indicated that LY364947 significantly counteracted the reduction in BG shedding induced by both TGF-β1 and TGF-β2. Inhibition of ALK-5 in the TGF-β1-mediated pathway restored BG shedding nearly to control levels (90% and 95% after 24 and 48 h, respectively; Fig. 13A). Nevertheless, inhibition of ALK-5 in the TGF-β2-mediated pathway was incomplete (85% and 80% after 24 and 48 h, respectively; Fig. 13B), indicating a possible involvement of additional pathways.



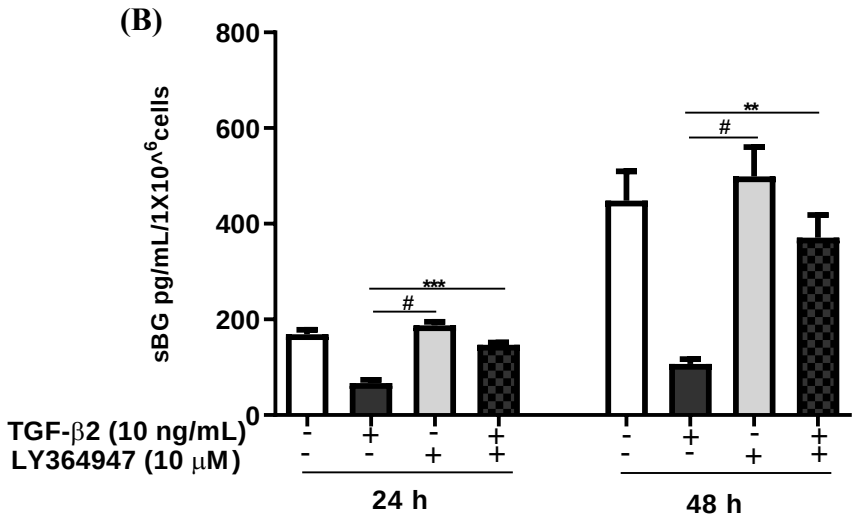


Figure 13. TGF-β1/2-mediated reduction in BG shedding is TGF-β type 1 receptor (ALK-5)-dependent. Epithelial 12Z cells were pre-incubated in the absence or presence of ALK-4/5 inhibitor, LY364947 (10 μM), for 2 h prior to stimulation with either TGF-β1 (10 ng/mL) **(A)** or TGF-β2 (10 ng/mL) **(B)** for 24 and 48 h and supernatants analyzed by sBG ELISAs. LY364947 significantly abrogated the reduction in BG shedding induced by TGF-β1 and TGF-β2. Cells treated with DMSO (0.01%) were taken as controls. Each bar represents the mean ± SEM of 3 independent experiments performed in duplicate. Dunnett's and Tukey's tests were used for statistical analysis; $^{**}p < 0.01$; $^{***}p < 0.001$; $^{\#}p < 0.0001$; sBG, soluble betaglycan; LY364947, 4-[3-(2-pyridinyl)-1H-pyrazol-4-yl]-quinoline.

3.2.4 Involvement of SMAD- and non-SMAD-dependent pathways in BG shedding

In the canonical TGF-β signaling pathway, TGF-β ligands activate members of the SMAD transcription factor family (Moustakas et al., 2001), whereas in the non-canonical pathway, the ligands activate members of the mitogen activated protein (MAP) kinase signaling molecules, like p38, ERKs, JNK and PI3-K/Akt (Zhang, 2009).

Consequently, next we explored the involvement of SMAD and non-SMAD signaling in TGF-β-mediated effects in BG shedding using gene silencing as well as selective inhibitors. Here, SMAD2/SMAD3 double-silenced epithelial 12Z cells were stimulated with either TGF-β1 or TGF-β2 and sBG quantified. The efficiency of each siRNA gene silencing was confirmed by RT-qPCR (Fig. 14A). The SMAD2/SMAD3 double gene knockdown

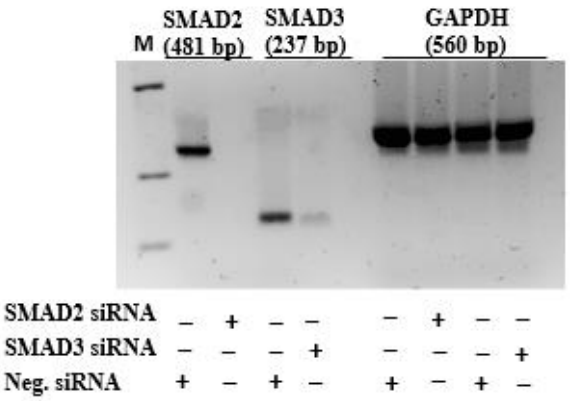
significantly abrogated the TGF-β-dependent effects in regulation of BG shedding (Fig. 14B). Particularly, SMAD2/SMAD3 double gene silencing completely restored BG shedding (100%) in the TGF-β1-treated cells and up to 85% in the TGF-β2-treated cells (Fig. 14B).

To investigate the involvement of the non-canonical pathways, epithelial 12Z cells were pre-treated with the MAPK/ERK kinase and PI3-kinases selective inhibitors, UO126 and LY294002, respectively, prior to TGF-β1 or TGF-β2 treatment. Inhibition of the MAPK/ERK kinase and PI3-kinases pathways had no effect on TGF-β-mediated reduction in BG shedding (Fig. 14C), thus supporting the involvement of only the canonical SMAD-signaling.

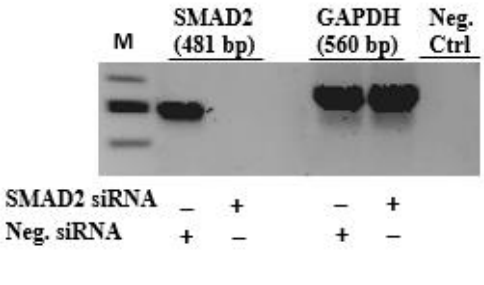
After confirming the involvement of SMADs in the TGF-β-mediated effects on BG shedding, we subsequently investigated the individual contribution of each SMAD using SMAD2 and SMAD3 gene knockdown. For this, SMAD2 and SMAD3 single-silenced epithelial 12Z cells were treated with TGF-β1 or TGF-β2 and sBG quantified using ELISAs. Results presented in Figure 15A-B indicate that silencing of SMAD3 but not of SMAD2 abolished the TGF-β-mediated reduction in BG shedding. Particularly, about 100% and 85% inhibition of TGF-β1-dependent (Fig. 15A) and TGF-β2-dependent (Fig. 15B) effects, respectively, were observed following the SMAD3 gene knockdown. Altogether, these results indicate that the canonical TGF-β pathway involving TGF-β/ALK-5/SMAD3 signaling is required in TGF-β-mediated reduction in BG shedding in endometriotic epithelial cells.

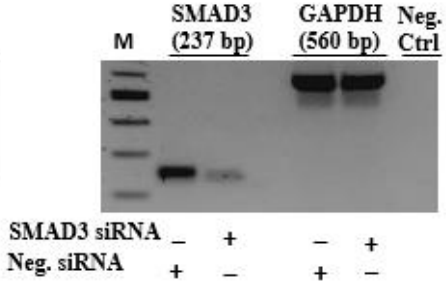
(A)

(i) SMAD2/3 double gene knockdown

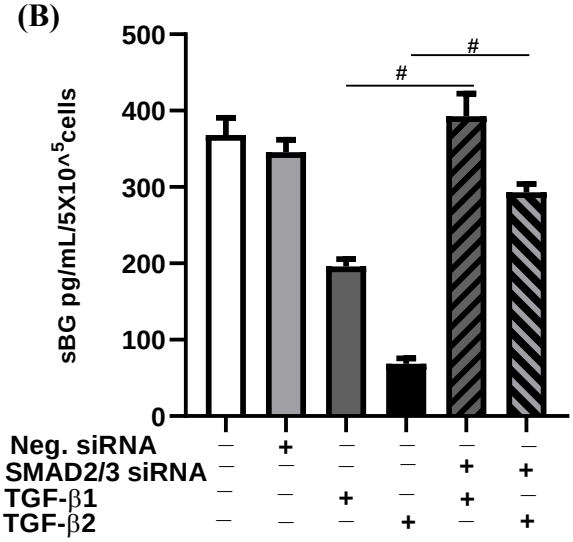


(ii) SMAD2/3 single gene knockdowns





(B)



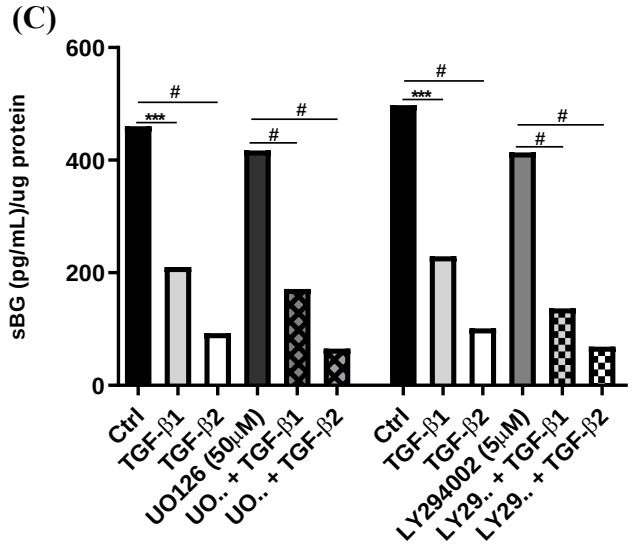


Figure 14. TGF-β1/2-mediated reduction in BG shedding is SMAD-dependent. A control RT-qPCR of the knockdown efficiency for each siRNA was performed to ascertain silencing of SMAD2 and SMAD3 double and single genes **(A)**. SMAD2/3 double-silenced **(B)** and UO126- (50 μM) or LY294002 (5 μM)-treated **(C)** 12Z cells were stimulated with TGF-β1 or TGF-β2 (10 ng/mL) for 48 h and supernatants analyzed by sBG ELISAs. SMAD knockdown significantly abrogated the TGF-β1 and TGF-β2-dependent reduction in BG shedding **(B)**, whereas inhibition of non-SMAD-dependent pathways had no effects **(C)**. Untreated cells and cells treated with Neg. siRNA or those treated with DMSO (0.01%) were used as controls. Each bar represents the mean ± SEM of 3 independent experiments performed in duplicates. Dunnett's and Tukey's tests were used for statistical analysis; ; $^{***}p < 0.001$; $^{\#}p < 0.0001$; M, DNA ladder; siRNA, small interfering RNA; Neg. siRNA, control siRNA; Neg. Ctrl, negative control; bp, base pairs; sBG, soluble betaglycan; Ctrl, control; UO126, 1,4-Diamino-2,3-dicyano-1,4-bis(o-aminophenylmercapto)butadiene; LY294002, 2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one.

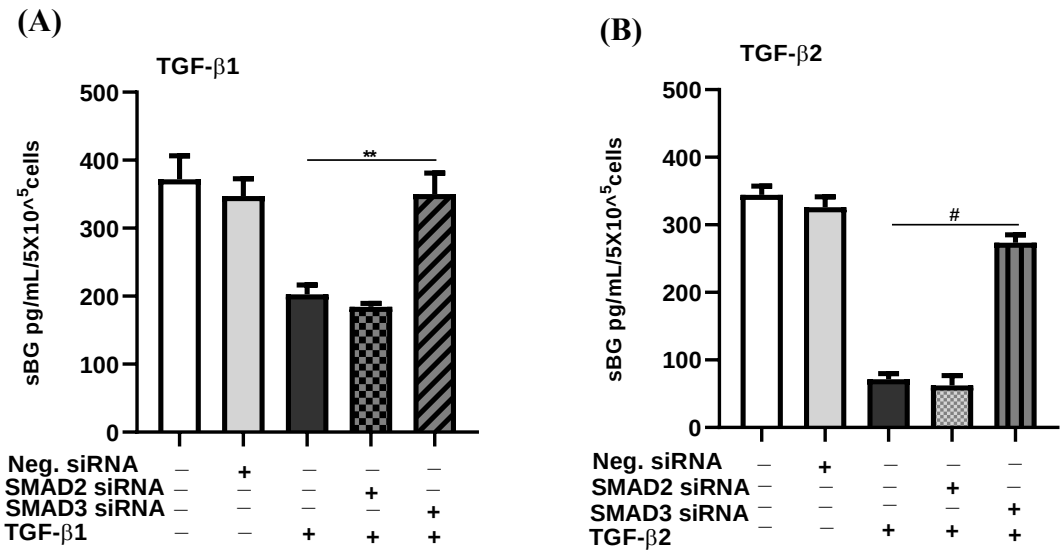


Figure 15. TGF-β1/2-mediated reduction in BG shedding is SMAD3- but not SMAD2-dependent. SMAD2 and SMAD3 single-silenced 12Z cells were stimulated with 10 ng/mL TGF-β1 **(A)** or TGF-β2 **(B)** for 48 h and supernatants analyzed by sBG ELISAs. Analysis of the gene knockdowns indicated that silencing of SMAD3 gene but not of SMAD2 gene abolished the TGF-β1- and TGF-β2-mediated suppression in BG shedding. Untreated cells and cells treated with Neg. siRNA were used as controls. Each bar represents the mean ± SEM of 3 independent experiments performed in duplicate. Dunnett's and Tukey's tests were used for statistical analysis; $^{**}p < 0.01$; $^{\#}p < 0.0001$; siRNA, small interfering RNA; Neg. siRNA, control siRNA; sBG, soluble betaglycan.

3.3 Activin A/inhibin A and BG shedding

3.3.1 Influence of activin A and inhibin A on BG shedding

Inhibin binds to activin type II receptors (ACVR2) via its β-subunits and functionally antagonizes activin activity by inhibiting recruitment of the ACVR2; nevertheless, to attain high inhibitory potency, inhibin requires presence of membrane-bound BG (Li et al., 2017). We investigated whether activin A and inhibin A modulate BG shedding in endometrial cells. Firstly, we determined time and concentration dependency following activin A and inhibin A treatment. To test this, epithelial 12Z cells and stromal THESC cells were stimulated with increasing concentrations of activin A and inhibin A and supernatants analyzed using sBG ELISAs.

A significant time- and concentration-dependent decrease in BG shedding was observed following stimulation of 12Z cells with varying concentrations of activin A. Approximately 10% - 60% decrease with 5 - 50 ng/mL activin A after 24, 48 and 72 h was observed (Fig. 16A). Likewise, about 10% - 45% decrease with 5 - 50 ng/mL activin A after 24, 48 and 72 h was noted in THESC cells (Fig. 16B). Generally, a stronger reduction of BG shedding in activin A-treated epithelial 12Z cells compared with the stromal THESC cells was observed (Fig. 16A, B).

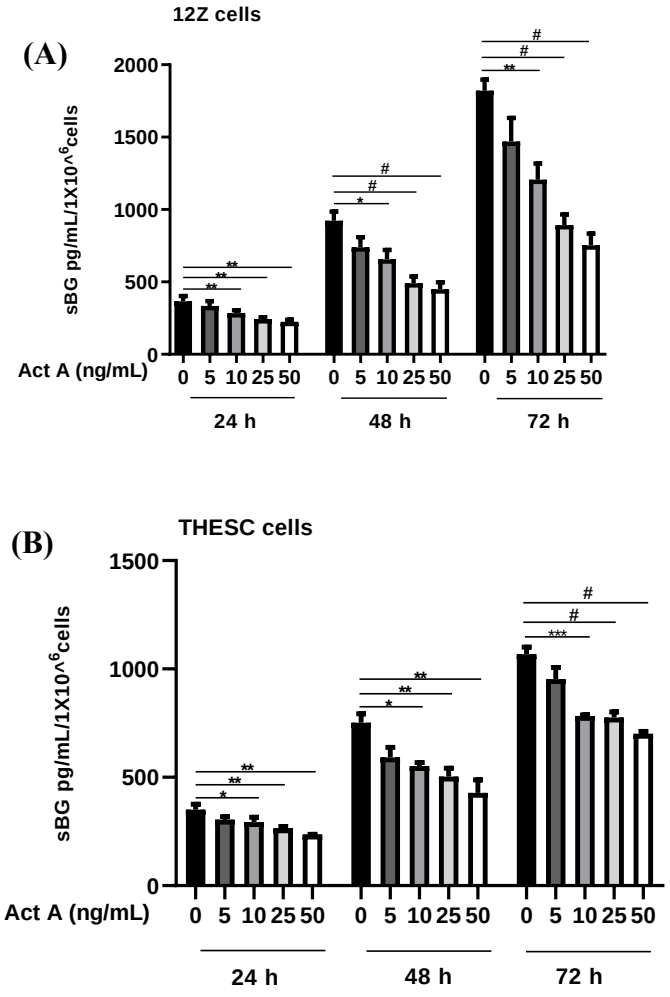
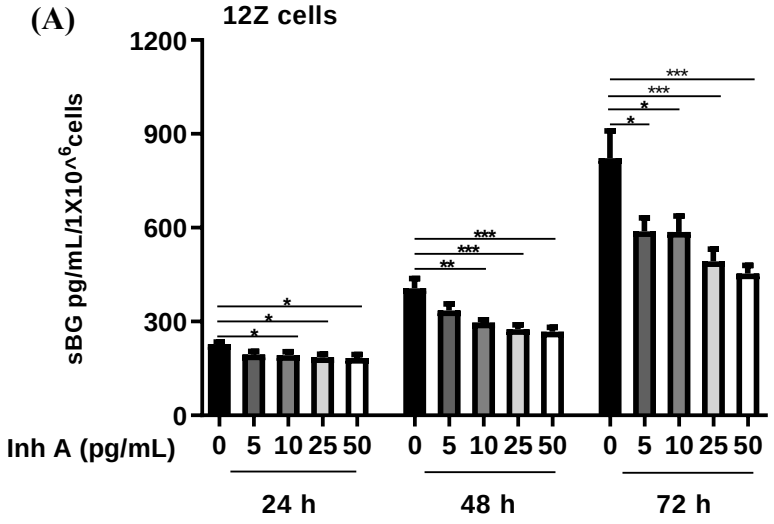


Figure 16. Time- and concentration-dependent effects of activin A on BG shedding in epithelial and stromal cells. Epithelial 12Z **(A)** and stromal THESC cells **(B)** were stimulated

with increasing concentrations of activin A (5 - 50 ng/mL) for 24, 48 and 72 h and supernatants analyzed by sBG ELISAs. Activin A treatment significantly reduced BG shedding in a time- and concentration-dependent manner in both epithelial and stromal cells. Untreated cells were used as controls. Each bar represents the mean ± SEM of 3 independent experiments performed in duplicate. Dunnett's test was used for statistical analysis; *$p \leq 0.05$; **$p < 0.01$; ***$p < 0.001$; $^{\#}p < 0.0001$; sBG, soluble betaglycan; Act A, activin A.

Treatment of endometriotic 12Z cell with inhibin A did not show a substantial reduction in BG shedding following inhibin A treatment after 24 h (Fig. 17A). In stromal THESC cells, about 5% - 40% decrease with 5 - 50 ng/mL inhibin A treatment was observed after 48 h (Fig. 17B). There were no substantial effects in BG shedding with concentrations below 5 ng/mL for activin A and inhibin A except for inhibin A treatment after 72 h in epithelial 12Z cells (Figs. 16A, 17A). Moreover, in THESC cells, no significant effects were observed with concentrations below 5 ng/mL for both activin A and inhibin A (Figs. 16B, 17B). Collectively, our results demonstrate that activin A and inhibin A treatment significantly reduce BG shedding in a time- and concentration-dependent manner in human endometrial cells.



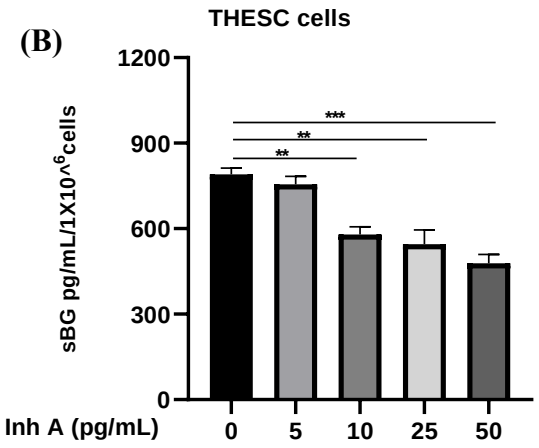


Figure 17. Time- and concentration-dependent effects of inhibin A on BG shedding in epithelial and stromal cells. Epithelial 12Z **(A)** and stromal THESC cells **(B)** were stimulated with increasing concentrations of inhibin A (5 - 50 ng/mL) for 24, 48 and 72 h and supernatants analyzed by sBG ELISAs. Inhibin A treatment significantly reduced BG shedding in epithelial cells in a time- and concentration-dependent manner **(A)** and in stromal cells in a concentration-dependent manner after 48 h **(B)**. Untreated cells were used as controls. Each bar represents the mean ± SEM of 3 independent experiments performed in duplicate. Dunnett's test was used for statistical analysis; *$p \leq 0.05$; **$p < 0.01$; ***$p < 0.001$; sBG, soluble betaglycan; Inh A, inhibin A.

3.3.2 Influence of activin A and inhibin A on BG shedding in different cell types

Besides the epithelial endometriotic 12Z and stromal THESC cells, we also examined BG shedding in epithelial Ishikawa cells as well as primary human endometrial stromal cells. To address this, cells were treated with activin A or inhibin A and supernatants analyzed by BG ELISAs. Our findings showed that both activin A and inhibin A significantly reduced BG shedding in epithelial 12Z cells (~ 50% and 55%, respectively; Fig. 18A), Ishikawa cells (~ 60% and 55%, respectively; Fig. 18B), primary stromal cells (~ 20% and 25%, respectively; Fig. 18C) as well as stromal THESC cells (~ 35% and 45%, respectively; Fig. 18D).

Although a comparable extent in reduction of BG shedding was observed with either activin A or inhibin A treatment in the four cell types, epithelial cells exhibited a stronger reduction in BG shedding compared to stromal cells, 55% vs 31%, respectively (Fig. 18A-D). Moreover, untreated stromal THESC cells released more sBG in comparison with untreated

epithelial Ishikawa cells (1.5-fold) and primary stromal cells (2.3-fold). Overall, these results suggest that activin A and inhibin A significantly suppress BG shedding in both endometrial epithelial and stromal cells.

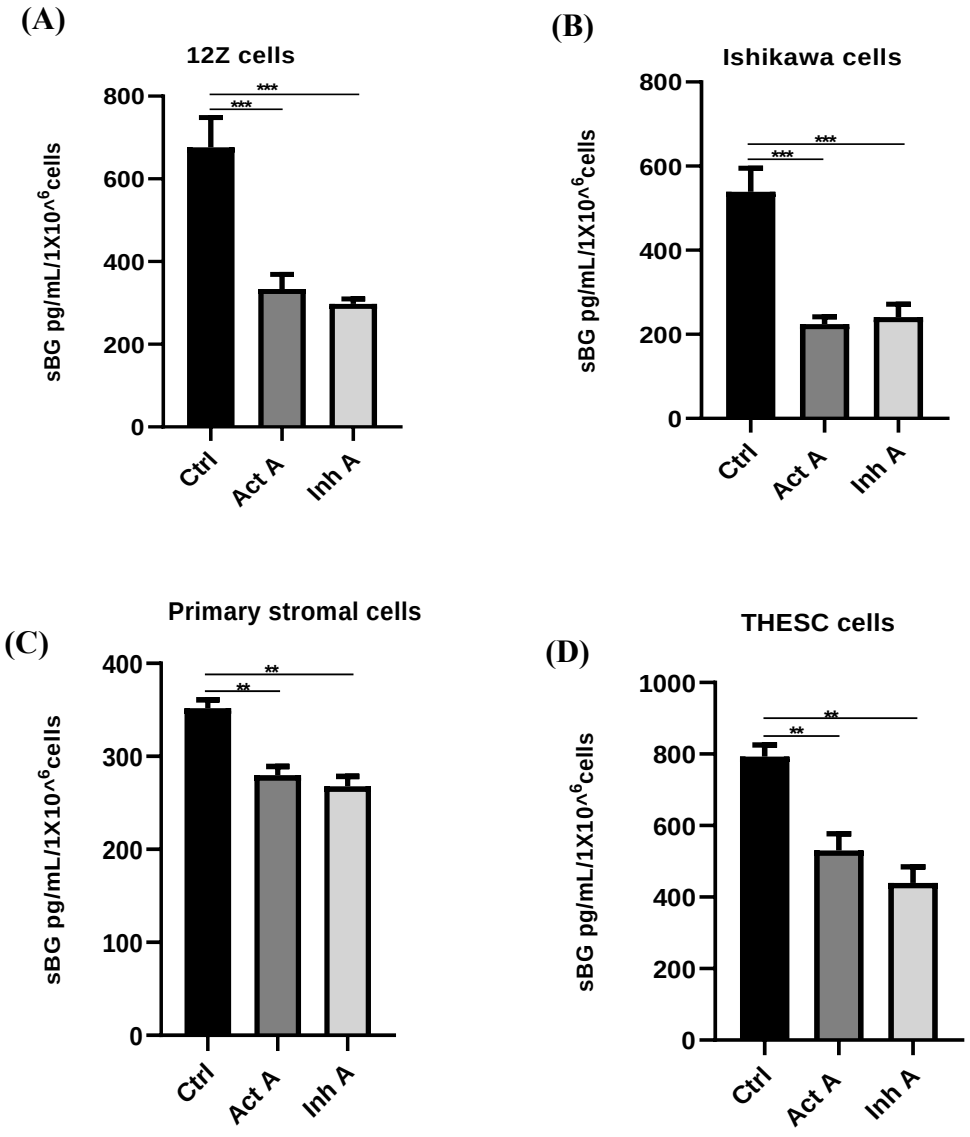
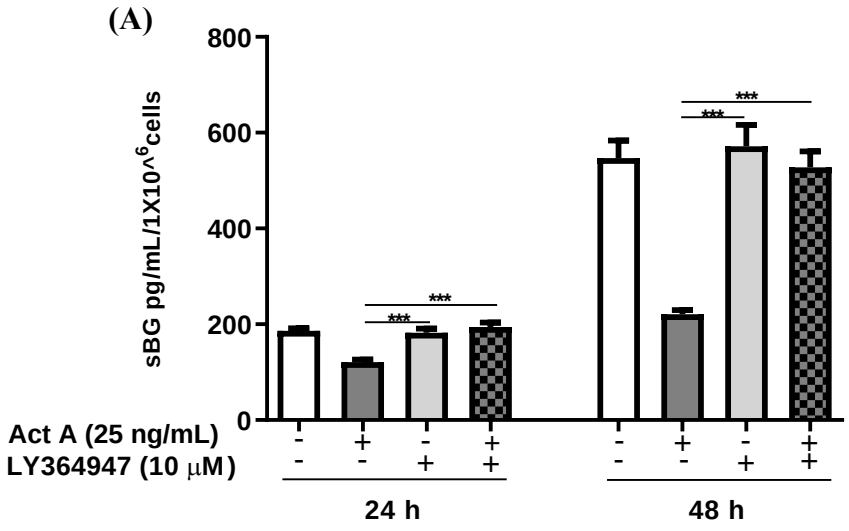


Figure 18. Activin A and inhibin A regulate BG shedding in both epithelial and stromal cells. Endometriotic epithelial 12Z **(A)**, endometrial epithelial Ishikawa **(B)**, primary human endometrial stromal **(C)**, and endometrial stromal THESC **(D)** cells were treated with activin A or inhibin A (25 ng/mL) for 48 h and supernatants analyzed by sBG ELISAs. Both activin A and inhibin A significantly attenuated BG shedding in all four cell types. Untreated cells were used as controls. Each bar represents the mean ± SEM of 3 independent experiments performed in duplicate. Dunnett's test was used for statistical analysis; $^{**}p < 0.01$; $^{***}p < 0.001$; sBG, soluble betaglycan; Ctrl, control; Act A, activin A; Inh A, inhibin A.

3.3.3 Involvement of activin receptor type-1B (ALK-4) in BG shedding

We explored the signaling mechanisms involved in the activin A-mediated reduction in BG shedding in endometriotic epithelial cells. Akin to other members of the TGF-β family, signaling of dimeric activin occurs through type I and type II serine-threonine kinase receptors (Cui et al., 2019). Consequently, we investigated the involvement of the activin receptor type-1B (ACVRB1 or ALK-4) in the activin A-mediated reduction in BG shedding. To test this, endometriotic 12Z cells were pre-treated with LY364947, which selectively inhibits ALK-4/5, prior to stimulation with activin A. Our results indicated that pretreatment with LY364947 completely (100%) abrogated the reduction in BG shedding induced by activin A after 24 and 48 h (Fig. 19A). Besides the ALK-4 inhibitor, we also evaluated the effects of follistatin, an activin binding and neutralizing protein, on activin A-mediated reduction in BG shedding. Contrary to ALK-4 inhibitor, pretreatment of 12Z cells with follistatin prior to activin A treatment had no effect on the activin A-induced regulation in BG shedding (Fig. 19B). Hence, phosphorylation and activation of ACVRB1 is required for the activin A-mediated decrease in BG shedding.



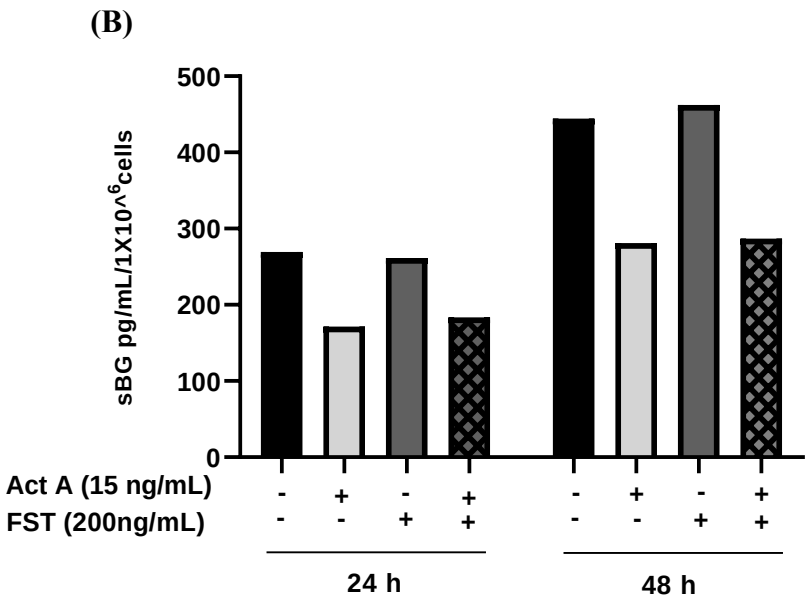


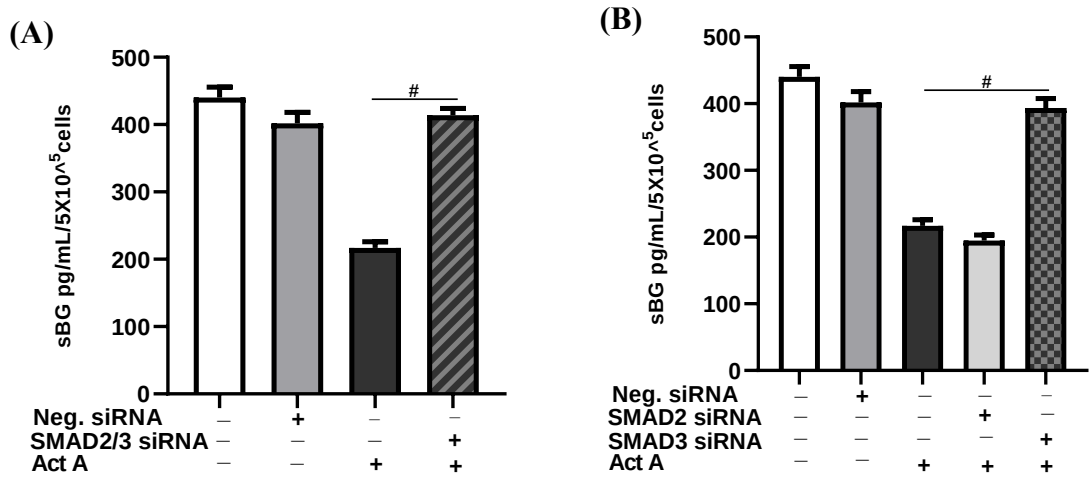
Figure 19. Activin A-mediated reduction in BG shedding is TGF-β type 1 receptor (ALK-4)-dependent but follistatin-independent. Epithelial 12Z cells were pre-incubated in the absence or presence of ALK-4/5 inhibitor, LY364947 (10 µM) **(A)**, or follistatin (200 ng/mL) **(B)** for 2 h prior to stimulation with activin A (25 ng/mL or 15 ng/mL) for 24 and 48 h and supernatants analyzed by sBG ELISAs. LY364947 completely abolished the reduction in BG shedding induced by activin A, whereas follistatin had no effect. Untreated cells or cells treated with DMSO (0.01%) were used as controls. Each bar represents the mean ± SEM of 3 independent experiments performed in duplicate. Dunnett's and Tukey's tests were used for statistical analysis; ***$p < 0.001$; sBG, soluble betaglycan; Act A, activin A; FST, follistatin.

3.3.4 Involvement of SMAD2/3 in activin A-mediated reduction in BG shedding

Binding of activin A instigates recruitment and activation of type II activin receptors and activin receptor type I that subsequently result in phosphorylation and activation of SMAD2/3 in the canonical pathway (Cui et al., 2019). Accordingly, we explored the involvement of the canonical SMAD signaling pathway in activin A-mediated modulation of BG shedding in endometriotic cells. To address this, both SMAD2 and SMAD3 genes were silenced using siRNA and the SMAD2/SMAD3 double-silenced epithelial 12Z cells treated with activin A and sBG quantified by ELISAs. Our findings demonstrated that

SMAD2/SMAD3 double gene knockdown completely counteracted the activin A-dependent reduction in BG shedding (Fig. 20A).

Subsequently, we investigated the individual contribution of each SMAD using SMAD2 and SMAD3 siRNA silencing and a SMAD3 inhibitor, SIS3, in epithelial 12Z cells followed by treatment with activin A and quantification of sBG. Examination of individual knockdowns revealed that silencing of SMAD3 but not SMAD2 abrogated the activin A-mediated reduction in BG shedding (Fig. 20B). Precisely, approximately 98% inhibition of activin A-mediated effects was observed following SMAD3 gene knockdown (Fig. 20B).



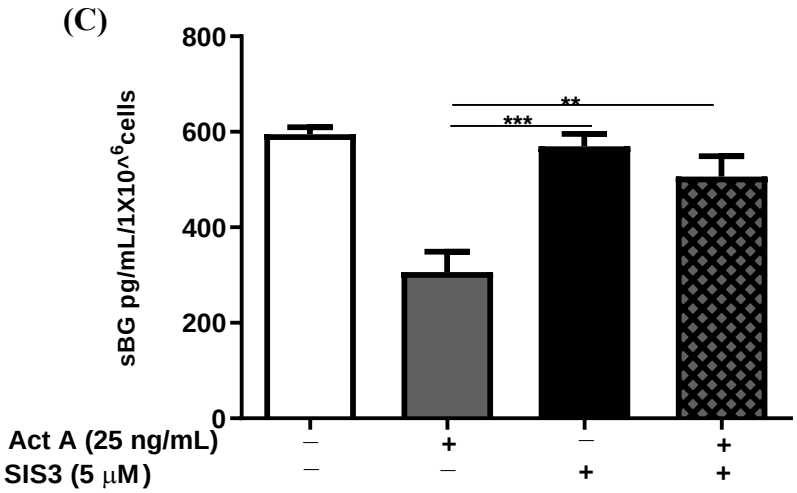


Figure 20. Activin A-mediated reduction in BG shedding is SMAD3- but not SMAD2-dependent. SMAD2/3 double-silenced **(A)** and single-silenced **(B)** 12Z cells were stimulated with activin A (25 ng/mL) for 48 h and supernatants analyzed by sBG ELISAs. SMAD2/SMAD3 double gene knockdown significantly abrogated the activin A-dependent reduction in BG shedding **(A)**. Single gene knockdown revealed that silencing of the SMAD3 gene and not of the SMAD2 gene reduced the activin A-mediated effects **(B)**. These results were further corroborated using the specific inhibitor of SMAD3, SIS3 (5 μM) **(C)**. Untreated cells and cells treated with Neg. siRNA or DMSO (0.01%) were used as controls. Each bar represents the mean ± SEM of 3 independent experiments performed in duplicate. Dunnett's and Tukey's tests were used for statistical analysis; $^{**}p < 0.01$; $^{***}p < 0.001$; $^{\#}p < 0.0001$; siRNA, small interfering RNA; Neg. siRNA, control siRNA; sBG, soluble betaglycan; Act A, activin A.

To further validate these results, we analyzed the effects of the specific inhibitor of SMAD3, SIS3, on activin A-mediated reduction in BG shedding (Fig. 20C). To address this, epithelial 12Z cells were pretreated with the SIS3 inhibitor prior to stimulation with activin A. Analysis of results indicated that SMAD3 inhibition significantly abolished (approximately 85% inhibition) the effect of activin A in reducing BG shedding (Fig. 20C). Concisely, these results imply that the canonical TGF-β pathway involving activin A/ACVRB1/SMAD3 signaling is required in activin A-mediated reduction in BG shedding.

3.4 Influence of recombinant betaglycan on cell viability

A study by Criswell et al. (2008) reported decreased growth rates of murine breast cancer cells following down-regulation of BG expression. Contrary, Turley et al. (2007) using non-small-cell lung carcinoma (NSCLC) cells and Bilandzic et al. (2009) using an immortalized ovarian granulosa cell line (COV434) and a human ovarian granulosa-like tumor cell line (KGN) reported no effect on cell proliferation following upregulation of BG expression. Thus, in the current study, we analyzed the effects of BG on viability of endometriotic epithelial cells. The cell viability assay using the cell counting kit-8 (CCK-8) was performed to determine the effects of increasing concentrations of recombinant BG on viability of 12Z cells after 24 h. No effect on the number of viable cells was observed at the concentrations tested (Fig. 21). Additionally, all concentrations exhibited a viability above 98% indicating that recombinant BG was without any effects on cell viability (Fig. 21).

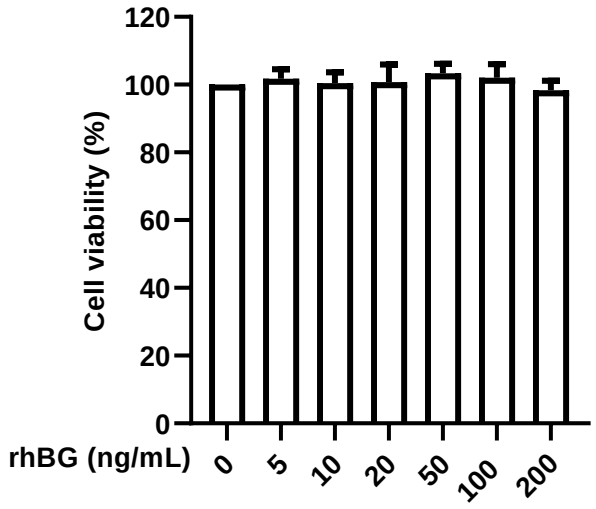
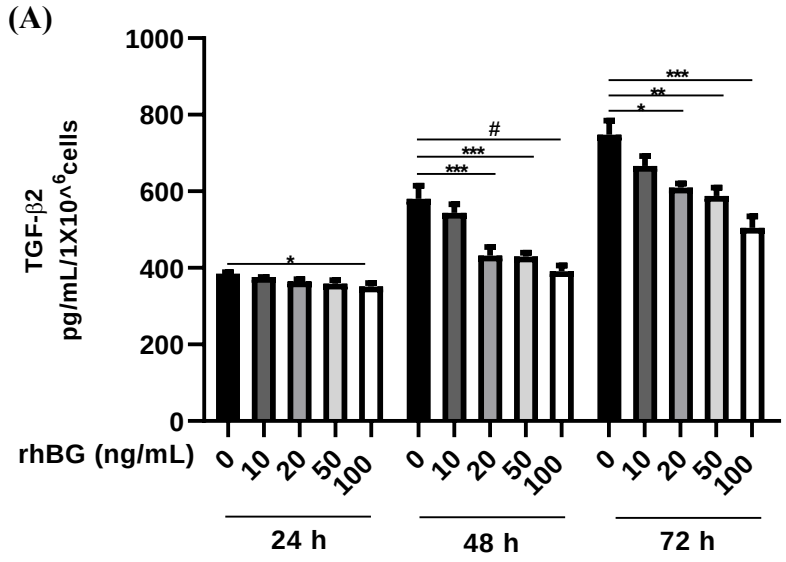


Figure 21. Concentration-dependent effects of recombinant BG (rhBG) on cell viability. Endometriotic epithelial 12Z cells were treated with increasing concentrations of recombinant BG (5 - 200 ng/mL) and analyzed for cell viability after 24 h using the CCK-8 assay kit. BG treatment had no effect on the number of viable cells at the concentrations tested. Untreated cells were used as control and the mean OD of the control was set to 100%. Each bar represents the mean ± SEM of 3 independent experiments performed in duplicate. Dunnett's test was used for statistical analysis; rhBG, recombinant betaglycan.

3.5 Effects of recombinant BG on TGF-β1 and TGF-β2 secretion

Previous studies reported that restoration of BG expression in numerous cancer types can block tumor progression, partly through production of sBG which binds to and neutralizes TGF-β activity, hence antagonizing the tumor promoting effects of TGF-β in late-stage cancers (Bandyopadhyay et al., 1999; 2002b; Nishida et al., 2018). Accordingly, we next focused on the influence of exogenous BG on secretion of TGF-β1 and TGF-β2. For this, epithelial 12Z cells were treated with increasing concentrations of recombinant BG (rhBG) and the levels of TGF-β1 and TGF-β2 quantified using ELISAs. Both TGF-β1 (~ 450 pg/mL/1x10^6 cells after 48 h) and TGF-β2 (~ 650 pg/mL/1x10^6 cells after 48 h) were detected in culture supernatants from 12Z cells (Fig. 22).

Our findings demonstrated a moderate time- and concentration-dependent reduction in secretion of TGF-β2 (~35% decrease with 20 - 100 ng/mL rhBG after 48 h and ~25% decrease after 72 h; Fig. 22A). Likewise, about 30% decrease in TGF-β1 secretion was noted after 48 h treatment with 20 - 100 ng/mL rhBG (Fig. 22B). Lower concentrations of rhBG had no effect on secretion of either TGF-β1 or TGF-β2 (Fig. 22A-B). Ergo, these results further confirm the role of BG in binding and antagonizing TGF-βs.



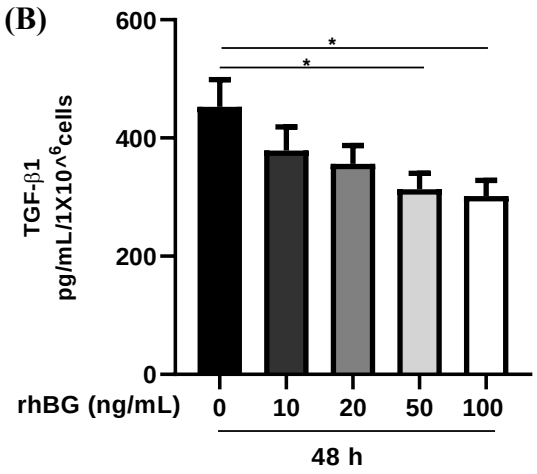


Figure 22. Time- and concentration-dependent effects of recombinant BG (rhBG) on TGF-β2 and TGF-β1 secretion. Endometriotic epithelial 12Z cells were treated with increasing concentrations of rhBG (10 - 100 ng/mL) for 24, 48 and 72 h and supernatants analyzed by TGF-β2 ELISA **(A)**. 12Z cells were also treated with increasing concentrations of rhBG (10 - 100 ng/mL) and analyzed for TGF-β1 secretion after 48 h **(B)**. BG treatment significantly decreased TGF-β2 levels in a time- and concentration-dependent manner **(A)** and moderately reduced TGF-β1 secretion in a concentration-dependent manner **(B)**. Untreated cells were used as control. Each bar represents the mean ± SEM of 3 independent experiments performed in duplicate. Dunnett's test was used for statistical analysis; *$p \leq 0.05$; **$p < 0.01$; ***$p < 0.001$; $^{\#}p < 0.0001$; BG, betaglycan.

3.6 Modulation of BG shedding by matrix metalloproteinases (MMPs)

A previous study proposed that membrane-type matrix metalloproteinases (MT-MMPs) and tissue inhibitor of metalloproteinases (TIMPs) regulate BG shedding (Velasco-Loyden et al., 2004). Consequently, we investigated the contribution of MMPs in BG shedding using the broad-range MMP inhibitor, GM6001, as well as recombinant TIMPs (rhTIMPs). Epithelial 12Z cells and stromal THESC cells were treated with GM6001 and sBG quantified by ELISAs. Our data indicated a 35% and 40% decrease in BG shedding in 12Z and THESC cells, respectively (Fig. 23A-B).

Besides the synthetic pan-MMP inhibitor, we also examined the effects of TIMP1, 2 and 3, which are core endogenous inhibitors of MMPs *in vivo* (Baker et al., 2002). For this,

endometriotic 12Z cells were treated with increasing concentrations of recombinant TIMP1/2 (10 ng/mL – 200 ng/mL) and TIMP3 (2.5 nM – 10 nM) and supernatants analyzed by sBG ELISAs. Stromal THESC cells were also treated with increasing concentrations of recombinant TIMP3 (2.5 nM – 10 nM). TIMP1 and TIMP2 had no substantial effects on BG shedding at the concentrations tested (Fig. 24A, B).

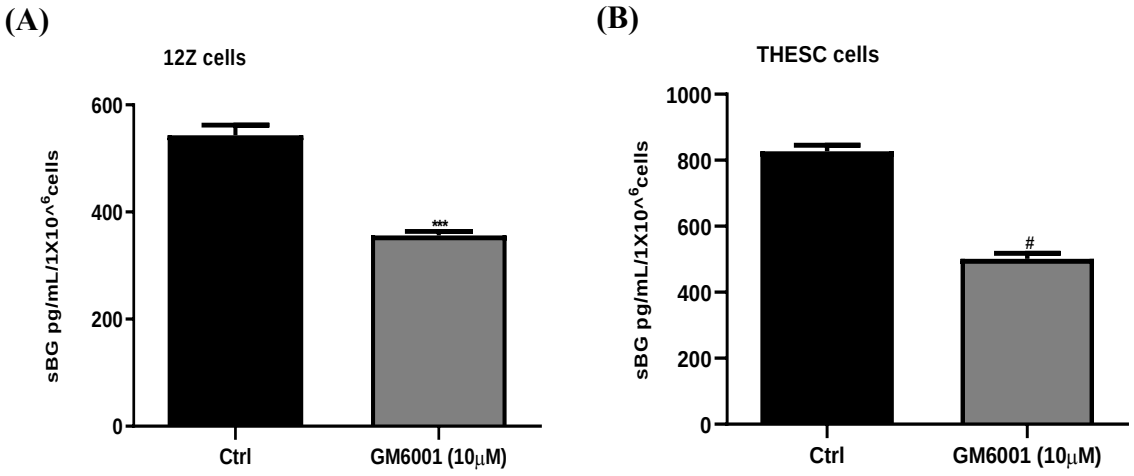


Figure 23. BG shedding is attenuated by the broad-spectrum MMP inhibitor, GM6001. Epithelial 12Z **(A)** and stromal THESC **(B)** cells were treated with GM6001 (10 μM) for 48 h and supernatants analyzed using sBG ELISAs. GM6001 significantly attenuated BG shedding in both epithelial 12Z and stromal THESC cells. Cells treated with DMSO (0.01%) were used as controls. Each bar represents the mean ± SEM of 3 independent experiments performed in duplicate. Student's t-test was used for statistical analysis; ***$p < 0.001$; $^{\#}p < 0.0001$; sBG, soluble betaglycan; Ctrl, control.

On the contrary, approximately 25% decrease in BG shedding was observed with 10 nM rhTIMP3, whereas lower concentrations had no significant effects (Fig. 24C). Furthermore, in stromal THESC cells, a 22% - 30% decrease in sBG levels was noted after treatment with 2.5 nM - 10 nM rhTIMP3, with a tendency towards plateauing being exhibited beyond 2.5 nM (Fig. 24D). Succinctly, these data show that inhibition of MMPs reduces BG shedding thus confirming the involvement of MMPs in modulating BG shedding.

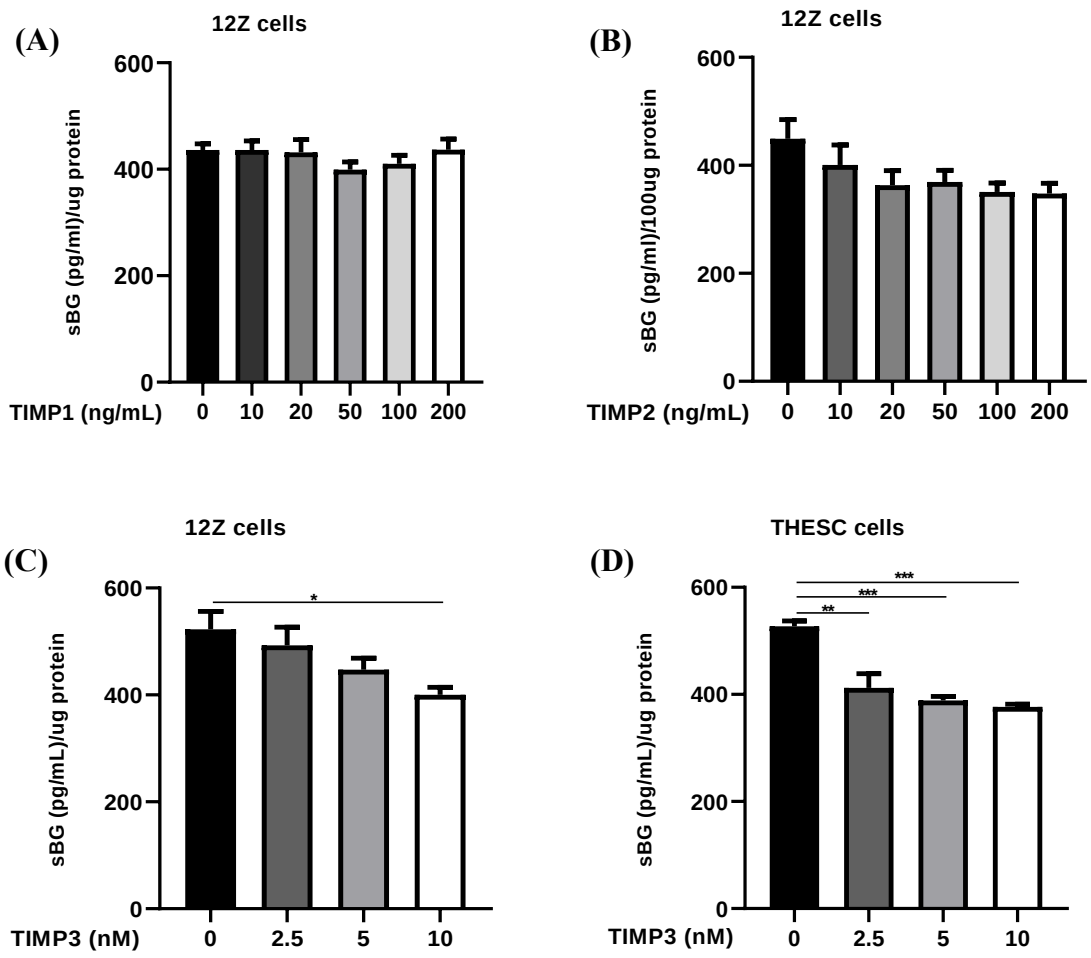


Figure 24. TIMP3 reduces BG shedding in epithelial and stromal cells. Epithelial 12Z **(A - C)** and stromal THESC **(D)** cells were treated with increasing concentrations of recombinant TIMP1/2 (10 – 200 ng/mL) or TIMP3 (2.5 –10 nM) for 48 h and supernatants analyzed by sBG ELISAs. Recombinant TIMP1 and TIMP2 had no substantial effects on BG shedding **(A, B)**, whereas TIMP3 significantly and concentration-dependently attenuated BG shedding in both epithelial **(C)** and stromal cells **(D)**. Untreated cells were used as controls. Each bar represents the mean ± SEM of 3 independent experiments performed in duplicate. Dunnett's test was used for statistical analysis; *$p \leq 0.05$; **$p < 0.01$; ***$p < 0.001$; sBG, soluble betaglycan.

3.7 Influence of TGF-βs, activin A & inhibin A on MMP2/3 & ADAM12 secretion

3.7.1 Influence of TGF-βs on MMP2 and MMP3 secretion

Previous findings propose that aberrant regulation of MMPs such as MMP2, MMP3 and MMP9 may be one of the prime causes of endometrial lesion formation (Balkowiec et al., 2018). Significantly higher levels of MMP2 (Huang et al., 2004) and MMP3 (Gilabert-Estellés et al., 2007) in sera and peritoneal fluid of endometriosis patients relative to healthy controls were previously reported.

Thus, we evaluated the effects of TGF-β1 and TGF-β2 on secretion of MMP2 and MMP3 in endometriotic 12Z cells. 12Z cells were stimulated with TGF-β1/2 and the concentrations of total MMP2 determined by ELISA. Both MMP2 (~ 6 ng/mL/1x10^6 cells) and MMP3 (~ 25 pg/mL/1x10^6 cells) were detected in culture supernatants from 12Z cells after 48 h (Figs. 25, 26). Our results revealed that both TGF-β1 and TGF-β2 significantly induced MMP2 secretion in a time-dependent manner (Fig. 25A). Strikingly, in 12Z cells, approximately 2-fold and 1.5-fold increase after 24 h and 6.5-fold and 4-fold increase after 48 h as well as a 5.5-fold and 3-fold increase in MMP2 secretion after 72 h following stimulation with TGF-β1 and TGF-β2, respectively, was observed (Fig. 25A). Furthermore, for stromal THESC cells, an approximately 5-fold and 3-fold increase in MMP2 secretion following stimulation with TGF-β1 and TGF-β2, respectively, after 48 h was observed (Fig. 25B).

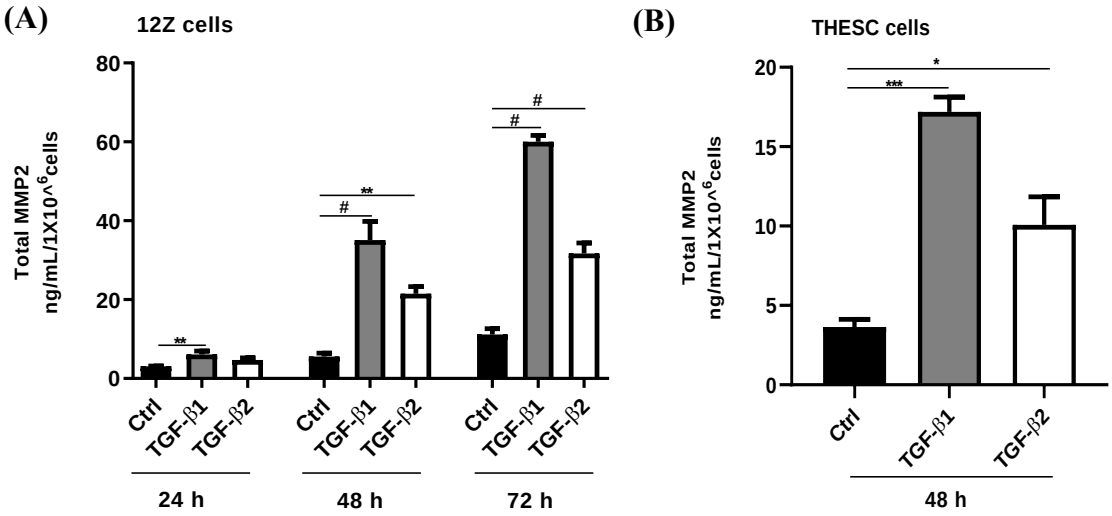


Figure 25. Time-dependent effects of TGF-β1 and TGF-β2 on MMP2 secretion. Endometriotic epithelial 12Z cells were stimulated with TGF-β1 or TGF-β2 (10 ng/mL) for 24, 48 and 72 h and supernatants analyzed by MMP2 ELISAs **(A)**. Stromal THESC cells were also stimulated with TGF-β1 or TGF-β2 (10 ng/mL) for 48 h and supernatants analyzed using MMP2 ELISAs **(B)**. Both TGF-β1 and -β2 stimulation of the epithelial 12Z cells significantly enhanced MMP2 secretion in a time-dependent manner **(A)**. TGF-β1 and -β2 stimulation of the stromal cells equally promoted MMP2 secretion **(B)**. Untreated cells were used as controls. Each bar represents the mean ± SEM of 3 independent experiments performed in duplicate. Dunnett's test was used for statistical analysis; *$p \leq 0.05$; **$p < 0.01$; ***$p < 0.001$; $^{\#}p < 0.0001$; Ctrl, control.

Examination of the MMP3 ELISA results revealed that TGF-β1 but not TGF-β2 significantly and time-dependently induced MMP3 secretion in the endometriotic 12Z cells (Fig. 26). Following TGF-β1 stimulation, a 1.5-, 3.5- and 5-fold increase in MMP3 secretion after 24, 48 and 72 h, respectively, was noted (Fig. 26). A comparison between the levels of secreted MMP2 and MMP3 in untreated 12Z cells indicated that the 12Z cells secrete about 250 times more MMP2 than MMP3 (Figs. 25, 26). Collectively, these results suggest that in endometriotic cells TGF-β1 enhances both MMP2 and MMP3 secretion, whereas TGF-β2 enhances MMP2 secretion only.

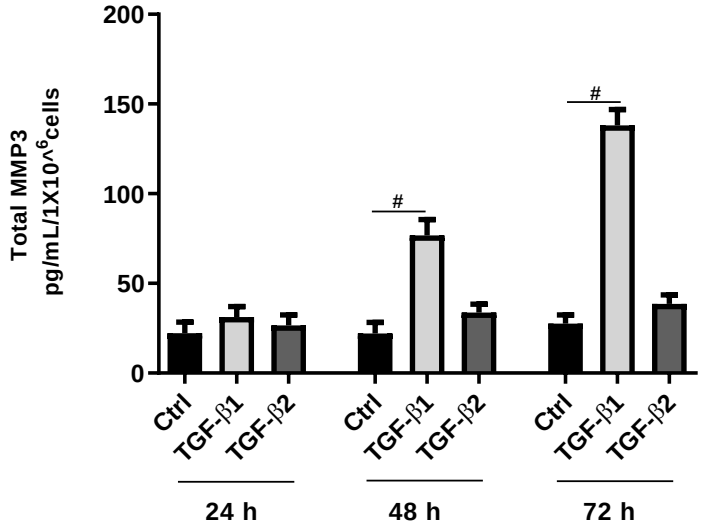


Figure 26. Time-dependent effects of TGF-β1 and TGF-β2 treatment on MMP3 secretion. Epithelial 12Z cells were stimulated with TGF-β1 or TGF-β2 (10 ng/mL) for 24, 48 and 72 h, respectively, and supernatants analyzed by MMP3 ELISAs. TGF-β1 but not TGF-β2 stimulation of the epithelial 12Z cells significantly increased MMP3 secretion in a time-dependent manner. Untreated cells were used as controls. Each bar represents the mean ± SEM of 3 independent experiments performed in duplicate. Dunnett's test was used for statistical analysis; $^{\#}p < 0.0001$; Ctrl, control.

3.7.2 Effects of TGF-β1 and TGF-β2 on ADAM12 secretion

ADAM12 is associated with a number of signaling pathways including the TGF-β pathway which is essential in cell proliferation, EMT, fibrosis as well as metastasis in certain cells including hepatic and renal cells (Le Pabic et al., 2003; Atfi et al., 2007; Ramdas et al., 2013). To gain insight into the regulation of ADAM12 by TGF-β in endometrial cells, we analyzed the effects of TGF-β1 and TGF-β2 on secretion of ADAM12 in epithelial 12Z and stromal THESC cells. Unlike THESC, 12Z cells were not found to secrete detectable levels of ADAM12 and thus a western blot was performed. Both 12Z and THESC cells were stimulated with TGF-β and the levels of ADAM12 determined by western blot and ELISAs. Stimulation of 12Z cells had no effect on expression of ADAM12 (data not shown). Contrary, evaluation of the ADAM12 ELISA results revealed that both TGF-β1 and TGF-β2 induced

ADAM12 secretion in THESC cells (Fig. 27). Following TGF-β1 and -β2 stimulation, about a 2-fold increase after 48 h and a 4- and 3-fold increase after 72 h in ADAM12 secretion, respectively, was noted (Fig. 27). Collectively, these results suggest that both TGF-β1 and TGF-β2 significantly augment secretion of ADAM12 in endometrial stromal cells but have no effect on endometriotic epithelial cells.

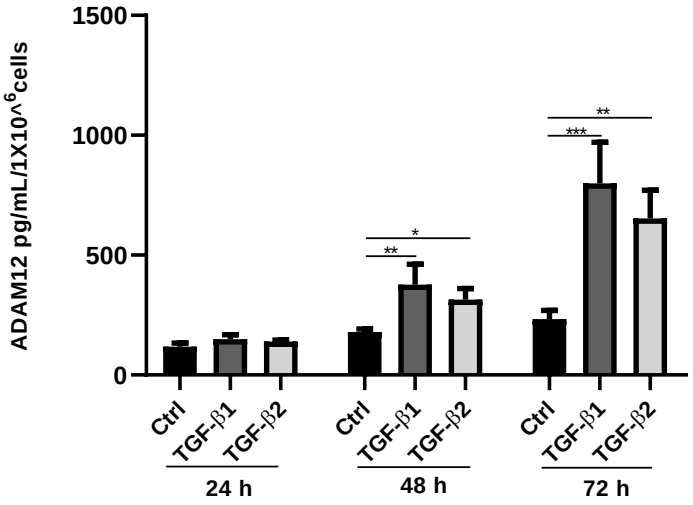


Figure 27. Time-dependent effects of TGF-β1 and TGF-β2 on ADAM12 secretion. Stromal THESC cells were stimulated with TGF-β1 or TGF-β2 (10 ng/mL) for 24, 48 and 72 h and supernatants analyzed by ADAM12 ELISAs. Both TGF-β1 and TGF-β2 stimulation of the THESC cells significantly enhanced ADAM12 secretion in a time-dependent manner. Untreated cells were used as controls. Each bar represents the mean ± SEM of 3 independent experiments performed in duplicate. Dunnett's test was used for statistical analysis; *$p \leq 0.05$; **$p < 0.01$; ***$p < 0.001$; Ctrl, control.

3.7.3 Influence of activin A and inhibin A on MMP2 and MMP3 secretion

Decidualization is requisite to successful blastocyst implantation and is promoted by several paracrine agents including activin A (Jones et al., 2006). Activin A and inhibin A are involved in regulation of embryo implantation and trophoblast invasion mainly via their effects on MMPs, which are principal mediators of decidualization. Maternal decidua-derived activin A was shown to enhance endometrial production of proMMP2, 3, 7, 9, and active MMP2 (Jones et al., 2006). Thus, we investigated the effects of activin A and inhibin A on secretion

of MMP2 and MMP3 in endometriotic cells. 12Z cells were stimulated with increasing concentrations of activin A and inhibin A and the levels of total MMP2 and MMP3 determined by ELISAs.

Our results revealed that activin A significantly induced MMP2 secretion in a concentration- and time-dependent manner (Fig. 28). Approximately 2-fold increase after 24 h stimulation with 25 ng/mL and 50 ng/mL activin A was observed, respectively. Following stimulation with 5, 10, 25 and 50 ng/mL activin A for 48 h, an approximately 2-, 3-, 4-, and 5-fold increase, respectively, in MMP2 secretion was noted. Additionally, a ~ 2-fold increase in MMP2 secretion was observed following stimulation with 10 - 50 ng/mL activin A after 72 h (Fig. 28). Conversely, inhibin A stimulation of the cells had no effect on MMP2 secretion (Fig. 29A, B). Moreover, simultaneous addition of inhibin A and activin A resulted in a ~ 20% decrease in the activin A-induced MMP2 secretion (Fig. 29B).

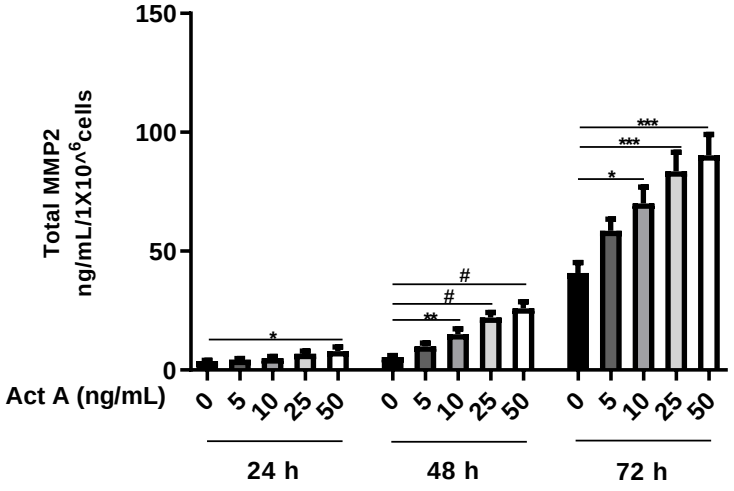


Figure 28. Time and concentration-dependent effects of activin A treatment on MMP2 secretion. Endometriotic 12Z cells were stimulated with increasing concentrations of activin A (5 - 50 ng/mL) for 24, 48 and 72 h and supernatants analyzed by MMP2 ELISAs. Activin A treatment significantly promoted MMP2 secretion in a time- and concentration-dependent manner. Untreated cells were used as controls. Each bar represents the mean ± SEM of 3 independent experiments performed in duplicate. Dunnett's test was used for statistical analysis; *$p \leq 0.05$; **$p < 0.01$; ***$p < 0.001$; $^{\#}p < 0.0001$; Act A, activin A.

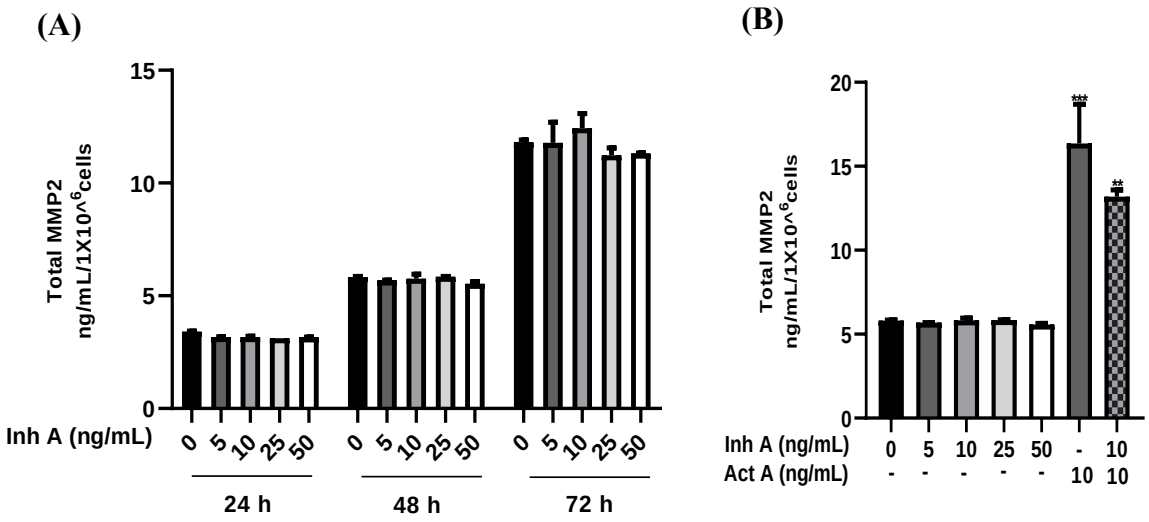


Figure 29. Effects of inhibin A treatment on MMP2 secretion. Endometriotic 12Z cells were stimulated with increasing concentrations of inhibin A (5 - 50 ng/mL) for 24, 48 and 72 h and supernatants analyzed by MMP2 ELISAs **(A)**. The cells were also treated with inhibin A with or without activin A (each 10 ng/mL) for 48 h and supernatants analyzed **(B)**. Inhibin A treatment of the epithelial cells had no effect on MMP2 secretion **(A, B)**. Simultaneous addition of inhibin A and activin A resulted in a moderate reduction in the activin A-mediated increase in MMP2 secretion **(B)**. Untreated cells were used as controls. Each bar represents the mean ± SEM of 3 independent experiments performed in duplicate. Dunnett's test was used for statistical analysis; $^{**}p < 0.01$; $^{***}p < 0.001$; Inh A, inhibin A; Act A, activin A.

Furthermore, activin A stimulation of the 12Z cells resulted in a significant time- and concentration-dependent increase in MMP3 secretion (Fig. 30). Approximately 2-fold increase in MMP3 secretion after 48 h and a ~ 3- and 4-fold increase after 72 h were noted with 25 ng/mL and 50 ng/mL activin A stimulation, respectively (Fig. 30). On the contrary, inhibin A stimulation of the 12Z cells had no effect on MMP3 secretion (data not shown). Taken together, these results imply that in endometriotic 12Z cells activin A promotes secretion of both MMP2 and MMP3 whereas inhibin A has no effect.

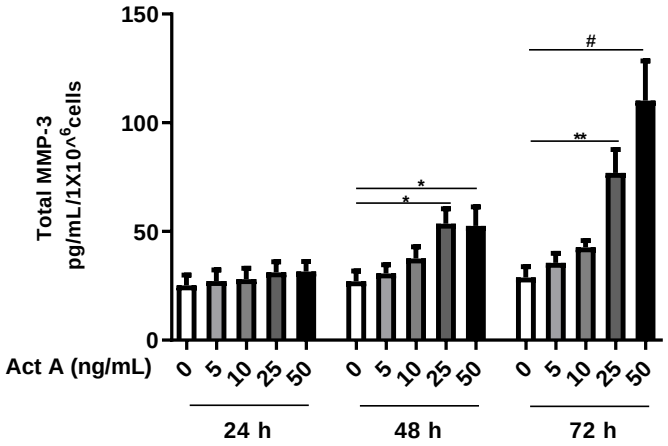
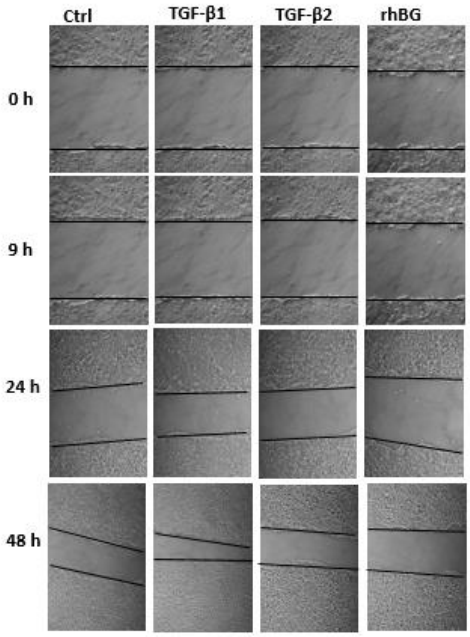


Figure 30. Time and concentration-dependent effects of activin A treatment on MMP3 secretion. Endometriotic 12Z cells were stimulated with increasing concentrations of activin A (5 - 50 ng/mL) for 24, 48 and 72 h and supernatants analyzed by MMP3 ELISAs. Activin A stimulation of the 12Z cells significantly augmented MMP3 secretion in a time- and concentration-dependent manner. Untreated cells were used as controls. Each bar represents the mean ± SEM of 3 independent experiments performed in duplicate. Dunnett's test was used for statistical analysis; *$p \leq 0.05$; **$p < 0.01$; $^{\#}p < 0.0001$; Act A, activin A.

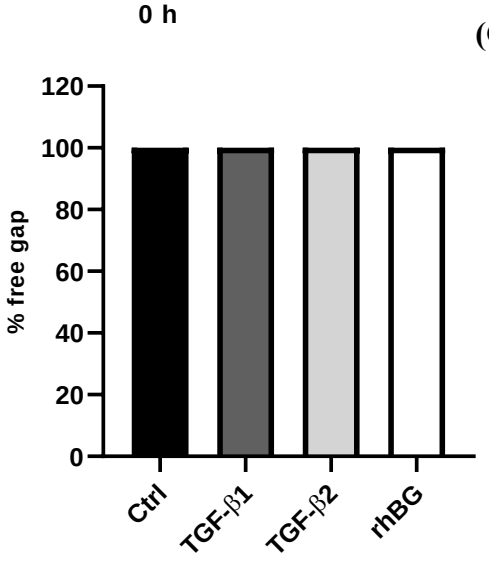
3.8 Effects of TGF-β1/2 and BG on wound healing

Context-dependent dysregulation of TGF-β signaling has been implicated in several wound healing pathologies (Barrientos et al., 2008; Kiritsi and Nyström, 2018). Within the endometrium, TGFβ superfamily members like activin A and TGF-βs have been proposed to promote the scar-less endometrial repair that occurs after menstruation (Kaitu'u-Lino et al., 2009; Omwandho et al., 2010). Accordingly, we investigated the influence of TGF-β1, TGF-β2 and BG on wound healing in endometriotic 12Z cells using the scratch assay. For this, 12Z cells were cultured to 100% confluency and a wound induced using a yellow pipette tip prior to stimulation with either TGF-β1, TGF-β2 or BG for 9, 24 and 48 h.

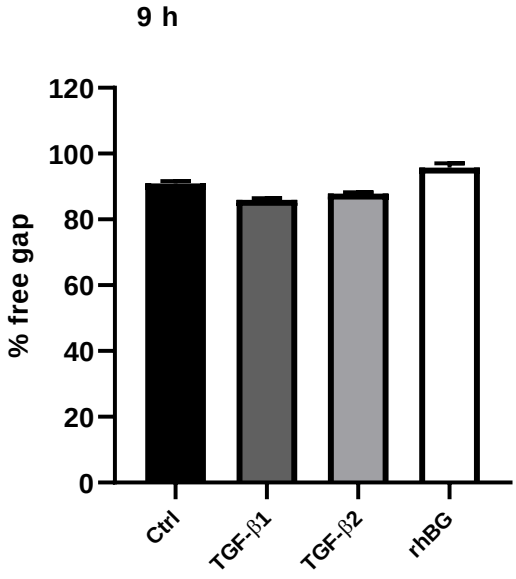
(A)


Ctrl
TGF-β1
TGF-β2
rhBG
0 h
9 h
24 h
48 h

(B)


0 h
% free gap
120
100
80
60
40
20
0
Ctrl
TGF-β1
TGF-β2
rhBG

(C)


9 h
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TGF-β2
rhBG

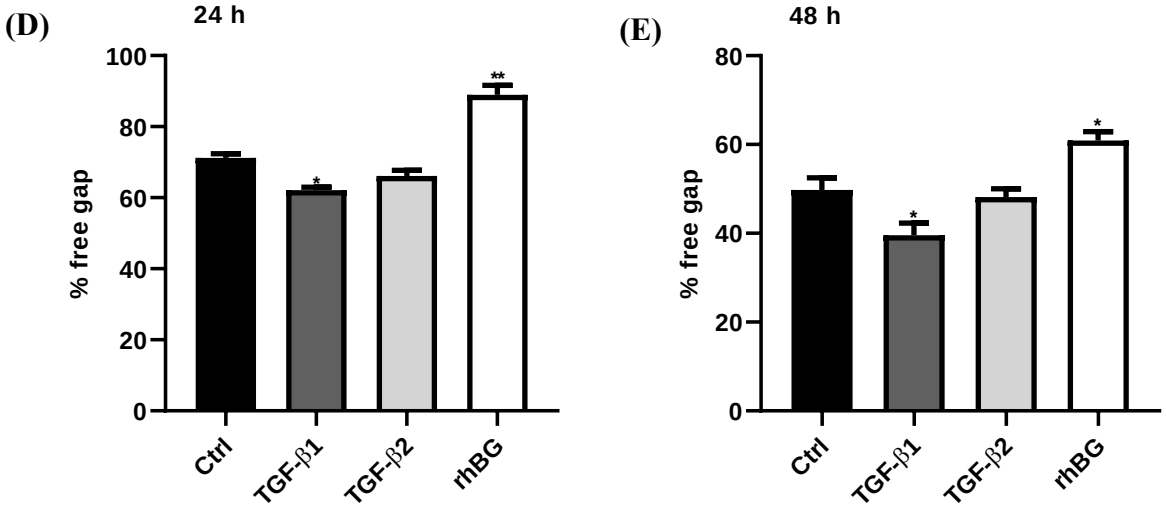


Figure 31. Effects of TGF-β1/2 and BG on wound healing of 12Z cells monitored by taking images at different time points. Representative images of the wound healing assay at the four time points **(A)**. Results are presented as percentage free gap following treatment with recombinant TGF-β1/2 (10 ng/mL) or BG (25 ng/mL) and analysis using ImageJ after 0 **(B)**, 9 **(C)**, 24 **(D)** and 48 **(E)** h. TGF-β1 but not TGF-β2 augmented migration of the endometriotic cells, whereas rhBG impeded migration of the 12Z cells **(D, E)**. Untreated cells were used as control. Each bar represents the mean ± SEM of 3 independent experiments performed in duplicate. Dunnett's test was used for statistical analysis; *$p \leq 0.05$; **$p < 0.01$; Ctrl, control; rhBG, recombinant betaglycan.

No significant differences were observed in the rate of wound closure of 12Z cells after 9 h (Fig. 31C). Conversely, compared to the untreated cells, a moderate increase of ~13% and 21% after 24 and 48 h, respectively, in wound closure with TGF-β1 treatment was noted, whereas no significant effects were observed with TGF-β2 (Fig. 31D, E). Additionally, a ~ 25% reduction in the rate of wound closure was observed following rhBG treatment for 24 and 48 h, respectively (Fig. 31D, E). Consequently, these data suggest that TGF-β1 promotes migration of endometriotic cells, while BG impedes migration of these cells.

3.9 Soluble BG levels in serum and endocervical mucus

3.9.1 Soluble BG levels in serum

Although BG has been demonstrated to participate in multiple diseases (Gatza et al., 2010) including endometrial cancer (Florio et al., 2005; Zakrzewski et al., 2011; 2020), its role in the pathophysiology of endometriosis remains unclear. Hence, we investigated the utility of serum/endocervical mucus sBG levels as a non-invasive diagnostic biomarker for endometriosis.

Soluble BG levels were determined in sera from 238 patients with and without endometriosis using sBG ELISA (Table 15). The protein was detected in all (100%) analyzed serum samples. Firstly, we investigated the dependence of sBG levels on the menstrual cycle phase. Correlation analysis of serum samples exhibited no significant associations between sBG levels and menstrual cycle days (Table 17A, 19). Although serum sBG levels were not significantly different between the three phases of menstrual cycle, a trend was observed of lower sBG concentrations in the secretory and menstruation phases relative to the proliferative phase ($p = 0.09$; Table 17A).

Subsequently, the subjects were grouped into two namely women without contraception either with or without endometriosis and women using hormonal contraception with or without endometriosis. There were no significant variations in serum sBG levels between patients with and without endometriosis, both in women without contraception as well as in women using contraception (Table 17B). Nevertheless, women with endometriosis but without contraception had elevated levels of serum sBG (~ 22 %) compared to those without endometriosis who were under contraception ($p = 0.04$; Table 17B). Further analysis of the ELISA results exhibited no significant correlations between serum sBG levels and age, fertility or pain parameters like dysmenorrhea, dyspareunia, dyschezia and dysuria (Table 19).

Table 17. Soluble betaglycan levels in serum

(A)	**Proliferative**	**Secretory**	**Menstruation**
n	47	72	26
Median age	28 ± 1.1	30 ± 0.9	30.5 ± 1.6
Mean sBG (ng/mL)	61.8 ± 3.2	53.7 ± 2.8	52.7 ± 4.8
Range (ng/mL)	12 - 114	11 - 98	9 - 87
P	ns	ns	ns

(B)	**Without contraception**		**With Contraception**	
	EM [a]	**w/o EM [b]**	**EM [c]**	**w/o EM [d]**
n	98	29	68	43
Median age	35 ± 0.7	27 ± 1.5	30 ± 0.9	23 ± 1.0
Mean sBG (ng/mL)	59.6 ± 2.2	60.3 ± 4.3	50.3 ± 3.1	48.7 ± 3.3
Range (ng/mL)	11 - 98	12 – 114	11 - 87	9 - 79
P	ns	Ns	ns	0.04 [a,d]

n = 238; Data are median ± SEM or mean ± SEM; SEM, standard error of the mean; sBG, soluble betaglycan; EM, endometriosis; w/o EM, without endometriosis; ns, not significant; e.g. 0.04 [a,d] means EM without contraception compared to w/o EM with contraception. Analysis was performed using the Kruskal-Wallis test and the Mann-Whitney U-test.

3.9.2 Soluble BG levels in endocervical mucus

Soluble BG can be detected in the extracellular matrix as well as body fluids like milk, serum and plasma (Andres et al., 1989; Zhang et al., 2001; Grgurevic et al., 2020). Thus, besides serum we also evaluated for the first time sBG levels in endocervical mucus samples. In total, 182 samples from 100 patients with endometriosis and 82 subjects without endometriosis (controls) were analyzed with ELISAs. Soluble BG was detectable in 96% and 99% of endocervical mucus samples from endometriosis and controls, respectively. We evaluated the dependency of endocervical mucus sBG levels on the menstrual cycle phase. Comparable to serum sBG, cycle independency was also found in endocervical mucus samples (Table 18A, 19).

We further evaluated endocervical mucus sBG levels in disease verses disease-free cases. Notably, the mean endocervical mucus sBG levels were significantly lower in endometriosis patients relative to controls, both in patients with and without contraception (Table 18B). Approximately 2-fold difference in endometriosis patients without contraception and ~3-fold

difference in endometriosis patients using contraception was noted (Table 18B). Although not statistically significant ($p = 0.1058$), a trend was observed of lower endocervical mucus sBG levels in patients using contraception compared to those who were not using contraception (Table 18B). Further stratification of the groups based on menstrual phases demonstrated a similar reduction in sBG levels (~ 55%) in endometriosis relative to non-endometriosis cases during the secretory phase, whereas no significant differences were observed in women in the proliferative phase (Table 18C).

Table 18. Soluble betaglycan levels in endocervical mucus

(A)	**Pro**	**Sec**
n	46	79
Median age	29 ± 1.0	30 ± 0.8
Mean sBG (pg/mL)	2186 ± 407	1940 ± 267
Range (pg/mL)	0 - 11335	0 - 10862
P	Ns	ns

(B)	**Without contraception**		**With Contraception**	
	EM [a]	**w/o EM [b]**	**EM [c]**	**w/o EM [d]**
n	70	51	30	31
Median age	31 ± 0.8	29 ± 1.0	33 ± 1.3	23 ± 1.4
Mean sBG (pg/mL)	1735 ± 243	3419 ± 454	912 ± 523	2573 ± 187
Range (pg/mL)	0 - 7865	0 - 11335	0 - 4369	297 - 13036
P	0.0103 **[a,b]**		0.0007 **[c,d]**	

(C)	**With endometriosis**		**Without endometriosis**	
	Pro [a]	**Sec [b]**	**Pro [c]**	**Sec [d]**
n	27	43	19	36
Median age	30 ± 1.3	34 ± 1.0	28 ± 1.5	28 ± 0.9
Mean sBG (pg/mL)	1796 ± 418	1239 ± 260	2740 ± 782	2778 ± 463
Range (pg/mL)	0 - 7865	0 - 7473	260 - 11335	0 - 10862
p	ns	Ns	ns	0.0233 **[b,d]**

n = 182; Data are median ± SEM or mean ± SEM; SEM, standard error of the mean; sBG, soluble betaglycan; EM, endometriosis; w/o EM, without endometriosis; pro, proliferative; sec, secretory; ns, not significant; e.g. 0.0103 [a,b] means EM versus w/o EM in patients without contraception. Analysis was performed using the Mann-Whitney U-test and the Kruskal-Wallis test.

No significant associations were observed between endocervical mucus sBG levels and age, fertility or pain parameters like dysmenorrhea, dyspareunia, dyschezia and dysuria (Table 19). The utility of endocervical mucus sBG as a potential non-invasive diagnostic biomarker for endometriosis was calculated with a cut-off of 1052 pg/mL and demonstrated a sensitivity of 61% and a specificity of 63% (area under the curve = 0.66, 95% CI = 0.5797 to 0.7411, likelihood ratio = 1.667, p = 0.0002) (Fig. 32) suggesting that sBG is only a moderate sensitive/specific diagnostic marker for endometriosis. Taken together, these results imply that sBG levels in serum are similar in women with and without endometriosis, but are significantly decreased in endocervical mucus of patients with endometriosis compared to controls.

Table 19. Correlations of cycle day, age, fertility and pain parameters with serum and endocervical mucus sBG levels

			Serum sBG			
	n		**Spearman r**	**Mean sBG (ng/mL)**		***P***
		BMI (kg/m^2)		**Without pain**	**With pain**	
Cycle day	145	23.1	-0.05			ns
Age	238	23.5	0.14			0.04
Fertility	112	24.0	0.07			ns
Dysmenorrhea	221	23.5		54.0	55.9	ns
Dyspareunia	214	23.4		56.3	53.6	ns
Dyschezia	223	23.5		56.5	50.2	ns
Dysuria	228	23.5		56.2	52.3	ns
			Endocervical mucus sBG			
	n		**Spearman r**	**Mean sBG (pg/mL)**		***P***
		BMI (kg/m^2)		**Without pain**	**With pain**	
Cycle day	125	22.6	-0.10			ns
Age	182	22.9	-0.09			ns
Fertility	85	24.0	0.07			ns
Dysmenorrhea	181	22.9		2812	2110	ns
Dyspareunia	181	22.9		2128	1965	ns
Dyschezia	182	22.9		2438	1800	ns
Dysuria	181	22.9		2304	1906	ns

Cycle day (Days 1 - 32); without pain (pain scale = 0 - 3); with pain (pain scale 4-10); sBG, soluble betaglycan; ns, not significant. Analysis was performed using the spearman r test and the Mann-Whitney U-test.

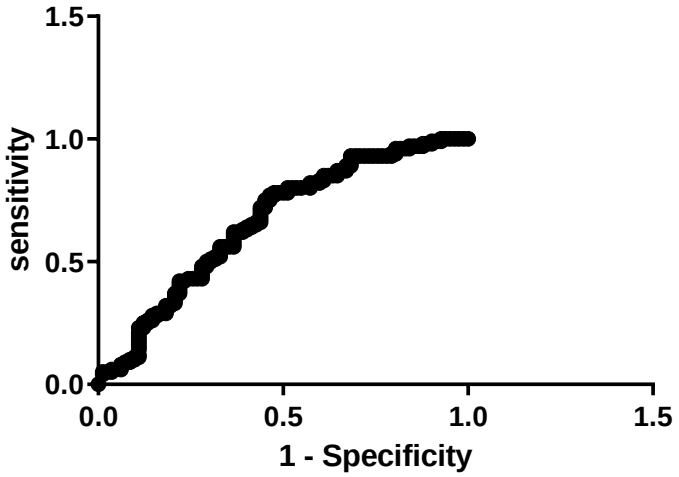


Figure 32. ROC curve in endometriosis patients vs. controls. The area under the curve (AUC) value is 0.66 (CI = 0.5797 - 0.7411, likelihood ratio = 1.667, p = 0.0002) from endometriosis patients (n = 100) and controls (n = 82); ROC, receiver operating characteristic; CI, confidence interval.

3.10 ADAM12 localization in the uterus, and serum & endocervical mucus levels

3.10.1 Immunohistochemical staining of ADAM12

In an attempt to determine which endometrial cells contribute to ADAM12 secretion, localization of ADAM12 in the human uterus was investigated. Biopsies of tissues (Table 16) were analyzed by immunohistochemistry and the pattern of localization of ADAM12 in eutopic and ectopic endometrium analyzed using anti-ADAM12 antibody.

Evaluation of immunohistochemistry results revealed that ADAM12 localizes mainly to the luminal cells and epithelial cells of the glandular portion of the endometrial layer as well as the smooth muscle cells and adenomyotic glands of the myometrial layer (Fig. 33A, B, E). Comparable glandular staining in the basalis as well as functionalis layers was noted. A pronounced staining of endometriotic epithelial cells at the three endometriotic entities: deep infiltrating, ovarian and peritoneal (Fig. 34A-C) as well as endothelial cells of blood vessels

(Fig. 33A) was observed for some tissues. Nonetheless, only very minimal to no staining was observed for stromal cells (Fig. 33). Glandular expression of ADAM12 was observed in both proliferative and secretory phases of the menstrual cycle (Fig. 33A, C).

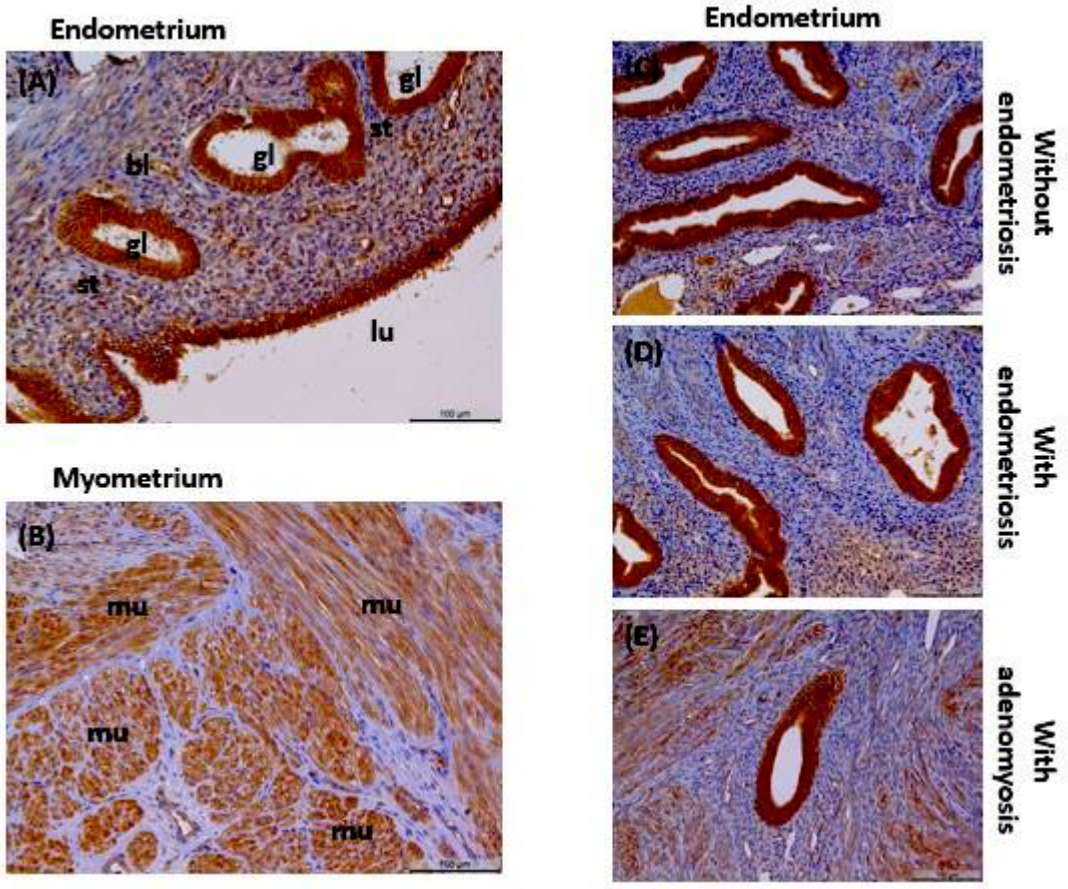


Figure 33. Representative microphotographs of ADAM12 staining in the uterus. Detection of ADAM12 (brown stain) in the endometrium **(A)** and myometrium **(B)**. Strong staining of the endometrial and apical lumen glands **(A)**, smooth muscle cells **(A, B)** and blood vessels **(A)** and no staining of stromal cells **(A-E)**. Endometrial staining was detected in women without endometriosis **(C)**, patients with endometriosis **(D)** as well as patients with adenomyosis **(E)**. Counterstaining (blue stain) was performed with hematoxylin. gl, gland; lu, lumen; st, stromal cells; bl, blood vessel; mu, muscle cells. Magnification 20x. Scale bars represent 100 μm.

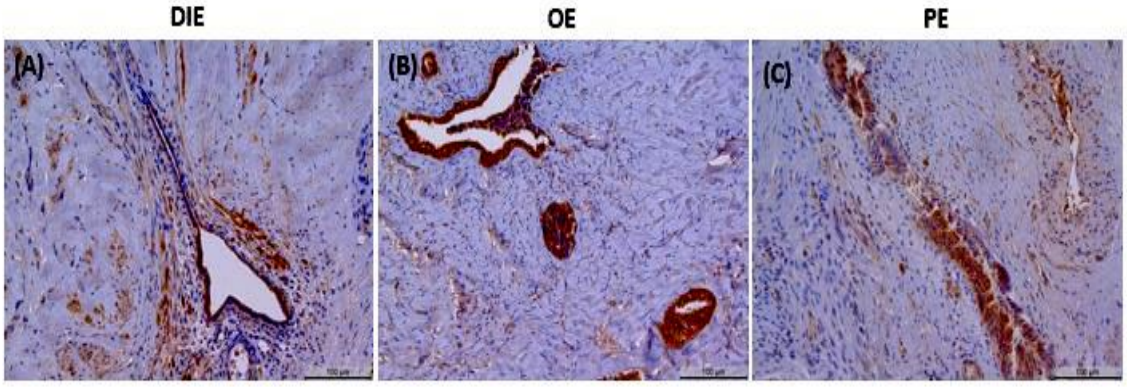


Figure 34. Representative microphotographs of ADAM12 staining at ectopic sites. Detection of ADAM12 (brown stain) in endometrial tissue located at rectovaginal septum **(A)**, ovarian tissue **(B)** and uterosacral ligament **(C)**. No staining of stromal cells was noted. Counterstaining (blue stain) was performed with hematoxylin. DIE, deep infiltrating endometriosis; OE, ovarian endometriosis; PE, peritoneal endometriosis. Magnification 200x. Scale bars represent 100 μm.

Quantification of staining intensity using HSCORE was performed and compared between the two phases of the menstrual cycle. No cycle-specific differences in ADAM12 expression in either endometrial or myometrial staining were observed (Table 20A, B). Further grouping of the patients based on disease state demonstrated similar endometrial and myometrial ADAM12 staining between women with and without endometriosis and those with adenomyosis (Table 21). Analysis of endometrial ADAM12 staining at ectopic sites revealed no differences in staining intensity in patients with deep infiltrating, ovarian and peritoneal endometriosis (Table 22). Thus, our results show that ADAM12 is highly expressed in the uterus although the expression is neither cycle dependent nor different in disease states.

Table 20. HSCORE comparison of endometrium and myometrium with and without endometriosis and adenomyosis during proliferative and secretory phases

	Healthy Endo, (Control)		Endo with endometriosis		Endo with adenomyosis	
(A) Endometrium	**Pro**	**Sec**	**Pro**	**Sec**	**Pro**	**Sec**
Mean HSCORE	99.2	78.8	76.6	97.1	91.7	53.0
SEM	20.8	23.7	18.8	15.5	21.7	22.3
n	15	9	12	12	10	5
Median age	40.0±2.5	44.0±1.5	39.5±3.7	42.5±1.5	45.5±1.9	45.0±2.5
p- value		ns	ns	ns	ns	ns
(B) Myometrium	**Pro**	**Sec**	**Pro**	**Sec**	**Pro**	**Sec**
Mean HSCORE	133.1	65.7	75.4	110.5	107.9	85.3
SEM	16.8	37.2	29.8	11.9	18.2	24.0
n	12	6	7	11	14	7
Median age	44±2.1	44.5±1.7	45.0±3.9	41.0±1.3	44.5±1.7	45±1.8
p- value		ns	ns	ns	ns	ns

Data are median ± SEM; SEM, standard error of the mean; Endo, endometrium; Pro, proliferative phase; Sec, secretory phase; ns, not significant. Analysis was performed using the Mann-Whitney U-test and the Kruskal-Wallis test.

Table 21. HSCORE comparison of endometrium and myometrium with and without endometriosis and adenomyosis

	Without endometriosis (Control)		With endometriosis		With adenomyosis	
	Endo[a]	**Myo[b]**	**Endo[c]**	**Myo[d]**	**Endo[e]**	**Myo[f]**
Mean HSCORE	97.3	108.5	86.8	96.8	78.8	103.2
SEM	15	15.4	12.1	13.8	16.5	13.2
All samples (n)	26	22	24	18	15	23
Median age	42 ± 1.6	44.5 ± 1.3	42 ± 1.9	42 ± 1.9	45 ± 1.5	45 ± 1.2
p- value					ns[a,c,e]	ns[b,d,f]

Data are median ± SEM; SEM, standard error of the mean; Endo, endometrium; Myo, myometrium; ns, not significant; e.g. ns[a,c,e] means comparison of endometrial staining in patients without and with endometriosis and adenomyosis. Analysis was performed using the Kruskal-Wallis test.

Table 22. HSCORE comparison of endometrium and smooth muscle staining of DIE, OE and PE

	DIE		OE		PE	
	Endo[a]	**SM[b]**	**Endo[c]**	**SM[d]**	**Endo[e]**	**SM[f]**
Mean HSCORE	137.4	49.2	137.0	55	143.7	61.1
SEM	18.8	8.6	20.2	9.2	12.3	9.8
n	24	24	18	18	21	21
Median age	45 ± 1.8	45 ± 1.8	42.0 ± 1.3	42.0 ± 1.3	44.5 ± 1.0	44.5 ± 1.0
p- value					ns[a,c,e]	ns[b,d,f]

Data are median ± SEM; SEM, standard error of the mean; Endo, endometrium; SM, smooth muscle cells; ns, not significant; e.g. ns[a,c,e] means comparison of endometrial staining in patients with DIE, OE and PE. DIE, deep infiltrating endometriosis; OE, ovarian endometriosis; PE, peritoneal endometriosis. Analysis was performed using the Kruskal-Wallis test.

3.10.2 ADAM12 concentrations in serum and endocervical mucus

Several ADAMs like ADAM17, ADAM12 and ADAM9 have been detected in tissue and body fluids of patients with specific malignant or pre-malignant conditions (Roy et al., 2020) with several studies implicating ADAM12 in tumor growth and development (Ruff et al., 2015). The diagnostic and prognostic utility of ADAM12 has been established in numerous diseases such as breast cancer, liver fibrogenesis, cardiac hypertrophy as well in adverse outcomes in pregnancy like pre-eclampsia and fetal trisomy 18 (Nyren-Erickson et al., 2013). Consequently, we investigated the levels of ADAM12 in serum and endocervical mucus in order to determine its potential as a non-invasive diagnostic biomarker for endometriosis. ADAM12 levels in serum (n = 293) and endocervical mucus (n = 79) of patients with and without endometriosis were determined using ELISAs (Table 23).

Comparison of serum ADAM12 levels during the different phases of the menstrual cycle demonstrated no difference in ADAM12 concentrations across the cycle (Table 23A). However, the levels were modestly but not significantly lower during the proliferative phase relative to the other phases. Serum and endocervical mucus ADAM12 concentrations were similar in patients with endometriosis and in controls (Table 23A, B). Notably, serum ADAM12 levels were shown to be significantly higher compared to endocervical mucus ADAM12 levels when paired serum and mucus samples were evaluated (Table 23C).

Table 23. ADAM12 levels in serum and endocervical mucus

		n	Median age	Mean ADAM12 (pg/mL)	Range (pg/mL)	*p*
(A) Serum (n = 293)	EM	179	34 ± 0.6	1997 ± 361.3	0 - 28023	
	w/o EM	114	28 ± 0.8	2334 ± 462.7	0 - 20905	ns
	Menstruation	55	32 ± 1.1	2219 ± 674.4	0 - 22267	
	Proliferative	69	33 ± 1.0	1366 ± 366.9	0 - 20279	
	Secretory	96	31 ± 0.7	2725 ± 539.3	0 - 20905	
	Contraception	56	30 ± 1.0	1968 ± 695.2	0 - 24969	ns
(B) Endocervical mucus (n = 79)	EM	35	33 ± 1.2	194.9 ± 55.4	0 - 1447	
	w/o EM	44	27 ± 1.2	109.6 ± 31.7	0 - 724	ns
(C) Paired serum and mucus	Serum	79	29 ± 0.9	3239 ± 647.8	0 - 20905	
	Endocervical mucus	79	29 ± 0.9	147.4 ± 30.4	0 - 1447	<0.0001

Data are median ± SEM or mean ± SEM; SEM, standard error of the mean; EM, endometriosis; w/o EM, without endometriosis; ns, not significant. Analysis was performed using the Mann-Whitney U-test and the Kruskal-Wallis test.

4 DISCUSSION

In the current study, it was shown for the first time that TGF-β1/2/3 and activin A suppress shedding of BG through the ALK-4/5–SMAD3 axis in human endometriotic cells and that the shedding process is modulated by MMPs. It was observed that inhibin A also reduces BG shedding in endometrial cells and that recombinant BG treatment significantly reduced secretion of TGF-βs as well as the rate of wound closure in endometriotic cells but has no effect on their viability. Additionally, our findings indicated that TGF-β1 significantly enhanced secretion of MMP2 and MMP3 and promoted the rate of wound closure in endometriotic cells. On the other hand, TGF-β2 promoted secretion of MMP2 only and had no effect on the rate of wound closure in endometriotic cells. Activin A significantly enhanced secretion of MMP2 and MMP3 while inhibin A had no effect. Surprisingly follistatin did not block activin A. Remarkably, sBG levels were significantly reduced in the endocervical mucus of patients with endometriosis compared to controls, whereas serum sBG levels were similar between endometriosis patients and controls. Analysis of ADAM12 localization in the uterus revealed that the protein localizes mainly on the luminal and glandular epithelial cells as well as the smooth muscle fibers of eutopic and ectopic endometrium. Additionally, no significant differences were observed between the levels of ADAM12 in endometriosis patients relative to controls in both serum and endocervical mucus samples.

BG serves as a TGF-β accessory receptor or as an antagonist in its soluble form (López-Casillas et al., 1991; 1993). Previous studies showed that BG functions as a tumor suppressor in numerous cell types including endometrial cancer (Gatza et al., 2010) although its significance in the pathophysiology of endometriosis remains unknown.

4.1 Impact of TGF-β on BG shedding

4.1.1 Influence of TGF-β on BG shedding in endometrial cells

BG was originally identified as a non-essential ligand accessory receptor that bound TGF-β ligands and enhanced signaling, particularly of TGF-β2 (López-Casillas et al., 1991). Using Sertoli cells, we recently demonstrated that BG is crucial in TGF-β2 but not in TGF-β1 signaling (Kudipudi et al., 2019).

In the present study, we showed that TGF-β1, TGF-β2 and TGF-β3 significantly suppressed shedding of BG in both endometrial epithelial and stromal cells. Moreover, we demonstrated that in 12Z, Ishikiwa, THESC and primary human endometrial stromal cells, the reduction in shedding of BG was remarkably stronger with TGF-β2 and TGF-β3 relative to TGF-β1. This is possibly due to the observation that TGF-β2 requires recruitment and presentation to TBRII via BG since TGF-β2 has only a weak intrinsic affinity for TBRII in the absence of BG, unlike TGF-β1 or TGF-β3 (López-Casillas et al., 1991; Vilchis-Landeros et al., 2001; Velasco-Loyden et al., 2004). The mature TGF-β isoform amino acid sequences show considerable conservation thus - TGF-β3 is 78-80% identical to TGF-β1 and 80-82% to TGF-β2, whereas TGF-β2 is 70-74% similar to TGF-β1. The main difference between the three isoforms is only in very few amino acids (TGF-β1/3 - Arg^{25}, $Val^{92/94}$; TGF-β2 -Lys^{25}, Ile^{92}, Lys^{91} and Ile^{92}, Lys^{97}, Glu^{99}) that determine binding specificity to TBRII (Derynck et al., 1988; Cordeiro, 2002). A study by Cui and Shuler (2000) suggested that BG mediates the effects of TGF-β3 during palatal fusion since the regulation of BG expression was observed to occur exclusively in the medial edge epithelium during fusion of the palate in mice embryos in *in vivo* and *in vitro* models. Besides TGF-β2 and TGF-β3, BG was also shown to tether TGF-β1 to the surface of cancer cell exosomes where it promoted TGF-β1-mediated myofibroblast differentiation (Webber et al., 2010). Transcriptionally, TGF-β1 was observed to downregulate BG mRNA levels via suppression of the proximal promoter of the BG gene in ovarian and breast cancer cells (Hempel et al., 2008). In gonadal and adrenal cell lines, both TGF-β1 and TGF-β2 were reported to downregulate expression of the BG gene, thereby indirectly reducing cellular sensitivity to inhibin action (Farnworth et al., 2007; Looyenga et al., 2010).

4.1.2 Mechanisms involved in TGF-β-mediated regulation of BG shedding

In the canonical TGF-β signaling pathway, the TGF-β ligands phosphorylate and activate members of the SMAD transcription factor family. TGF-βs and activins signal through SMAD2/3 while BMPs require SMAD1/5/8 (Moustakas et al., 2001). In the current study, we demonstrated for the first time that the canonical TGF-β pathway involving TGF-β/ALK-5/SMAD3 signaling is required in TGF-β-mediated reduction in shedding of BG in

endometriotic cells. TGF-β1 and TGF-β2 treatment of SMAD3- but not of SMAD2-silenced endometriotic cells exhibited increased BG shedding, suggesting non-redundant SMAD3-dependent regulation of BG shedding. Upon TGF-β ligands activation, both SMAD2 and SMAD3 show C-terminal phosphorylation of Ser-Ser-X-Ser motifs, which promotes their oligomerization with SMAD4 (Liu et al., 2016). Although SMAD2 and SMAD3 share an overall 92% identity at the amino acid sequence level, major structural differences between the two transcription factors have been reported (Dennler et al., 1999; Liu et al., 2016). For instance, SMAD3 acts as a transcription factor by binding to a TGF-β-responsive sequence within the promoter region of plasminogen activator inhibitor-1 (PAI-1) termed CAGA box whereas SMAD2 does not (Dennler et al., 1998). Moreover, in the N-terminal mad homology 1 (MH1) domain, SMAD2 has two short peptide inserts, amino acids 21-30 and 79-108, which impose steric constraints that hinder SMAD2 from binding to DNA. Contrary, SMAD3 readily binds DNA in complex with SMAD4 (Dennler et al., 1999). Following TGF-β stimulation, the adaptor protein embryonic liver fodrin (ELF) associates with receptor-linked SMAD3 and SMAD4, and not SMAD2, an interaction which facilitates nuclear translocation of SMAD3/4 and TGF-β-dependent transcriptional responses (Tang et al., 2003). Overall, these observations suggest that SMAD2 and SMAD3 may have different subsets of target genes involved hence distinct responses among TGF-β pleiotropic effects (Petersen et al., 2010).

The distinct phenotypes of SMAD2/3 deficient mice are indicative also of non-compensatory functions. SMAD3 mutant mice die between 1 - 8 months postnatally owing to a key defect in immune function while symptomatic mice develop bacterial abscesses near the mucosal surface, exhibit thymic involution and enlarged lymph nodes (Yang et al., 1999). On the other hand, SMAD2 knockout results in early embryonic lethality and the mutants show inability to form an organized egg cylinder and fail to develop mesoderm, and approximately 20% of SMAD2 heterozygous embryos develop severe gastrulation defects and lack mandibles or eyes (Nomura and Li, 1998). The differential regulation of transcription of TGF-β1 target genes for instance in SMAD2/3-deficient mice fibroblast cells are indicative of non-compensatory functions (Major and Jones, 2004). However, redundancy in SMAD2/3 roles was also previously demonstrated in mice T cells (Takimoto et al., 2010). SMAD3 is a key

modulator of TGF-β activity particularly in mediating anti-proliferative effects of TGF-β and is implicated as a potential effector for TGF-β in regulating immune system function (Datto et al., 1999).

In the non-canonical signaling pathway, TGF-β ligands activate members of the MAP kinase signaling molecules and the PI3K/Akt pathway (Zhang, 2009). In the present study, inhibition of the MAPK/ERK kinase and PI3K signaling using selective inhibitors had no effect on TGF-β-mediated suppression of BG shedding, thus corroborating the involvement of canonical SMAD-signaling.

4.2 Impact of activin A and inhibin A on BG shedding

4.2.1 Action of activin A/inhibin A on BG shedding in endometrial cells

Activin and inhibin share comparable β subunits, with activins occurring as ββ homodimers, whereas inhibins are αβ heterodimers. Apart from TGF-βs, BG also binds inhibin with high affinity and is a key determinant of inhibin potency on pituitary LβT2 gonadotrope cells (Escalona et al., 2009). BG's most significant role is to modulate activin signaling through direct interaction with inhibin (Namwanje and Brown, 2016). Binding of inhibin to BG and activin type II receptors results in formation of a stable complex that sequesters activin type II receptors and lessens their accessibility to propagate activin signaling (Lewis et al., 2000). Hence, we investigated whether activin A and inhibin A might modulate BG shedding in endometrial cells.

Our study demonstrated a significant time- and concentration-dependent reduction in BG shedding with activin A and inhibin A treatment in both endometrial epithelial and stromal cells. Although we observed reduced BG shedding following activin A treatment in endometrial cells, these findings are opposite to our previous observations using TN4A5 Sertoli cells where activin A treatment significantly induced BG shedding (Kudipudi et al., 2019). Accordingly, the functional impact of activin A on BG shedding seem to be both cell-type- and probably context-dependent. TGF-βs have a greater affinity for BG compared to inhibin although a considerable overlap in the binding sites for TGF-βs and inhibin has been observed within the membrane proximal domain of BG (Farnworth et al., 2006; Looyenga et al., 2010). TGF-β was reported to block the access of inhibin A to BG in LβT2 gonadotrope

cells, thus directly attenuating the inhibin-mediated antagonism of activin effects (Ethier et al., 2002). Interestingly, recombinant inhibin-A was reported to antagonize TGF-β2 signaling by causing endocytic clathrin-independent internalization of cell surface BG in adrenocortical cells (Looyenga et al., 2010).

4.2.2 Mechanisms involved in activin A-mediated regulation of BG shedding

Activin binds to two type II serine-threonine kinase receptors, ACVRIIA and ACVRIIB, resulting in recruitment and association with two type I activin receptors that are subsequently phosphorylated and activated (Appiah Adu-Gyamfi et al., 2020). The activin type I receptors are activin receptor type-1B (ACVRIB or ALK-4) and activin receptor type-1C (ACVRIC or ALK-7).

In the present study, we investigated the involvement of ALK-4, generally regarded as the principal activin type I receptor (Li et al., 2017), in activin A-mediated reduction in BG shedding. Our results indicated that suppression of BG shedding is mediated by ALK-4 since the ALK-4/5 inhibitor, LY364947, significantly abrogated this effect. Activins are antagonized by follistatin, an activin-binding protein that influences the accessibility of activins to their respective receptors (Harrington et al., 2006). In our study, pretreatment of cells with follistatin had no effect on the activin A-induced regulation of BG shedding at the tested concentration suggesting that follistatin is not involved in activin A-mediated regulation of BG shedding in endometriotic cells. A previous review by Namwanje and Brown (2016) reported that follistatin inhibits accessibility of activins to their receptors thereby attenuating activin-mediated follicle-stimulating hormone (FSH) release by the anterior pituitary gland. Conversely, using Sertoli cells and testicular germ cells, Mather et al. (1993) demonstrated that regulation of activin activity by follistatin is cell- and tissue-specific.

The fully assembled hexameric Activin-ACVRII-ACVRI complex then initiates SMAD- or non-SMAD-mediated signaling by phosphorylating regulatory SMAD2/SMAD3 or non-SMAD proteins near their carboxyl termini (Appiah Adu-Gyamfi et al., 2020). In the present study, we explored the involvement of the canonical SMAD signaling pathway in activin A-mediated modulation of BG shedding. Our *in vitro* SMAD2/SMAD3 gene knockdown

experiment demonstrated that silencing of SMAD3 but not SMAD2 blocked the activin A-mediated suppression of BG shedding. These results were further corroborated using the specific inhibitor of SMAD3 (SIS3) which confirmed involvement of SMAD3 in activin A-mediated suppression of BG shedding.

Normally, phosphorylated SMAD3 then binds to the common SMAD4 prior to translocation of the complex to the nucleus (Appiah Adu-Gyamfi et al., 2020). Using SMAD3 null mice embryonic fibroblasts (MEFs), Datto et al. (1999) demonstrated that these cells lose the ability to form SMAD-containing DNA binding complexes and are incapable of inducing transcription from the TGF-β-responsive promoter construct, 3TP-lux. Additionally, using primary dermal fibroblasts, the authors showed that SMAD3 is essential for induction of endogenous PAI-1. Whilst SMAD2 was shown in the context of overexpression to activate 3TP-lux and PAI-1 in human breast-carcinoma cell line, MDA-MB468 (Lagna et al., 1996), no compensation by SMAD2 was observed in the SMAD3 null mice embryonic fibroblasts (Datto et al., 1999). Association of the SMAD complex and transcription coactivators with activin-responsive elements (AREs) then leads to transcription of hundreds of genes (Appiah Adu-Gyamfi et al., 2020), in our case we speculated about genes encoding matrix metalloproteinases.

4.3 Role of betaglycan in cell viability and TGF-β secretion

4.3.1 Influence of BG on cell viability

Previous studies with both *in vitro* and *in vivo* cancer models indicated that BG is a vital modulator of cell growth, migration, angiogenesis, along with apoptosis (Gatza et al., 2010; Bilandzic and Stenvers, 2011). Thus, in the current study, we investigated the influence of varying concentrations of recombinant BG on viability of endometriotic epithelial cells and demonstrated no effect on the number of viable cells. Similarly, restoration of BG expression in prostate cancer DU145 cells was reported to inhibit migration and invasion but had no effect on cell proliferation in a study by Turley et al. (2007). Additionally, BG was reported to have no effect on cell proliferation or apoptosis of two human granulosa cell tumors (GCT) cell lines, COV434 and KGN (Bilandzic et al., 2009). Expression of BG in non-small cell lung cancer (NSCLC) cells expressing normal to low levels of BG had no effect on cell

proliferation (Finger et al., 2008). Moreover, in a NSCLC *in vivo* xenograft assay, over-expression of BG resulted in decreased tumor incidence, tumor growth, as well as tumor invasiveness.

Contrary, a study by Bilandzic et al. (2013) reported that re-expression of BG in GCT cells, which lack BG, altered the NFκB-TGF-β2 cross talk and decreased cell survival. In the same study, a TGF-β2/BG-mediated pro-apoptotic pathway was found following suppression of NFκB or SMAD2/3. A study on human and murine breast cancer cells reported that tumorigenic MDA-231 and PMTLuc cells with downregulated BG expression exhibited reduced proliferation and enhanced apoptosis relative to controls, and these growth changes were linked to modulation of NF-κB activity (Criswell et al., 2008). BG-mediated induction of apoptosis was found to be p38 MAPK-dependent and was suggested to be mediated by the cytoplasmic BG domain since expression of the extracellular BG domain could not mimic the induction of apoptosis observed with full-length BG in ccRCC (Margulis et al., 2008).

4.3.2 Influence of BG on TGF-β1 and TGF-β2 secretion

Unlike membrane-bound BG which functions as an accessory protein and enhances signaling, sBG acts as a decoy for TGF-β ligands, thus antagonizing TGF-β signaling (López-Casillas et al., 1991; Vilchis-Landeros et al., 2001; Bilandzic and Stenvers, 2011). In our study, recombinant BG moderately reduced TGF-β1 and TGF-β2 secretion. Although we observed a similar magnitude in the effects of BG on TGF-β1 and TGF-β2, Vilchis-Landeros et al. (2001) reported a 10-fold higher anti-TGF-β potency of recombinant sBG against TGF-β2 compared to TGF-β1 in COS-1 and Mv1Lu cell lines. In HEK293 cells, interaction between BG and β-arrestin2 facilitated co-internalization and downregulation of TBRI, TBRII, and BG leading to reduced TGF-β signaling (Chen et al., 2003). Contrary, the class I PDZ binding domain of the cytoplasmic BG domain was shown to interact with GIPC to form a complex that stimulated TGF-β signaling (Blobe et al., 2001a).

4.4 Modulation of BG shedding by MMPs

Our hypothesis that MMPs modulate shedding of BG in endometrial cells is supported by findings from our inhibition studies. Inhibition of MMPs using GM6001 and TIMP3 resulted

in significant attenuation of BG shedding in both epithelial and stromal endometrial cells. Although the mechanism of BG cleavage is still elusive, a study by Velasco-Loyden et al. (2004) indicated that BG shedding is regulated by pervanadate, a tyrosine phosphatase inhibitor, and is partly mediated by MT1-MMP and/or MT3-MMP. Moreover, plasmin, a serine proteinase, was shown to cleave the extracellular domain of BG (Mendoza et al., 2009).

TAPI-2, a tumor necrosis factor-converting enzyme (TACE) and MMP inhibitor, was found to suppress BG shedding (Velasco-Loyden et al., 2004; Blair et al., 2011), suggesting involvement of MMPs in the shedding process which is consistent with our previous findings using prepubertal rat Sertoli cell line 93RS2 (Kudipudi et al., 2019) and our present findings using endometrial cells. In the past, sBG levels were shown to correlate with mBG levels implying constitutive shedding (Andres et al., 1989; López-Casillas et al., 1991), nonetheless, little is known with regards to BG ectodomain shedding and regulation, hence further studies are warranted. Markedly, the transmembrane-cytoplasmic BG fragment that remains after ectodomain shedding was shown to be stable and is a target for the intramembrane protease, ϒ-secretase complex, which releases a cytoplasmic fragment that is involved in TGF-β2 signaling (Blair et al., 2011). Future studies should attempt to determine the exact MMPs involved in BG shedding.

4.5 Role of TGF-βs, activin A and inhibin A on MMP2/3 secretion

Apart from extracellular matrix (ECM) turnover, MMPs are also involved in numerous physiological processes including inflammation, angiogenesis, ovulation, menstruation, decidualization, and embryogenesis (Marbaix et al., 1996; Jones et al., 2006; Incorvaia et al., 2007; Amălinei et al., 2010). Moreover, they also play an integral role in cyclic changes in structure and thickness of the endometrium. Altered expression of numerous MMPs is associated with several pathological processes like fibrosis, endometriosis, adenomyosis, and cancer (Amălinei et al., 2010; Giannandrea and Parks, 2014; Szóstek-Mioduchowska et al., 2020). In endometriosis, a dysregulated profile of MMP and TIMP expression has been reported in a number of studies (Balkowiec et al., 2018) with some suggesting suppression of endometriosis development following inhibition of MMP expression (Bruner-Tran et al.,

2002; Mönckedieck et al., 2009). Therefore, a balance between activation and inhibition of MMPs is crucial in maintaining tissue homeostasis.

Both *in vitro* and *in vivo* studies indicated that TGF-βs regulate MMPs (Abdallah et al., 2006; Collette et al., 2006; Soni et al., 2019). TGF-β1 was observed to be a key regulator of MMPs and TIMPs in human and murine endometrial cells and tissue (Bruner et al., 1999; Bruner-Tran et al., 2002). Previously, we demonstrated that TGF-β1 and TGF-β2 promoted secretion of MMP2 and MMP9 in endometrial and endometriotic cells (Mecha et al., 2015). In the present study, we further extended these data, demonstrating that both TGF-β1 and activin A stimulated MMP3 secretion in endometriotic cells, and that the increase in both MMP2 and MMP3 secretion was concentration- and time-dependent. Contrary to TGF-β1, TGF-β2 did not have any effect on secretion of MMP3 indicating a functional difference between the two isoforms. Collectively, these results suggest that TGF-β1 may exacerbate its pro-fibrotic action by enhancing expression of MMPs in endometriosis. This is consistent with a possible role of TGF-βs in ECM remodeling and fibrosis via MMP regulation (Barrientos et al., 2008). Excessive deposition of ECM components like collagen type I and fibronectin by MMPs around endometrial glands and stroma is a characteristic feature of endometrosis as was demonstrated by Szóstek-Mioduchowska et al. (2020) using an equine model. In kidney disease, MMPs are associated with initiation and progression of kidney fibrosis via tubular cell EMT along with activation of resident fibroblasts, endothelial-mesenchymal transition as well as pericyte-myofibroblast trans-differentiation (Zhao et al., 2013). A similar concentration-dependent increase in the expression and production of MMP3 by TGF-β1 was reported in other cell types including human corneal epithelial cells, consistent with its role in wound healing of the eye (Kim et al., 2004).

Overexpression, enhanced secretion and activity of MMP2 and MMP3 has previously been implicated in pathogenesis of endometriosis (Gilabert-Estellés et al., 2007; Banu et al., 2008; Lv et al., 2015; Balkowiec et al., 2018). In our study, inhibin A stimulation of the cells had no effect on secretion of either MMP2 or MMP3 although inhibin was shown to moderately inhibit the activin A-induced secretion of MMP2. A study by Jones et al. (2006) also demonstrated enhanced endometrial secretion of pro-MMP2, 3 and active MMP2 with activin A stimulation whereas inhibin A antagonized these actions in regulating

decidualization and trophoblast invasion. Similar observations were made in a study in which exogenous activin A enhanced mRNA expression and gelatinolytic activity of MMP2 in mouse peritoneal macrophages (Ogawa et al., 2000). Additionally, stimulation of HTR8/SVneo immortalized human extravillous cytotrophoblast (EVT) cells and primary EVT cells with activin A increased mRNA expression and protein production of MMP2 which was correlated with enhanced trophoblast cell invasion (Li et al., 2015).

4.6 Contribution of TGF-β1/2 and BG to wound healing

TGF-βs are implicated in migration and invasion of cells in physiological processes like embryo implantation (Latifi et al., 2019) as well as in pathological processes like cancer and endometriosis (Omwandho et al., 2010; Young et al., 2017; Soni et al., 2019). Increasing evidence suggests that although benign, endometriosis exhibits properties similar to that of cancer such as uncontrolled growth, cell migration and invasion, neo-vascularization and reduced apoptosis (Omwandho et al., 2010; Vercellini et al., 2014; Zondervan et al., 2018). The invasive properties of endometrial cells are associated with heightened proteolytic activity and remodeling of the ECM (Di Carlo et al., 2009). In our study, TGF-β1 but not TGF-β2 promoted wound closure in endometriotic cells which is in agreement with previous studies implicating TGF-β1 in ECM remodeling, cell migration and wound healing in various cell types (Kopecki et al., 2007; Finnson et al., 2013; Young et al., 2017). Furthermore, some members of the TGF-β superfamily have previously been reported to mediate wound healing and restoration of the endometrium following menstruation (Kaitu'u-Lino et al., 2009; Omwandho et al., 2010; Finnson et al., 2013).

In our current study, recombinant BG moderately reduced the rate of wound closure in endometriotic cells possibly via suppression of migration. Similarly, a study by Bilandzic et al., (2009) on human ovarian granulosa cell tumors (GCT) showed that BG significantly inhibited cellular invasion and wound healing of GCT-derived COV434 and KGN cells. Interestingly, the authors observed that the inhibitory effect of BG on wound healing was strongly linked to the inhibin-binding region of BG. Moreover, silencing of inhibin A mRNA expression blocked the BG-mediated inhibition of wound healing and invasion (Bilandzic et al., 2009). Previous reports showed that BG reduced migration and invasion of various cancer

cell models (Turley et al., 2007; Finger et al., 2008; Gordon et al., 2008; Mythreye and Blobe, 2009; Lee et al., 2010; Nishida et al., 2018).

Using breast cancer cells, Lee et al. (2010) demonstrated that BG interacts with GIPC to inhibit TGF-β-mediated SMAD signaling and migration. Loss of BG was observed to stimulate formation of lamellipodium and activation of FAK-PI3K-Akt signaling pathway promoting ccRCC cell migration (Nishida et al., 2018). Mythreye and Blobe (2009) proposed that GAG chains modifications may also contribute to the ability of BG to regulate cell migration, since a BG mutant lacking the GAG chains failed to completely induce alterations in the actin cytoskeleton or block migration of ovarian cells. In the same study, the authors suggested that the ability of BG to regulate migration was dependent on its interaction with β-arrestin2, and occurred through activation of a protein involved in regulation of the cell cycle (Cdc42), alteration of actin cytoskeleton, as well as suppression of motility in both normal and cancerous ovarian epithelial cells (Mythreye and Blobe, 2009).

In prostate cancer cells, restoration of BG expression inhibited both basal and TGF-β-mediated cell migration and invasion (Turley et al., 2007). A study by Finger et al. (2008) on lung cancer cells reported that expression of BG in NSCLC cells expressing normal to low levels of BG inhibited cell motility and invasion whereas knockdown of BG expression resulted in opposite effects. The BG-mediated effect on migration and invasion was due, in part, to generation of sBG since treatment with conditioned media from cells over-expressing BG was able to block invasion in NSCLC cells (Finger et al., 2008). Downregulation of BG expression in pancreatic cancer cells enhanced cell motility and invasion in response to TGF-β stimulation. In the same study, the authors observed that the cytoplasmic domain of BG was not required in mediating the effects of BG on motility and invasion since a BG mutant lacking this domain (TβRIII-Δcyto) inhibited basal motility and invasion to the same extent as full length BG in pancreatic cancer cells (Gordon et al., 2008).

4.7 Soluble BG levels in serum and endocervical mucus

Having demonstrated the essential roles of BG in TGF-β functions in an *in vitro* endometriotic model, we further investigated the potential role of BG in pathophysiology of endometriosis using human serum and endocervical mucus samples. Increasing evidence

suggested a role of BG in multiple diseases including human cancers (Gatza et al., 2010). Currently, no non-invasive diagnostic tool that can be used to accurately diagnose endometriosis is available in clinical practice (Agarwal et al., 2019). Thus, we investigated the utility of serum/endocervical mucus sBG levels as a non-invasive diagnostic biomarker for endometriosis. This type of biomarker(s) would offer a cheap, easily accessible and less invasive diagnostic tool for clinicians, which may assist early identification of endometriosis patients.

Using endocervical mucus, we recently found that clusterin levels are modestly higher in endometriosis cases compared to controls but are significantly reduced in patients with endometriosis receiving contraception compared to cases with endometriosis but not using contraception (Konrad et al., 2016). In the present study, we further evaluated, for the first time, serum and endocervical mucus sBG levels in endometriosis. Since endometriosis implants in the pelvic cavity secrete substances in the peritoneal fluid that bathe the ovary and enter the uterine cavity via the oviducts, endometriotic markers may influence the entire reproductive system, including the cervical mucus (Grande et al., 2017). We identified detectable levels of sBG in endocervical mucus indicating that BG is synthesized and secreted by endocervical cells.

We found no significant correlations between serum or endocervical mucus sBG levels and menstrual phases, consistent with reports of a recent study on plasma sBG levels in breast cancer patients (Grgurevic et al., 2020). Although we did not detect differences between serum sBG levels of endometriosis and controls, the levels of endocervical mucus sBG were significantly lower in endometriosis compared to controls. It is plausible that reduced sBG levels in endocervical mucus of endometriosis patients may indicate enhanced TGF-β function characterized by increased inflammation, cell migration and invasion, angiogenesis as well as reduced apoptosis since sBG is known to antagonize TGF-β signaling (Nishida et al., 2018; Huang et al., 2019). This suggests that reduced levels of sBG in endocervical mucus of endometriosis patients may in part be linked to chronic inflammation reported in endometriosis. Indeed, a chronic and sustained inflammatory process is a major attribute of endometriosis that makes pain a cardinal symptom of this condition (Vercellini et al., 2014; Thézénas et al., 2020).

Currently, available therapy for endometriosis is by surgery and/or hormone therapies that are mainly contraceptive (Halis et al., 2010; Vercellini et al., 2014). For robust assessment of potential biomarkers for endometriosis, their expression levels in patients receiving hormone medications should also be investigated (Falcone and Lebovic, 2011). Thus, in our present study we also included patients who were under hormonal contraception. The rationale behind including these patients was to determine whether hormonal therapy has any influence on the levels of sBG. Despite the fact that these patients were on hormonal contraception, we noticed suppression of BG levels in endocervical mucus of endometriosis patients compared to controls and no differences in serum sBG levels which are similar to our observations made in patients without hormonal contraception.

BG was identified as a tumor suppressor in many tissue types and down-regulation or loss of BG expression at the mRNA or protein level correlated with increased tumor progression and poor prognosis (Gatza et al., 2010; Stenvers and Findlay, 2010; Zakrzewski et al., 2011; Nishida et al., 2018; Grgurevic et al., 2020). Decrease or loss of BG expression occurs via several molecular mechanisms which include loss of heterozygosity at the BG gene locus and epigenetic silencing (Dong et al., 2007; Sharifi et al., 2007; Cooper et al., 2010). This is frequently associated with a loss of sensitivity to anti-proliferative TGF-β- and/or inhibin-mediated activity, reduced NF-kB-mediated control of inflammation and immune response, in addition to increased cell migration and invasion, angiogenesis, and tumor progression along with reduced apoptosis (Criswell et al., 2008; Bilandzic et al., 2009; Gatza et., 2010; Lee et al., 2010; Nishida et al., 2018; Huang et al., 2019).

In vivo studies revealed that restoration of BG expression in some cancer types can hinder tumor progression, partly via release of sBG which binds to and neutralizes TGF-β activity, antagonizing the tumor promoting effects of TGF-β especially in late stage cancers (Gatza et al., 2010; Nishida et al., 2018). The therapeutic potential of sBG was demonstrated through administration of sBG to a mouse model of type 2 diabetes (Juárez et al., 2007) as well as in lung (Finger et al., 2008), breast and colon (Bandyopadhyay et al., 1999; 2002a; 2002b), renal (Nishida et al., 2018), prostate (Bandyopadhyay et al., 2005) and glioma (Naumann et al., 2008) *in vivo* cancer models where sBG was demonstrated to have anti-cancer properties.

The major symptoms of endometriosis include chronic abdominal pain, dysmenorrhoea, dysuria, dyschezia, and dyspareunia along with infertility (Taylor et al., 2012; Vercellini et al., 2014). In the present study, we did not detect any associations between serum/endocervical mucus sBG levels and pain or infertility. Since attenuated BG expression may contribute directly to many inflammatory conditions as well as chronic diseases (Gatza et al., 2010), dysregulation of BG actions may significantly impact TGF-β, inhibin or activin signaling and action and thus endometrial health and reproductive function.

4.8 ADAM12 endometrial expression and serum/endocervical mucus levels

ADAM12 is a membrane-anchored metalloprotease associated with activation/inactivation of growth factors like HB-EGF and IGF binding proteins that play a central role in wound healing (Harsha et al., 2008). In our study, both TGF-β1 and TGF-β2 significantly augmented secretion of ADAM12 in endometrial stromal cells. Likewise, TGF-β stimulation resulted in a significant up-regulation of ADAM10, 12, 17, and 19 gene expression in renal cells as well as in preclinical models of hypertension-induced renal damage and glomerulonephritis (Ramdas et al., 2013). In the same study, exogenous overexpression of the miR-29 family inhibited the TGF-β-mediated up-regulation of ADAM12 and ADAM19 gene expression indicating that these ADAMs play an essential role in renal fibrosis. In human hepatic stellate cells (HSCs), ADAM12 but not ADAM9 expression was up-regulated by TGF-β1 via the PI3K and MEK signaling pathways and both ADAM9 and ADAM12 expression was correlated with tumor aggressiveness and progression in liver cancers (Le Pabic et al., 2003). Interestingly, in human embryonic kidney cell lines 293T, HSCs, human rhabdomyosarcoma (RD) cells, mouse C2C12 cells, monkey kidney COS7 cells, and mink lung MvLu1 cells, ADAM12 was found to facilitate the activation of TGF-β signaling via mechanisms that included phosphorylation of SMAD2, interaction of SMAD2 with SMAD4, transcriptional activation, as well as accumulation of TBRII in early endosomal vesicles (Atfi et al., 2007). Increasing evidence suggested a role of ADAM12 in multiple diseases including human cancers (Nyren-Erickson et al., 2013). Having demonstrated the effect of TGF-β on ADAM12 secretion in an *in vitro* endometrial model, we further investigated the potential role of ADAM12 in the pathophysiology of endometriosis in human samples. The

immunohistochemistry results indicated that ADAM12 localizes mainly in the luminal and glandular epithelial cells as well as the smooth muscle cells in both eutopic and ectopic endometrium but is absent in stromal cells, proposing that ADAM12 may be involved in endometrial and reproductive functions. Equally, Van Sinderen et al. (2017) observed maximum localization of ADAM12 in endometrial glandular and luminal epithelium with very minimal/zero stromal staining. A study by Harsha et al. (2008) reported that ADAM12 was 5-fold up-regulated in chronic wounds compared to healthy skin, thus supporting our hypothesis that absence of stromal ADAM12 is important in the scar-less wound healing that ensues following menstruation. The immunostaining intensity was not different between either early- or mid-secretory phases of the menstrual cycle or between fertile and infertile women in a study by Van Sinderen et al. (2017). Similarly, in the current study, staining intensity was unchanged in either the different phases of the menstrual cycle or between patients with and in subjects without endometriosis.

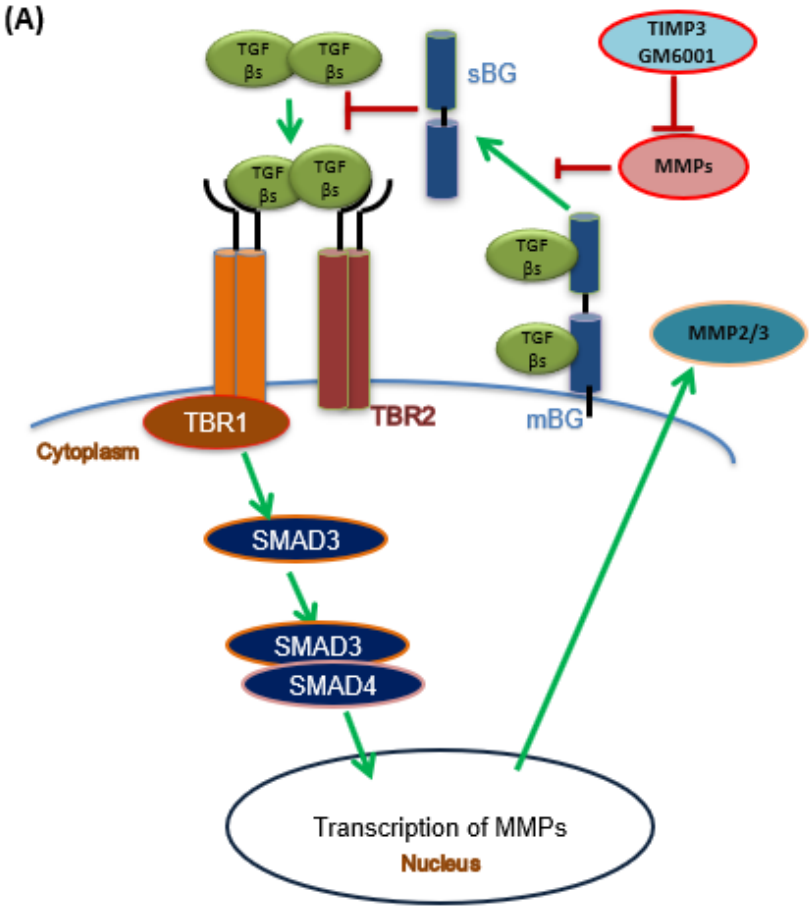
Akin to our immunohistochemistry findings, we did not observe significant differences between women with and without endometriosis in both serum and endocervical mucus samples. However, serum levels of ADAM12 exceeded those in the paired endocervical mucus suggestive of local production. Despite not seeing any differences in ADAM12 expression and secretion between endometriosis and non-endometriosis women, ADAM12 has previously been associated with processes like cell migration and adhesion, ECM remodeling as well as cell signaling (Nyren-Erickson et al., 2013). Moreover, ADAM12 expression is significant during decidualization and uterine remodeling as well as in several stages of early pregnancy such as placental development in mice and human models (Kim et al., 2006; Zhang et al., 2009; Sahraravand et al., 2011; Christians and Beristain, 2016).

4.9 Conclusion

Collectively, both our functional *in vitro* studies and patient data highlight several novel aspects of BG in the context of endometriosis. For the first time it was demonstrated that TGF-β and activin A suppress shedding of BG via the canonical SMAD-dependent pathways (Fig. 35). The findings also suggested that MMPs are involved in modulating BG shedding in both epithelial and stromal cells. Moreover, we demonstrated that recombinant BG

regulated secretion of TGF-β1/2 and the rate of wound healing. Using epithelial endometriotic cells, we also showed that TGF-β1/2 and activin A modulate secretion of MMP2 and MMP3. Lastly, we observed that endocervical sBG levels are dysregulated in patients with endometriosis relative to controls.

These novel findings may have a significant clinical impact in the search for alternative non-invasive diagnostic biomarkers for endometriosis in addition to treatment. Although speculative, and based on the results of our study, we propose that BG may represent a potential therapeutic agent and hence, further studies to elucidate its function in endometrial tissue and the peritoneal cavity and eventual association with endometriosis will undoubtedly provide valuable insights. Future studies will aim to recapitulate our present *in vitro* findings in an *in vivo* model and to determine the exact role of TGF-β-mediated effects on BG shedding in endometriosis.


(A)
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MMPs
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TGF βs
TGF βs
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MMP2/3
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TBR2
mBG
Cytoplasm
SMAD3
SMAD3
SMAD4
Transcription of MMPs
Nucleus

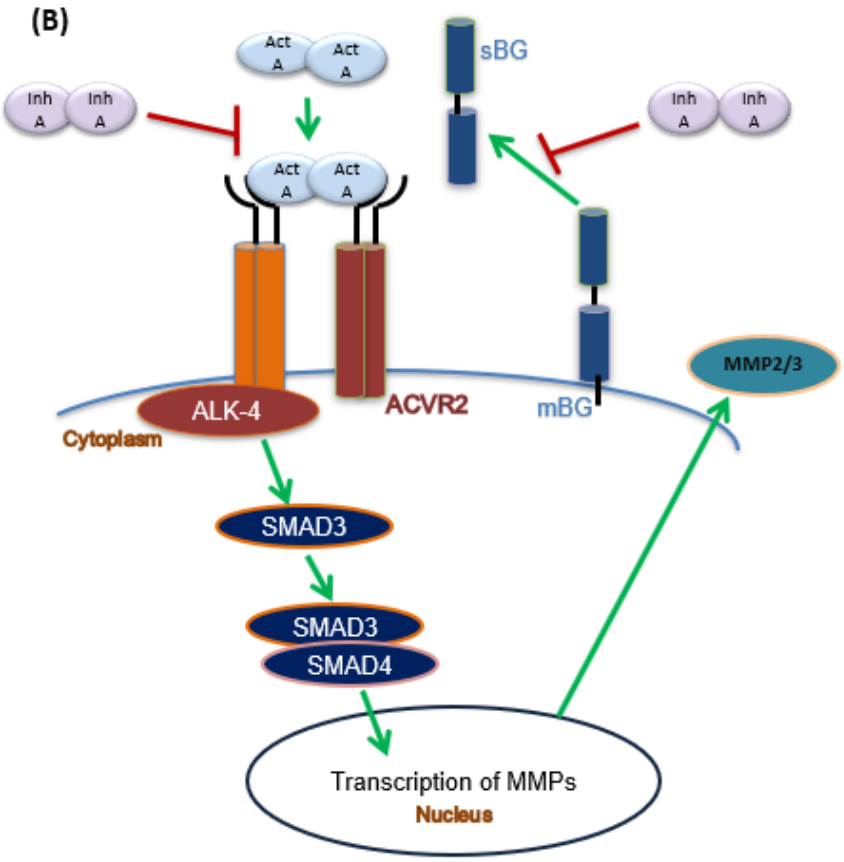

Figure 35. Scheme of TGF-β1/2 **(A)** and activin A **(B)** signaling under the influence of betaglycan (BG) and TIMP3 in human endometrial cells. Binding of both ligands to the TGF-β receptor complex results in phosphorylation of SMAD3 but not of SMAD2. TGF-β1/2 and activin A (act A) increase secretion of MMP2, but only TGF-β1 and activin A increase secretion of MMP3. Both TIMP3 and GM6001, a pan-MMP inhibitor, reduce shedding of BG via inhibition of MMPs. In contrast to TGF-β1 which promotes cell migration and hence wound healing, soluble BG (sBG) impedes cell migration. Inhibin A (inh A) suppresses shedding of BG and activin A-mediated increase in MMP2 secretion.

5 SUMMARY

Endometriosis is an estrogen-responsive disease defined as the presence of endometrial tissue at ectopic sites. TGF-β superfamily members are pleiotropic cytokines that exhibit cell-type-specific effects and regulate a plethora of diverse cellular responses. Perturbations in TGF-β superfamily components and signaling have been implicated in endometriosis. Betaglycan (BG) is a vital co-receptor and modulator of TGF-β superfamily. It functions as a tumor suppressor in numerous cancers. In the current investigation, we addressed the modulation of BG shedding by TGF-β, activin A and inhibin A and the molecular mechanisms involved using endometrial *in vitro* models. We also analyzed the influence of TGF-βs and BG on wound healing in endometriotic cells. Additionally, we investigated the utility of serum/endocervical mucus sBG and ADAM12 levels as potential non-invasive diagnostic biomarkers for endometriosis.

The results demonstrated that TGF-β1/2 and activin A suppress shedding of BG via the ALK-4/5–SMAD3 axis in human endometriotic cells. We observed that inhibin A also suppresses BG shedding. Notably, shedding was modulated by MMPs. Furthermore, recombinant BG significantly reduced secretion of TGF-β1/2 and the rate of wound closure in endometriotic cells but had no influence on their viability. Interestingly, TGF-β1 significantly augmented secretion of MMP2/3 and enhanced the rate of wound closure in endometriotic cells. Conversely, TGF-β2 promoted secretion of MMP2 only and had no effect on the rate of wound closure. Activin A but not inhibin A significantly enhanced secretion of MMP2/3. Remarkably, endocervical mucus but not serum sBG levels were significantly reduced in endometriosis patients compared to controls. TGF-β1/2 significantly augmented secretion of ADAM12 in endometrial stromal cells *in vitro*. ADAM12 localizes mainly to the luminal and glandular epithelial cells as well as smooth muscle cells of eutopic and ectopic endometrium. No significant differences were observed in ADAM12 levels in endometrial staining intensity as well as in serum/endocervical mucus samples from endometriosis patients relative to controls.

These findings provide new insights into the roles of BG in TGF-β signaling, wound healing, and in the pathophysiology of endometriosis. The strong reduction in BG shedding indicates that the TGF-β ligands require mBG for signaling, since it is particularly vital in establishing

the potency of its ligands on their target cells. We speculate that the reduced endocervical mucus sBG levels in endometriosis patients may indicate enhanced TGF-β functions, which may in part be linked to the chronic inflammation observed in endometriosis. Although speculative, we propose that sBG may represent a potential therapeutic agent and an early diagnostic biomarker and hence, further studies to elucidate its function in endometriosis will undoubtedly provide indispensable insights.

6 ZUSAMMENFASSUNG

Endometriose ist eine durch Östrogen dominierte Krankheit, die durch das Vorhandensein von Endometriumgewebe an ektopischen Stellen definiert ist. Die Mitglieder der TGF-β-Superfamilie sind pleiotrope Zytokine, die zelltypspezifische Wirkungen aufweisen und eine Fülle unterschiedlicher zellulärer Reaktionen regulieren. Störungen der Komponenten der TGF-β-Superfamilie und der Signalübertragung wurden mit Endometriose in Verbindung gebracht. Betaglykan (BG) ist ein wichtiger Co-Rezeptor und Modulator der TGF-β-Superfamilie. Es fungiert als Tumorsuppressor bei zahlreichen Krebsarten. In der aktuellen Untersuchung untersuchten wir die Modulation des BG-Sheddings durch TGF-β, Activin A und Inhibin A und die molekularen Mechanismen, die daran beteiligt sind, anhand von endometrialen *in vitro* Modellen. Außerdem wollten wir den Einfluss von TGF-β und BG auf die Wundheilung von endometriotischen Zellen analysieren. Darüber hinaus untersuchten wir den Stellenwert der sBG- und ADAM12-Spiegel im Serum/Endozervixschleim als potenzielle nicht-invasive diagnostische Biomarker für Endometriose.

Die Ergebnisse zeigten, dass TGF-β1/2 und Aktivin A die Freisetzung von BG über die ALK-4/5-SMAD3-Achse in menschlichen endometriotischen Zellen unterdrücken. Wir beobachteten, dass Inhibin A ebenfalls die Ablösung von BG unterdrückt. Bemerkenswert ist, dass der Ablösungsprozess durch MMPs moduliert wurde. Darüber hinaus stellten wir fest, dass rekombinantes BG die Sekretion von TGF-β1/2 und die Geschwindigkeit des Wundverschlusses von endometriotischen Zellen signifikant reduzierte, aber keinen Einfluss auf ihre Lebensfähigkeit hatte. Interessanterweise erhöhte TGF-β1 die Sekretion von MMP2/3 signifikant und förderte die Geschwindigkeit des Wundverschlusses endometriotischer Zellen. Umgekehrt erhöhte TGF-β2 nur die Sekretion von MMP2 und hatte keinen Einfluss auf die Geschwindigkeit des Wundverschlusses. Es wurde festgestellt, dass Aktivin A, nicht aber Inhibin A, die Sekretion von MMP2/3 signifikant erhöhte. Bemerkenswerterweise war der endozervikale Schleim, nicht aber der Serum-SBG-Spiegel bei Endometriose-Patientinnen im Vergleich zu den Kontrollen signifikant reduziert. TGF-β1/2 steigerte die Sekretion von ADAM12 in endometrialen Stromazellen *in vitro* signifikant. ADAM12 findet sich hauptsächlich in den luminalen und glandulären Epithelzellen sowie in

den glatten Muskelzellen des eutopen und ektopen Endometriums. Darüber hinaus wurden bei Endometriose-Patientinnen im Vergleich zu Kontrollpersonen keine signifikanten Unterschiede in der Intensität der Färbung des Endometriums sowie in den ADAM12-Spiegeln in Serum und Zervixschleim festgestellt.

Diese Ergebnisse liefern neue Erkenntnisse über die Rolle von BG bei der TGF-β-Signalübertragung, der Wundheilung und der Pathophysiologie der Endometriose. Die starke Verringerung der BG-Ausschüttung deutet darauf hin, dass die TGF-β-Liganden mBG für die Signalübertragung benötigen, da es besonders wichtig ist, um die Wirksamkeit seiner Liganden auf ihren Zielzellen herzustellen. Wir spekulieren, dass die verringerten sBG-Spiegel im Endozervikalschleim von Endometriose-Patientinnen auf verstärkte TGF-β-Funktionen hinweisen, die zum Teil mit der bei Endometriose beobachteten chronischen Entzündung zusammenhängen könnte. Obwohl spekulativ, schlagen wir vor, dass sBG ein potenzieller therapeutischer Wirkstoff und Biomarker sein könnte, und daher werden weitere Studien zur Aufklärung seiner Funktion bei Endometriose zweifellos unverzichtbare Erkenntnisse liefern können.

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9 PUBLICATIONS & CONFERENCES

Mwaura N. Agnes holds a Bachelor and Master of Science degree in Biochemistry, both from the University of Nairobi (Kenya).

Publications

- **Mwaura, A. N**., Riaz, M. A., Maoga, J. B., Mecha, E., Omwandho, C. O., Scheiner-Bobis, G., ... & Konrad, L. (2022). Role of betaglycan in TGF-β signaling and wound healing in human endometriotic epithelial cells and in endometriosis. Biology, 11(4), 513.
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Conferences

- **Mwaura N. Agnes**, Georgios Scheiner-Bobis, Jane Maoga, Ezekiel Mecha, Charles O.A. Omwandho, Muhammad A. Riaz, Lutz Konrad. "TGF-β1/β2 mediate reduction in betaglycan shedding via ALK-5 and SMAD3 pathway in human endometrial cells." 14th GGL Annual conference, 29th - 30th September 2021.

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- **Mwaura N. Agnes**, Georgios Scheiner-Bobis, Lutz Konrad. "The role of a disintegrin and metalloproteinase-12 (ADAM12) in the pathophysiology of human endometriosis." 12th GGL Annual conference, 29th - 30th September 2019.
- **Mwaura, A. N.**, Scheiner-Bobis, G, Maoga, J, Mecha, E, Omwandho, C.O.A, Riaz, M.A, Konrad, L. "Analysis of ADAM12 in the endometrium and ADAM12 levels in serum and endocervical mucus of endometriosis." 5th Kirinyaga University Annual Virtual International Conference, 24th - 25th March 2022.
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10 EHRENWÖRTLICHE ERKLÄRUNG

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I declare that I have completed this dissertation single-handedly without the unauthorized help of a second party and only with the assistance acknowledged therein. I have appropriately acknowledged and referenced all text passages that are derived literally from or are based on the content of published or unpublished work of others, and all information that relates to verbal communications. I have abided by the principles of good scientific conduct laid down in the charter of the Justus Liebig University of Giessen in carrying out the investigations described in the dissertation.

Giessen, den 29-11-2022 Agnes Njoki Mwaura