Aus dem

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Biological and pathobiological processes of human endometrium: endometrial receptivity, glucose metabolism, and cell migration

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List of Abbreviations

2-NBDG	2-[N(7-nitrobenz-2-oxa-1,3-diaxol-4-yl)amino]-2-deoxyglucose
AA	Arachidonic acid
ANXA7	Annexin A7
BMP2	Bone morphogenetic protein 2
cAMP	8-Bromoadenosine-3',5'-cyclic monophosphate
CCEC	Clear cell endometrial cancer
CM	cAMP and MPA
COX	Cyclooxygenase
COX1	Cyclooxygenase 1
COX2	Cyclooxygenase 2
COX3	Cyclooxygenase 3
EBAF	Endometrial bleeding related factor
EC	Endometrial cancer
EEC	Endometrioid endometrial cancer
ELISA	Enzyme-linked immunosorbent assay
GYS1	Glycogen synthase-1
F-actin	Filamentous actin
Hb-egf	Heparin binding epidermal growth factor
HESCs	Human endometrial stromal cells
IGFBP1	Insulin-like growth factor binding protein 1
Ihh	Indian hedgehog
IVF-ET	*In vitro* fertilization and embryo transfer
LEFTY2	Left-right determination factor 2
MPA	Medroxyprogesterone 17-acetate
MMRd	Mismatch repair-deficient
NHE1	Na^+/H^+ exchanger 1
NT	Non-targeting
NSMP	No specific molecular profile
p53abn	p53-abnormal
PAK1	p21 protein-activated kinase 1

PC	Proprotein convertase
PGE_2	Prostaglandin E_2
PGH_2	Prostaglandin H_2
pGYS1	Phosphorylate glycogen synthase-1
PLA_2	Phospholipase A_2
POLEmut	Polymerase epsilon-mutated
PR	Progesterone receptors
PRL	Prolactin
Rac1	Ras-related C3 botulinum toxin substrate 1
RPE	Retinal pigment epithelial
RPL	Recurrent pregnancy loss
SEC	Serous endometrial cancer
SGLT1	Na^+ coupled glucose transporter-1
siRNA	Small interfering RNA
TGF-β	Transforming growth factor-beta
TH	T helper cells
UA	Urolithin A
UCS	Uterine carcinosarcoma
uNK	Uterine natural killer cell
WT	Wild type

1. Introduction

1.1. Endometrium and the immune system

A structural and physiological prerequisite for the survival of the human species is the endometrium. The endometrium is the only site, with very few exceptions, where the conceptus may successfully implant, mature, and develop into a baby until delivery (Vilella et al., 2021). Human endometrial tissue is highly regenerative, and undergoes shedding and regenerating each month during the women's reproductive years (Gargett, 2007; Jabbour et al., 2006). It is composed of two stratified layers that are sensitive to ovarian steroid hormones. These two layers comprise the superficial stratum functionalis and the permanent deeper stratum basalis which is attached to the myometrium (Ferenczy and Bergeron, 1991; Gargett et al., 2012). The uterus lumen is lined by simple columnar epithelial cells. Tubular glands extend from the surface epithelium to the endometrial-myometrial interface. The endometrial stroma, which lies beneath the luminal epithelium and surrounds the glands, is made up of a diverse range of cell types. It contains basal and spiral arteries, as well as endothelial cells and stem-like perivascular cells (Masuda et al., 2012). The major components are specialized fibroblast like stroma cells that derive from perivascular mesenchymal stem-like cells of the stratum basalis and differentiate into epithelial-like cells every menstrual cycle (Brosens et al., 1999).

Implantation of an embryo requires acquisition of immune tolerance during pregnancy at the maternal-foetal interface that is accomplished by a balanced action of effector and regulator immune cells (such as helper T (TH1) and (TH2) cells). Beyond that, infiltration of natural killer cells and antigen-presenting cells have been demonstrated to contribute to the remodelling of the spiral arteries in the decidua and this contribution turned out to be a prerequisite for maintenance of early pregnancy underscoring the pivotal role of endometrial immune cells in implantation (Lee et al., 2011; Masuda et al., 2012). Of note,

immune escape of endometrial cancer by immunoediting has been suggested to pursue processes similar to those involved in the immune tolerance against the embryo at the maternal-foetal interface (Bruno et al., 2020). The present cumulative thesis intends to describe the overlap of processes regulating endometrial receptivity on the one hand and progression of endometrial cancer on the other. A deeper understanding of endometrial cancer biology and of endometrial receptivity is a prerequisite to develop a fertility-sparing cancer treatment. The aim of the subsequent section of the thesis is to introduce the biology of endometrial carcinoma.

1.2. Endometrial carcinoma

Endometrial carcinoma is the most frequent gynaecological malignancy and reflects the fifth most prevalent cancer in developed countries, with epithelial cells lining the uterus as the source (Huang et al., 2019; Van Nyen et al., 2018). Endometrial carcinoma has become more common in many nations over the last few decades, a trend that is thought to be attributable to rising obesity, especially in childhood and adolescence, type-2 diabetes mellitus rates, and changes in female reproductive cycles such as early menarche or extended unopposed oestrogen stimulation, and late natural menopause (Lortet-Tieulent et al., 2018; Morice et al., 2016). Additional risk factors include nulliparity, an advanced age, family history of endometrial cancer, hereditary predisposition, insulin resistance hyperinsulinemia and hyperglycaemia are further important risk factors (Zabuliene et al., 2021).

Although there are presently no well-established endometrial cancer screening programs, endometrial hyperplasia is a recognized precursor lesion of the most prevalent kind of endometrial cancer, and it’s diagnosis can help to avoid the disease (Doherty et al., 2020; Sanderson et al., 2017). Endometrial cancer (EC) is divided into four groups with unique clinical, pathologic, and molecular characteristics: polymerase epsilon-mutated (POLEmut), p53-abnormal

(p53abn), mismatch repair-deficient (MMRd), and no specific molecular profile (NSMP). POLEmut variants comprise about 6-9%, MMRd 20-30%, NSMP 40-50%, and p53abn 13-18% of all ECs (Goulder and Gaillard, 2022; Huvila et al., 2021; Mitric and Bernardini, 2022; Vermij et al., 2020; Yen et al., 2020).

Histologically, EC had been classified into several types, including endometrioid EC (EEC), serous EC (SEC), clear cell EC (CCEC), mixed EC, and uterine carcinosarcoma (UCS), all of which differ in incidence, clinical characteristics, prognosis, and risk factors. EECs are oestrogen-dependent tumours that account for around 80% of all ECs patients are on average 62 years old at diagnosis. SECs and CCECs, on the other hand, are quite infrequent and account for around 10% and 3% of ECs, respectively (Urick and Bell, 2019). They are typically irresponsive to oestrogen and are identified later in life (Setiawan et al., 2013). UCS tumours, which include both epithelial and connective tissue cells, account for less than 2% of all ECs (Dedes et al., 2011; Urick and Bell, 2019). Early diagnosed and low-grade tumours have a higher 5-year survival rate than high-grade and metastatic tumours that are detected late in their progression, since early detected tumours are more sensitive to therapy and have better outcomes (Urick and Bell, 2019).

Treatment of gynaecological tumours comprises hysterectomy and bilateral salpingo-oophorectomy. Beyond surgery, gonadotoxic effects of chemotherapy and radiotherapy put fertility at risk or even loss of reproductive function and ovarian hormone production (Obermair et al., 2020; Schuurman et al., 2021). In adolescents and young adult women with gynaecological malignancies, maintaining fertility is a crucial aspect in preserving a high quality of life. The loss of fertility after cancer treatment has been described by young women as being almost as heart-breaking as the cancer diagnosis itself. Some cancer patients report feeling excluded from the fertility decision-making process, which has a substantial psychological and social influence on quality of life (Knez et al., 2021; Navarria et al., 2009; Yamazawa et al., 2007). Both radiotherapy and chemotherapy have been shown to be very harmful to a woman's ovarian

reserve and, as a result, her fertile lifespan (Knez et al., 2021; Obermair et al., 2020). The decrease in pregnancy rates could be due to cancer treatment-induced follicular depletion, but it is also plausible that uterine injury plays a component. When cancer survivors use their own cryopreserved oocytes that have not been subjected to cancer treatments, or they get oocyte donor embryo transfers, they have a substantially lower pregnancy success rate than women who have no history of cancer. In particular, implantation and clinical pregnancy rates were significantly lower in assisted reproductive technology users than in control women (Chen et al., 2022; Knez et al., 2021). Furthermore, cancer survivors have an increased risk of having an unfavourable pregnancy result, such as preterm delivery, low birth weight infants, and children who are too tiny for their gestational age. These findings raise the risk that anti-cancer drugs may cause lasting uterine damage, compromising the uterus' ability to establish and maintain pregnancy (Knez et al., 2021).

Endometrial cancer treatment options that preserve fertility are urgently required to assist women who wish to have children (Janda and Obermair, 2021; Lucchini et al., 2021). Young patients with good oncological prognosis who want to keep their fertility, therefore, may choose more conservative therapies. It is critical in these circumstances to carefully select the women for whom fertility sparing treatment is oncologically safe and to personalize the treatment to their specific needs. However, in an attempt to preserve fertility, women with endometrial cancer, may be subjected to unnecessary oncological risk and disease progression. Overtreatment, on the other hand, can result besides to infertility in a lifetime of adverse effects such as an increased risk of cardiovascular events, or even secondary cancers (Knez et al., 2021).

An unfulfilled wish for children and associated emotional distress is not only an important issue for adolescents and young adult women with endometrial cancer. About 10% of the German couples remain unintentionally childless (Wolf et al., 2012). A successful pregnancy requires the well attuned reciprocal

interaction of many processes, some of those occur during decidualization, as described in the next paragraphs.

1.3. Decidualization

In the 28 days menstrual cycle, the endometrium goes through changes such as growth, differentiation and regression (Curry and Osteen, 2003). The cycle is divided into two phases, the proliferative phase and the secretory phase. The proliferative phase follows the menstrual phase and lasts to the ovulation, and the secretory phase from ovulation to next menstruation (Monis and Tetrokalashvili, 2022).

Decidualization describes the remodelling of endometrial mesenchymal cells during the secretory phase of menstrual cycle (Coulam, 2016). In particular, decidualization is defined as differentiation of the elongated stromal fibroblasts into secretory, epithelioid-like decidual cells. In rodents, this process is initiated by the implanting blastocyst (Salker et al., 2012) while in humans decidualization is triggered independent of blastocyst signals by elevated progesterone levels starting 6 days after ovulation in the mid-secretory phase and reaches their peak in the terminal-luteal phase of the menstrual cycle (Coughlin, 2005; Gellersen and Brosens, 2014; Wilcox et al., 1999). Prolactin (PRL) and insulin-like growth factor binding protein 1 (IGFBP1) are abundant in amniotic fluid, and their source has been identified as the decidua (Riddick et al., 1978; Rutanen et al., 1986). These factors have been recognized as decidual phenotype markers and are commonly used to evaluate decidualization in endometrial stromal cell cultures (Gellersen and Brosens, 2014).

Simultaneously, spiral arteries in the superficial layer are generated by angiogenesis surrounding the stromal cells of the endometrium to supply blood to the conceptus (Gellersen and Brosens, 2014; Wilcox et al., 1999). Beyond

the morphological changes of human endometrial stromal cells and angiogenesis, decidualization is further associated with the secretory transformation of the uterine glands, recruitment of specialized uterine natural killer (uNK) cells (Schatz et al., 2016). Decidualization of the endometrium is a prerequisite for the successful implantation of the blastocyst as discussed in the next paragraphs (Brosens et al., 2002).

1.4. Implantation

Implantation is a process of invasion of the blastocyst into the deeper layer of the endometrium (Liu et al., 2014). In the process of embryo implantation in the endometrium, many genes are involved, such as progesterone (PR) receptors, Indian hedgehog (Ihh), bone morphogenetic protein 2 (BMP2), and heparin binding epidermal growth factor (Hb-egf). These genes are essential for endometrial luminal closure and blastocyst apposition, blastocyst attachment and implantation (Wang and Dey, 2006). During decidualization, the endometrium becomes receptive to embryo implantation (Brosens and Gellersen, 2006). This period is termed as “window of implantation” (WOI) (Dey et al., 2004) which is associated with a pro-inflammatory response. In human beings the WOI lasts for 4 days starting at about day 9 after ovulation (Bergh and Navot, 1992).

Implantation failure is the major cause of the lower rate of successful pregnancy outcome. It accounts for approximately 75% of pregnancies loss in human beings (Wang et al., 2003; Wilcox et al., 1988) although different technologies have been developed for the *in vitro* fertilization and embryo transfer (IVF-ET) to minimize these causes of infertility (Miller et al., 2012). Recurrent Implantation Failure is defined by an absence of implantation after 3 or more high quality embryo transfers (Thornhill et al., 2005). Further early pregnancy complications that include ectopic pregnancy, miscarriage, hyperemesis gravidarum and gestational trophoblastic disease, are common. Early miscarriage as defined as

pregnancy loss before 12 weeks occurs in one of five pregnancies (Lucas et al., 2016; Miller et al., 2012; Practice Committee of the American Society for Reproductive, 2012; van den Boogaard et al., 2010). In the UK, miscarriage alone accounts for over 50,000 inpatient admissions per annum (Radford and Hughes, 2015). Miscarriage may bring about significant physical and psychological morbidity to affected couples providing the impetus for deeper investigation of cellular processes regulating fertility (Lok and Neugebauer, 2007; Quenby et al., 2021).

One factor that is suggested to regulate ovulation, decidualization and embryo implantation is prostaglandin E_2 (PGE_2) (Vilella et al., 2013a). PGE_2 synthesis rises during the luteal phase of the menstrual cycle and early pregnancy (Ye et al., 2018b). Notably, an aberrant PGE_2 formation has been reported in women with repeated implantation failure (Achache et al., 2010; Qu et al., 2021). The next paragraphs introduce this prostanoid as well as two enzymes (cyclooxygenase and phospholipase A2) involved in PGE_2 synthesis and an upstream regulator (annexin A7) of prostaglandin synthesis.

1.5. Prostaglandin E_2

PGE_2 is one of the most abundant prostaglandins produced in the body, has been studied extensively, and has a wide range of biological functions including regulation of the immune response, regulation of blood pressure, gastrointestinal integrity, and most importantly in the context of this thesis, regulation of fertility (Legler et al., 2010; Ricciotti and FitzGerald, 2011). Abnormal PGE_2 synthesis or degradation has been linked to a variety of clinical disorders. PGE_2 is particularly interesting in the context of inflammation since it is involved in all of the processes that contribute to the traditional indications of inflammation: redness, swelling, and pain. Enhanced blood flow into the inflammatory region is caused by PGE_2-mediated arterial dilatation and increased microvascular permeability, resulting in redness and oedema (Funk,

2001; Ricciotti and FitzGerald, 2011). In addition, PGE_2 acts on peripheral sensory neurons as well as at central sites in the spinal cord and brain, causing pain (Funk, 2001).

1.6. Cyclooxygenase and Phospholipase A_2

Prostanoids, which include prostaglandin, prostacyclin, and thromboxane, require the enzyme cyclooxygenase (COX) for their synthesis. COX isoforms constitute the major regulatory and rate-limiting enzyme in these pathways. Through peroxidase activity, the COX enzyme transforms arachidonic acid (AA) to prostaglandin H_2 (PGH_2) a precursor of PGE_2 (Luo et al., 2015; Vilella et al., 2013b). Three COX genes encode three isoforms: COX1, COX2, and COX3. COX1 is found in a variety of tissues, including the stomach, bowel mucosa, and kidney (Mattia and Coluzzi, 2005). Physiologically, COX2 expression as part of the immune response is substantially lower than that of COX1. Inflammatory cytokines, growth hormones, carcinogens, and other substances can upregulate COX2. As a consequence, inflammation and cancer are linked to aberrant COX2 activation and prostaglandin generation (Kargi et al., 2013; Yu et al., 2016). COX3 is most recently identified COX isoform that is similar to COX1. Aspirin and other non-steroidal anti-inflammatory drugs (NSAIDs) can lower the synthesis of prostaglandins by blocking the COX enzymes (Fatemi and Popovic-Todorovic, 2013; Luo et al., 2015; Yu et al., 2016).

Phospholipase A_2 (PLA_2) isoform that releases arachidonic acid from phospholipids are a broad and diverse family of proteins that act upstream of COX (Ricciotti and FitzGerald, 2011). Based on structural homology and cellular location, the enzymes are divided into two primary subfamilies. Cytosolic PLA_2 ($cPLA_2$-a, $cPLA_2$, Type IV PLA_2) has been well reviewed (Scott et al., 1999). Upstream of PLA_2 activity, annexin A7 has the ability to dock in a Ca^{2+}-dependent manner to the phospholipid cell membrane and prevents binding of

PLA_2 to the membrane, thus inhibiting PGE_2 formation (Herr et al., 2001; Luo et al., 2015; Rick et al., 2005).

1.7. Annexins

Annexins are a widely distributed multigene superfamily of evolutionary conserved calcium dependent phospholipids binding proteins, which are expressed in almost all tissues and cell types (Benz and Hofmann, 1997; Gerke and Moss, 2002; Voelkl et al., 2014; Ye et al., 2018a). There are over 160 different annexin proteins found in protists, fungi, plants, and vertebrates (Luo et al., 2015; Moss and Morgan, 2004). In human beings, twelve annexin subfamilies have been discovered (annexin A1-annexin A11 and annexin 13) (Luo et al., 2015; Ye et al., 2018a). Annexin proteins are mainly distributed beneath the inner surface of the plasma membrane and participate in many important biological processes, including regulation of cytoskeletal activity, cell adhesion, pinocytosis, membrane receptor regulation, activation of cell membrane receptors, membrane transport, cell signalling and mitosis (Gerke et al., 2005; Liu et al., 2020; Ye et al., 2018a). The presence of several annexins in a variety of cell types shows that they play a crucial function in cell biology. Knockout mice have supplied a wealth of information about the activities of annexins A1, A2, A5, A6, A7, and A8 (Hannon et al., 2003; Luo et al., 2015).

Annexin A7 (synexin, ANXA7) was the first described and isolated annexin protein (Camors et al., 2005; Herr et al., 2001). It has a remarkable feature to exhibit a Ca^{2+}-dependent GTPase activity (Camors et al., 2005; Luo et al., 2015). ANXA7 is different from other members as it carries an exon of 22 amino acids (Camors et al., 2005; Rick et al., 2005) and alternative splicing generates two isoforms of 47 kDa and 51 kDa. The smaller (47 kDa) isoform is expressed in most of the tissues and the larger isoform is expressed in skeletal muscle, heart, myocardium and brain (Camors et al., 2005; Luo et al., 2015; Rick et al., 2005). Physiologically, ANXA7 binds to secretory vesicles and plays a role in

secretion control (Caohuy et al., 1996; Clemen et al., 1999; Kuijpers et al., 1992). Spherocytosis, inflammatory myopathies, cardiac remodelling, as well as cell survival and tumour progression, have all been linked to ANXA7 (Li et al., 2013; Voelkl et al., 2014). Beyond the ANXA7/PLA_2/COX/PGE_2 signalling, decidualization seems to be dependent on an adequate glucose fuelling of the endometrial stromal cells. As a matter of fact, endometrial cells grown *in vitro* with glucose concentrations less than 2.5 mM show reduced levels of decidualization (Frolova and Moley, 2011). Left-Right Determination Factor 2 (LEFTY2) seems to play a pivotal role herein. As discussed in the next paragraphs, LEFTY2 may also be a factor regulation progression of endometrial carcinoma.

1.8. LEFTY2 in decidualization and endometrial cancer

LEFTY2, a secreted ligand of the transforming growth factor-beta (β) class of proteins, was originally designed as an endometrial bleeding related factor (EBAF). LEFTY2 acts as a Nodal signalling pathway antagonist by interfering with both NODAL binding to the activin receptor and the development of a receptor complex after proteolytic processing of the released precursor (Salker et al., 2018; Schier, 2003). This cytokine is abundantly expressed by decidualizing stromal cells and to a lesser amount in the endometrial glands during the late secretory phase of the menstrual cycle, i.e., after the window of implantation closes and before menstruation (Tabibzadeh, 2005). In addition, LEFTY2 has been found in the endometrial fluid of fertile women, showing that LEFTY2 is secreted into the uterus lumen (Tang et al., 2005). In the mouse uterus, activation of the proprotein convertase (PC) 5/6, which processes LEFTY2, is activated in response to mechanical damage or artificial decidualization using oil (Salker et al., 2018; Tang et al., 2005). In certain cases with "unexplained infertility," endometrial LEFTY2 levels are raised during the receptive phase, suggesting that dysregulation of LEFTY2 plays an important role in infertility (Salker et al., 2011; Tabibzadeh et al., 2000).

In normal tissues, glycolysis and lactic acid fermentation is a natural response to hypoxia (Parks et al., 2013; Reshkin et al., 2014). Beyond that, in cancer cells, aerobic glycolysis and lactic acid fermentation is a major source of energy supply even under normoxic conditions, a phenomenon described first by Otto Warburg (Liberti and Locasale, 2016). The rate-limiting enzymes of glycolysis are pH-sensitive and hindered by cytosolic acidification, thus, maintaining an neutral cytosolic pH is essential for glycolytic flux (Boiteux and Hess, 1981). LEFTY2 reportedly works in part by inhibiting the Na^+/H^+ exchanger 1 (NHE1) (Salker et al., 2015). The latter is required for cytosolic pH homeostasis, suggesting a reduced glycolytic flow upon LEFTY2-mediated NHE1 inhibition. As a matter of fact, LEFTY2 has previously been found to be a stimulator of apoptosis and an inhibitor of cell proliferation and tumour growth (Cavallari et al., 2013; Miyata et al., 2012; Naidu et al., 2008; Sun et al., 2014).

The expression of the Na^+ coupled glucose transporter-1 (SGLT1) is reportedly upregulated in many tumour entities (Koepsell, 2017; Scafoglio et al., 2015; Wright et al., 2017). SGLT1 is required for cell survival during tumour hypoxia and high glucose utilization because it facilitates cellular glucose uptake against a high chemical glucose concentration gradient (Casneuf et al., 2008; Guo et al., 2011; Ren et al., 2013) suggesting pivotal roles in tumour biology. On the other hand, in both human beings and mice, it has recently been discovered that SGLT1 is present in the endometrium and plays a critical role in pregnancy outcome (Salker et al., 2017). Thus, one might, speculate that SGLT1-mediated glucose fuelling is crosslinked to LEFTY2-regulated glucose metabolism. The present thesis, therefore, studied the interaction between LEFTY2 and SGLT1 in endometrial cancer cells.

Besides glucose metabolism, there is growing evidence that the microbiome of the gut may influence neoplastic transformation, cancer progression or cancer immunity (Gopalakrishnan et al., 2018). The next paragraph describes potential tumoricidal actions of the gut microbiome factor Urolithin A.

1.9. Urolithin A

Rac1 (ras-related C3 botulinum toxin substrate 1) is a fundamental regulator of the actin cell cytoskeleton and belongs to the Rho GTPases family. It is overexpressed in many tumour entities (including endometrial cancer). Inhibiting its activity reportedly slows down tumour growth *in vitro* (Bosco et al., 2009; Paradiso et al., 2004; Parri and Chiarugi, 2010; Vega and Ridley, 2008). The downstream effector of Rac1, PAK1 (p21 protein-activated kinase 1), on the other hand, is a serine/threonine protein kinase that is substantially overexpressed in human malignancies (including endometrial cancer) and plays an important role in cell proliferation, cell survival, and cytoskeletal dynamics (Al-Harbi et al., 2021; Ye and Field, 2012). The microbiota derived Urolithins have been demonstrated to downregulate Rac1 *in vitro* suggesting their tumoricidal action (Mc Cormack et al., 2021). As a matter of fact, high dietary intake of the polyphenols that are the precursors of Urolithins might be associated with lower incidences of diabetes, neurodegenerative, cardiovascular disorders and also cancer (Cerdá et al., 2005). Polyphenols are present in grapes, almonds, strawberries, black currants, raspberries, green tea, and pomegranates. Ellagitannin and its hydrolysed form ellagic acid, have been assumed to be involved in the supposed health-promoting qualities of polyphenols (Espín et al., 2013). Because ellagic acid and ellagitannin have a low absorbance in the gut, they are metabolized by gut bacteria into various dibenzopyran-6-derivates such as 3,8-dihydroxy-6H-dibenzo[b,d]pyran-6- (Urolithin A), B, and C, with Urolithin A being the most detectable component in human plasma and urine (Cerdá et al., 2005).

Individuals' ability to manufacture these metabolites in the colon varies depending on the bacterial composition of the gut. There are three metabotype categories: metabotype A, which accounts for over 80% of those who can generate Urolithin A, metabotype B, who can produce Urolithin B and isourolithin A in addition to Urolithin A, and metabotype O, who have no Urolithins in their plasma or urine (Tomás-Barberán et al., 2014). In particular,

Gordonibacter, which produces Urolithin C and A, and *Ellagibacter isourolithinifaciensth*, which produces Urolithin B and isourolithin A, have been found as bacterial genera that may convert ellagic acid into Urolithins in the human gut (Cortés-Martín et al., 2019).

Furthermore, Urolithin B synthesis is elevated in people with a high number of pro-inflammatory bacteria, such as *Methanogenic archaea* from the Euryarchaeota phylum (*Methanobrevibacter* and *Methanosphaera* genera) and the *Gammaproteobacteria* class, suggesting that this metabolite is caused by dysbiosis microbiota in the intestine (Cortés-Martín et al., 2019). Notably, overweight adults reportedly are also more likely to belong to metabotype B (Kang et al., 2016) pointing to the strong influence of nutrition on the gut microbiome.

1.10. Aims

Due to its reported PGE_2 regulatory function, the first part of this thesis aimed to assess the impact of ANXA7 on embryo implantation and fertility.

The second part of this thesis aimed to unravel the function of LEFTY2 in cellular glucose uptake and associated cell cycle and cell death regulation of human endometrial cancer.

Since Urolithin A has been suggested to be the physiologically active metabolite of an ellagitannin-rich diet, the last part of the present thesis aimed to disclose the tumoristatic effects of Urolithin A in endometrial carcinoma *in vitro* and the underlying cellular mechanisms.

2. Results

2.1. Annexin A7 Regulates Endometrial Receptivity, Md Alauddin, Madhuri S. Salker, Anja T. Umbach, Janet Rajaxavier, Toshiyuki Okumura, Yogesh Singh, Anna Wagner, Sara Y. Brucker, Diethelm Wallwiener, Jan J. Brosens and Florian Lang, Frontiers in cell and developmental biology.

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Annexin A7 Regulates Endometrial Receptivity

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A limited window of receptivity is a prerequisite of reproductive success. Indispensable receptivity genes include cyclooxygenase 2 (COX2), an enzyme accomplishing formation of prostaglandin E_2 (PGE_2). A powerful regulator of PGE_2 formation is Annexin A7 (*ANXA7*). The present study thus explored whether ANXA7 impacts on implantation and fertility. Here we show that *ANXA7* is expressed in endometrial tissue and increases upon decidual transformation of human endometrial stromal cells (HESCs) in a time-dependent manner. Silencing ANXA7 significantly decreased the expression of *PRL* and *IGFBP1*, canonical decidual marker genes, but enhances COX2 and PGE_2 levels. Genetic knockout of *AnxA7* in mice significantly increases the number of implantation sites and litter sizes. Further, analysis of human endometrial biopsies showed that *ANXA7* transcript and protein levels are decreased during the midluteal window of implantation in women suffering from recurrent pregnancy loss (RPL) when compared to subfertile patients. Taken together, the data indicate that ANXA7 has a conserved role in regulating endometrial receptivity and implantation.

Keywords: endometrium, COX2, implantation, pregnancy, PGE_2

INTRODUCTION

Following the post-ovulatory rise in progesterone levels, human endometrial stromal cells (HESCs) undergo extensive biochemical and morphological reprogramming, a process known as decidualization, in preparation for pregnancy (Koot et al., 2012; Gellersen and Brosens, 2014). While this process is initiated during the mid-luteal phase of the cycle, the emergence of morphologically decidualized cells at the start of the late-luteal phase marks the end of the implantation window, defined as the limited period during which a developmentally competent blastocyst can implant (Cha et al., 2012; Koot et al., 2012; Gellersen and Brosens, 2014; Macklon and Brosens, 2014). Decidualization is thus essential for the establishment of uterine receptivity, post-implantation embryo survival, and placentation. Key factors involved in endometrial receptivity and embryo implantation include prostaglandin (PG) production (namely PGE_2) by cyclooxygenase 2 (COX2; PTGS2) (Cha et al., 2012; Ruan et al., 2012).

Prostaglandin synthesis starts with the formation of arachidonic acid (AA) from membrane phospholipids mediated by phospholipase A_2 (PLA_2) (Vilella et al., 2013). Subsequently, COX2,

via its peroxidase activity, converts AA into PGs, including PGE_2 (Leslie, 2004; Cha et al., 2012; Ruan et al., 2012; Vilella et al., 2013). In the endometrium, COX2 levels and PGE_2 production increase during the luteal phase of the menstrual cycle and during early pregnancy (Ye et al., 2018). Compromised endometrial prostaglandin synthesis is observed in women with repeated implantation failure (Achache et al., 2010). Importantly, loss of COX2 or PLA_2 can result in infertility due to abnormalities of ovulation, implantation, and decidualization in mice (Lim et al., 1997; Vilella et al., 2013; Salker et al., 2018).

As shown in other cell types, COX2, PLA_2 and thus PGE_2 formation are strictly controlled by multiple factors, including Annexin A7 (synexin or ANXA7) (Scott et al., 1999; Harper and Tyson-Capper, 2008). Annexins are a family of evolutionary calcium-dependent phospholipids binding proteins, which are found in almost all tissues and cell types (Benz and Hofmann, 1997; Gerke and Moss, 2002; Guo et al., 2013). In humans, twelve annexin subfamilies have been described (Annexin A1-11 and Annexin 13) (Benz and Hofmann, 1997; Gerke and Moss, 2002; Guo et al., 2013). These proteins are mainly distributed in the inner surface of the plasma membrane and have important roles in regulation of cytoskeletal activity, cell adhesion, membrane receptor regulation, membrane transport and mitosis (Liu et al., 2020). ANXA7 was the first annexin protein to be described (Benz and Hofmann, 1997; Gerke and Moss, 2002). It was first isolated as a factor that mediates aggregation of chromaffin granules and fusion of phospholipids membranes in the presence of Ca^{2+} and in Ca^{2+}-GTP-dependent secretion (Herr et al., 2001; Guo et al., 2013). ANXA7 differs from other members as it possesses and extra-long amino terminus (Camors et al., 2005; Rick et al., 2005) and alternative splicing generates two isoforms with molecular weights of 47 kDa and 51 kDa. Most tissues harbor the 47 kDa isoform, while the larger isoform is expressed in skeletal muscle, heart, and brain (Camors et al., 2005; Rick et al., 2005). ANXA7 has a unique architecture that allows it to dock in a Ca^{2+} dependent manner onto the phospholipid cell membrane; this prevents PLA_2 from binding to the membrane, thus inhibiting PGE_2 formation (Herr et al., 2001, 2003; Clemen et al., 2003; Rick et al., 2005; Schrickel et al., 2007; Lang et al., 2009, 2010; Guo et al., 2013). Consequently, ANXA7 influences many physiological processes including secretion, hormone release, cell survival, cell volume, cardiac remodeling, gastric acid secretion, and inflammation by inhibiting PLA_2 and PGE_2 formation (Lang et al., 2009; Luo et al., 2015). Overexpression of ANXA7 is associated with aggressive tumors by accelerating cell cycle progression and proliferation (Liu et al., 2020).

Knockout of *AnxA7* in mice was initially reported to be lethal on embryonic day 10 (Srivastava et al., 1999). A second attempt by Herr et al. (2001) yielded viable *AnxA7* knockout mice (Herr et al., 2001). In the mouse line described in the latter study, the neo cassette was inserted directly into exon 8 of the *AnxA7* gene (Herr et al., 2001). Srivastava et al. (1999) replaced part of intron 5 and exon 6, an exon transcribed selectively in striated muscle and brain. Hence, the results of the two knockout models might be due to alterations in the expression of other genes in the vicinity of the integration site.

We speculated that ANXA7 may modulate endometrial receptivity in view of its role in regulating prostaglandin synthesis. However, to the best of our knowledge, little is known about the impact of ANXA7 on early implantation events. The present study thus explored the impact of ANXA7 on embryo implantation and fertility. We examined the expression of ANXA7 upon decidual transformation of HESCs. We demonstrated that ANXA7 knockdown impairs the induction of cardinal decidual markers prolactin (PRL) and insulin-like growth factor binding protein 1 (IGFBP1), but enhances COX2 and PGE_2 levels in human endometrium. We report that litter sizes are significantly larger in $AnxA7^{-/-}$ knockout females when compared to wild-type littermates. Finally, we disclose that ANXA7 levels are higher in women subfertility (SF) when compared to women with recurrent pregnancy loss (RPL).

RESULTS

Expression of ANXA7 in Human Endometrium

According to the Human Protein Atlas, ANXA7 is expressed in the endometrial glands and stroma and luminal epithelium (Uhlen et al., 2005). To investigate *ANXA7* mRNA levels in cycling endometrium we performed *in silico* analysis of gene expression data obtained in human endometrium across the cycle (GEO Profiles ID: 24464767). Endometrial *ANXA7* transcript levels increased from the proliferative to the early secretory phase of the cycle (**Figure 1A**). To investigate whether ANXA7 plays a role in decidualization, primary HESCs were decidualized with 0.5 μM 8-br-cAMP and 1 μM MPA (CM) for a total of 8 days. ANXA7 expression was examined at both mRNA and protein levels. Analysis of independent primary cultures demonstrated that ANXA7 (at both mRNA and protein levels) remained low during the initial pro-inflammatory decidual phase (days 2–4; aligned to the implantation window) (Salker et al., 2012a; Lucas et al., 2020) before rising around days 6–8, which coincides with the emergence of specialized decidual cells (aligned to the refractory period *in vivo*) (Salker et al., 2012a; Lucas et al., 2020; **Figures 1B,C** and **Supplementary Figure S1**).

Impact of ANXA7 Knockdown on Decidual Marker Genes

Decidualization denotes the differentiation process by which HESCs acquire a specialized secretory phenotype. *PRL* and *IGFBP1* are canonical marker genes widely used to assess the quality of the decidual response in HESCs (Saleh et al., 2011). In order to test whether ANXA7 influences the expression of decidual marker genes, primary cultures were first transfected with NT or siRNA targeting ANXA7 and then decidualized with CM for 6 days. As seen in **Figures 2A,B** and **Supplementary Figure S2**, siRNA-mediated knockdown decreased ANXA7 expression at both transcript and protein level by 85.05% and 87.23%, respectively. ANXA7 knockdown also inhibited *PRL* and *IGFBP1* mRNA levels in decidualizing cultures as well

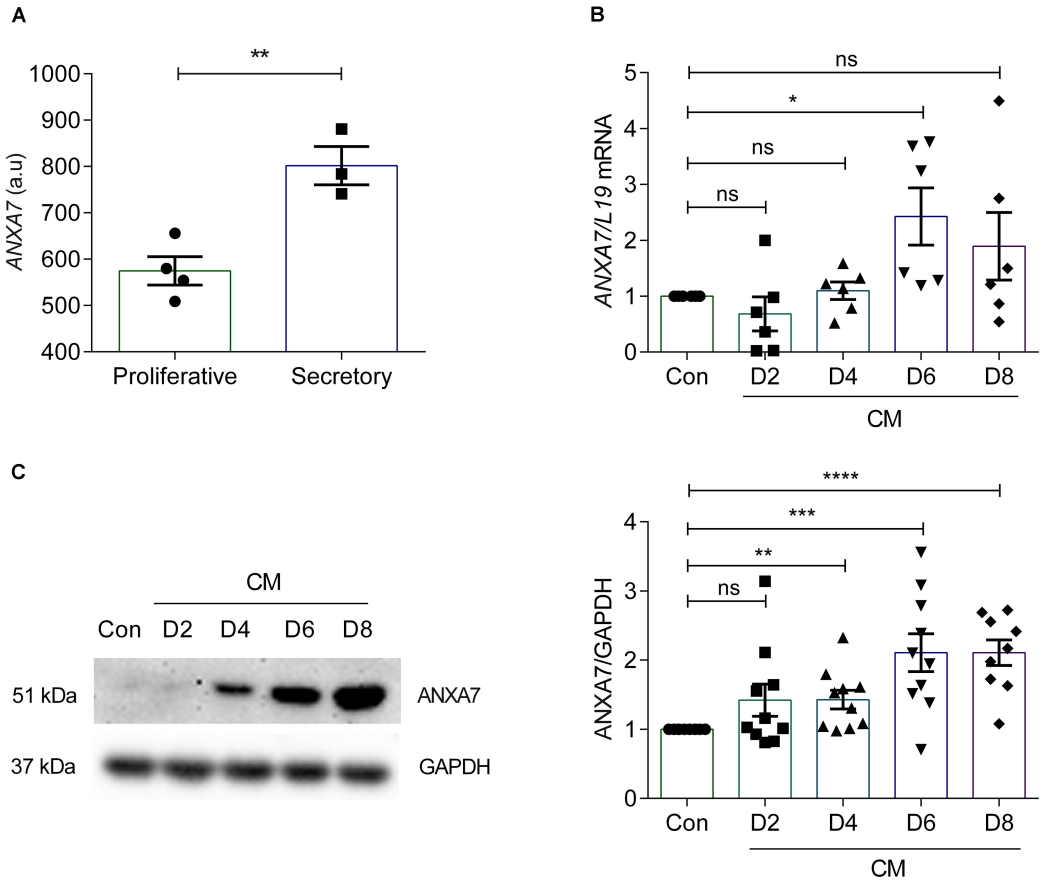

FIGURE 1 | ANXA7 level in human endometrium and human endometrial stromal cells (HESCs). **(A)** *ANXA7* gene expression is upregulated in secretory phase in human endometrium (Gene Expression Omnibus; GEO ID 24464767). **(B)** Arithmetic means ± SEM ($n = 6$) of *ANXA7* mRNA levels in HESCs, decidualized with 0.5 μM 8-Br-cAMP and 1 μM MPA (CM) up to 8 days. **(C)** Original Western blot (left) and arithmetic means ± SEM ($n = 10$; right) of *ANXA7* in HESCs decidualized with 0.5 μM 8-Br-cAMP and 1 μM MPA (CM) up to 8 days. Student's *t*-test was used to calculate statistical significance. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$.

as secreted PRL levels (**Figures 2C–E**). The differentiation-associated changes in the actin cytoskeleton by phalloidin staining of filamentous actin (F-actin) was also reduced in cells targeted with siRNA-ANXA7 (**Figure 2F**).

Downregulating ANXA7 Increases COX2 Levels

To investigate the effect of ANXA7 knockdown on COX2 and PGE_2, cultured HESCs were transfected with non-targeting (NT) or siRNA targeting ANXA7 and then decidualized for 6 days with CM. qRT-PCR analysis demonstrated that silencing of ANXA7 significantly upregulated *COX2* gene expression compared with the non-targeting control ($P < 0.05$; **Figure 3A**). Western blot results indicated that loss of ANXA7 upregulated COX2 protein level significantly (**Figure 3B** and **Supplementary Figure S3**). To test whether loss of ANXA7 increases PGE_2 levels, HESCs were transfected with NT or siRNA targeting *ANXA7* and then decidualized for 6 days. Cell culture supernatants were collected and PGE_2 levels measured using an ELISA kit. As shown in **Figure 3C**, loss ANXA7 increased PGE_2 levels significantly ($P < 0.001$).

Loss of AnxA7 Enhances Receptivity

In view of its role in PG synthesis, ANXA7 could be a putative regulator of endometrial receptivity. We first mined publicly available microarray data that profiled gene expression in periimplantation mouse uterine luminal epithelium (GEO ID: GSE44451). As seen in **Supplementary Figure S4**, *AnxA7* levels in wild type (WT) mice significantly increased upon transition of the receptive to the refractory phase ($P < 0.05$). To define the role of AnxA7 in regulating endometrial receptivity, pseudopregnancy was induced in $AnxA7^{-/-}$ and WT ($AnxA7^{+/+}$) female mice. Uterine horns were collected 5.0 days post coitus (dpc) and total RNA was extracted. The number of oocytes were the same in both groups (data not shown), thus ruling out "hyper-ovulation." As demonstrated in **Figure 4A**, several murine endometrial receptivity genes were up-regulated in the $AnxA7^{-/-}$ female mice, including *Cox2* transcripts. Decidualization marker *Prl8a2* tended to be lower

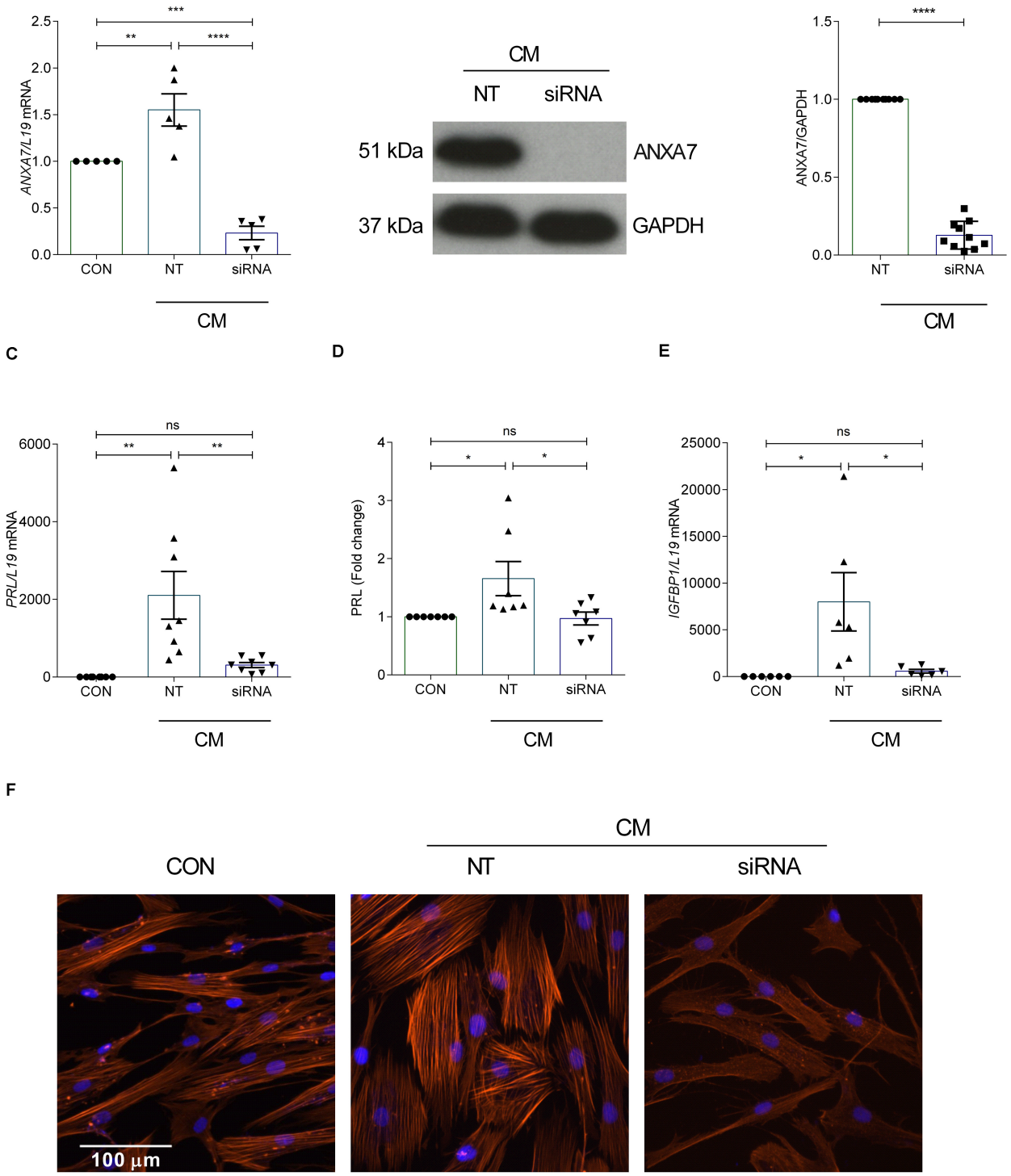

FIGURE 2 | Loss of ANXA7 reduces key decidual markers. HESCs were transfected by non-targeting (NT) or siRNA *ANXA7* and then decidualized for 6 days with 0.5 μM 8-Br-cAMP and 1 μM MPA (CM). **(A)** Arithmetic means ± SEM (n = 5) of *ANXA7* mRNA levels. **(B)** Original Western blot (left) and arithmetic means ± SEM (n = 10; right) of ANXA7 protein abundance. Arithmetic means ± SEM (n = 6–8) of **(C)** *PRL transcript* and **(D)** secreted PRL levels and **(E)** *IGFBP* expression levels in decidualizing HESCs. **(F)** Immunofluorescence images of F-actin (Phalloidin, red) in undifferentiated HESCs, decidualised or with siRNA targeting ANXA7. Nuclei are stained blue with DAPI (n = 3). Scale bar (100 μM). ANOVA or Student's t-test was used to calculate statistical significance when appropriate. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$.

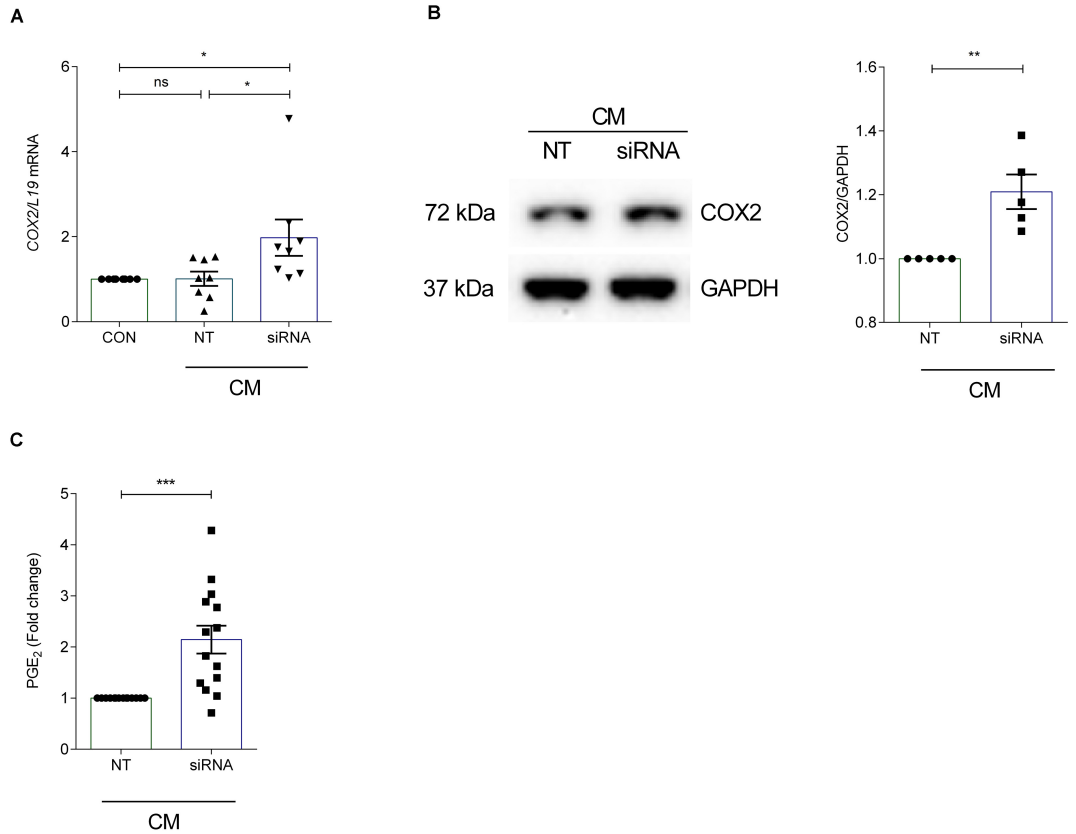

FIGURE 3 | Silencing of *ANXA7* upregulates COX2 and PGE_2 in HESCs. HESCs were transfected by non-targeting (NT) or siRNA targeting *ANXA7* and then decidualized for 6 days with 0.5 μM 8-Br-cAMP and 1 μM MPA (CM). **(A)** Arithmetic means ± SEM (n = 8) of *COX2* mRNA levels. **(B)** Original Western blot (left) and arithmetic means ± SEM (n = 5, right) of COX2 protein abundance in HESCs. **(C)** Arithmetic means ± SEM (n = 14) of PGE_2 levels measured by ELISA. ANOVA or Student's t-test was used to calculate statistical significance when appropriate. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

in $AnxA7^{-/-}$ female mice, although the difference did not reach statistical significance ($P = 0.06$). We further tested if loss of AnxA7 impacts on the number of implantation events and litter size. As seen in **Figure 4**, there were significantly more implantation sites (**Figure 4B**) and increased litter sizes knockout compared to WT mice (**Figure 4C**).

ANXA7 Regulates Endometrial Receptivity in Human Endometrium

To explore further the putative link between ANXA7 expression and endometrial receptivity, we explored midluteal endometrial RNA-sequencing data obtained from 10 subfertile patients and 10 women with a history of RPL (GEO Profiles ID: GSE65102). RPL is associated with a prolonged window of implantation, out-of-phase implantation, and heightened receptivity (i.e., superfertility), defined by short time-to-pregnancy (Teklenburg et al., 2010b; Salker et al., 2012a; Lucas et al., 2020; Ticconi et al., 2020). As shown in **Figure 4D**, endometrial *ANXA7* transcript levels were significantly higher in subfertile patients when compared to RPL subjects (**Supplementary Table S1**). The difference in endometrial ANXA7 protein expression between the clinical groups was also confirmed by western blot analysis (**Figure 4E** and **Supplementary Figure S5** and **Supplementary Table S2**).

DISCUSSION

Annexin A7 is a member of the calcium dependent phospholipids binding proteins and influences many functions and metabolic processes. In this study we reveal that ANXA7 is upregulated in the secretory phase of human endometrium and that *ANXA7* mRNA and protein levels are significantly upregulated in the HESCs decidualized with 8-Br-cAMP and MPA. We also show that endometrial cells express the 51 kDa isoform only. RNA-seq data on midluteal endometrial biopsies showed that *ANXA7* transcript levels varies between 90 and 204 transcripts per million, indicating moderate to high expression (Lucas et al., 2016) when compared to other tissues (Fagerberg et al., 2014). The present observations further show that receptivity is higher and litter size larger in gene-targeted female mice lacking functional AnxA7 ($AnxA7^{-/-}$) when compared to WT ($AnxA7^{+/+}$) mice. Finally, we show that endometrial *ANXA7* transcript and protein levels are significantly lower in RPL compared to subfertile patients. Importantly, RPL

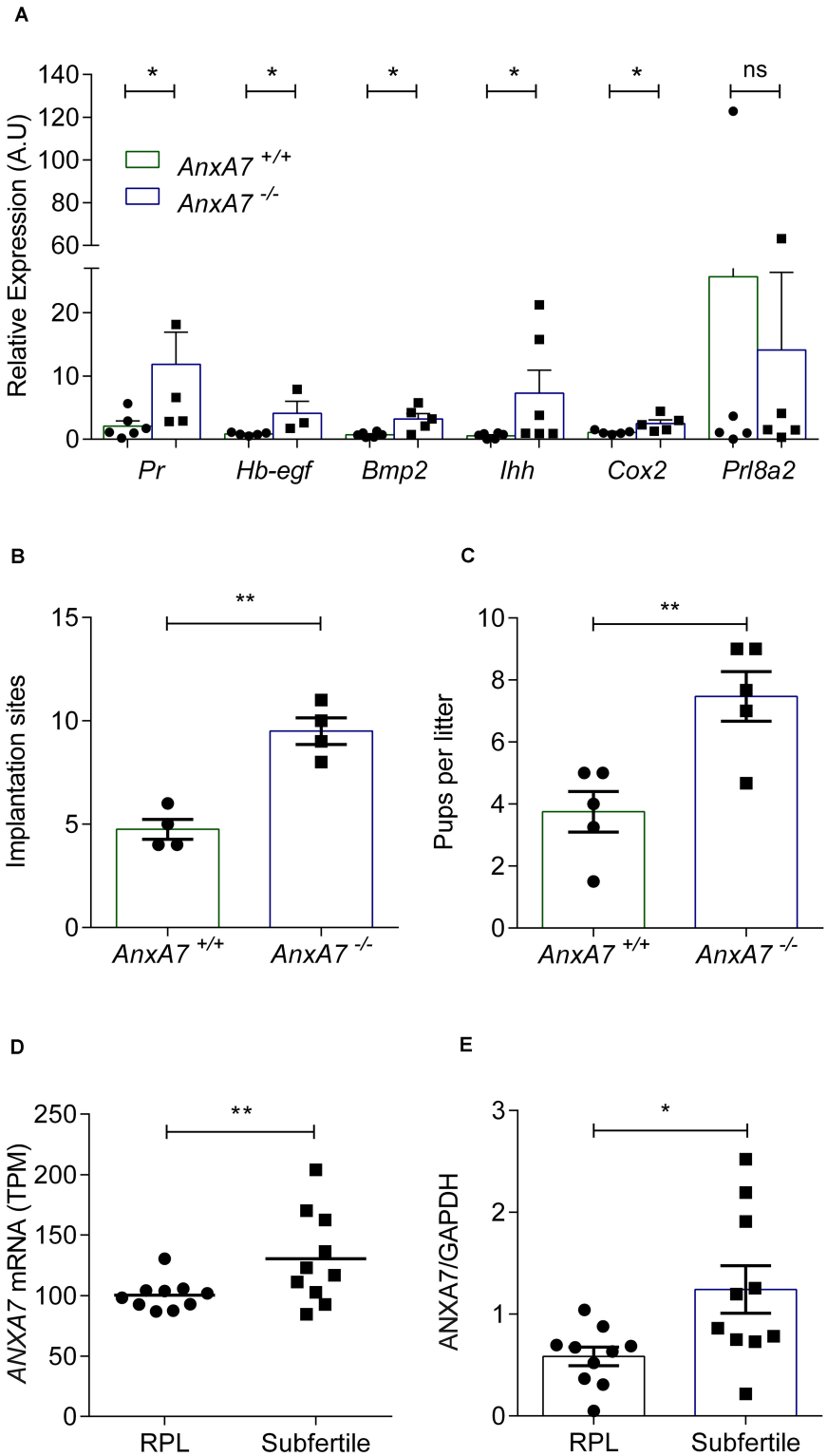

FIGURE 4 | Loss of ANXA7 enhances receptivity. **(A)** Mice were pseudo-mated and the uteri collected at day 5.0 d.p.c. Arithmetic means ± SEM (n = *AnxA7*$^{+/+}$; 6 and *AnxA7*$^{-/-}$ 5) of murine transcripts of *Pr* (Progesterone receptor), *Hb-egf* (Heparin binding -Egf), *Bmp2* (Bone morphogenetic protein), *Ihh* (Indian hedgehog), *Cox2* (Cyclooxygenase 2) and *Prl8a2* (Decidual PRL-related protein) in *AnxA7* knockout mice (*AnxA7*$^{-/-}$) and wild type littermates (*AnxA7*$^{+/+}$). *AnxA7*$^{-/-}$ female mice crossed with wild type males and wild type females with *AnxA7*$^{-/-}$ males. Arithmetic means ± SEM of **(B)** number of implantation sites at 8.5 d.p.c. (n = 4) and **(C)** pups per litter (n = 5). **(D)** Human *ANXA7* mRNA levels (GEO 65102) in RPL and Subfertile patients (n = 10). **(E)** Arithmetic means ± SEM (n = 10) of ANXA7 protein abundance in RPL and Subfertile patients patient samples. Demographic and clinical characteristics are presented in **Supplementary Tables S1, S2**. Student's t-test was used to calculate statistical significance where $^{*}P < 0.05$ and $^{**}P < 0.01$.

patients are more fertile, i.e., receptive toward the implanting embryo, than healthy women (Ticconi et al., 2020). The present study thus uncovers a novel molecular determinant of endometrial receptivity.

In response to elevated circulating progesterone levels and rising cellular cAMP levels, differentiation of the decidua takes place. Differentiating HESCs must transit through two distinct functional phenotypes for implantation to take place (Salker et al., 2012b; Lucas et al., 2013; Gellersen and Brosens, 2014). This decidual transitional pathway is characterized first by an acute auto-inflammatory phase, which is then followed by a profound anti-inflammatory response. The initial pro-inflammatory response renders the endometrium receptive to embryo implantation (Al-Sabbagh et al., 2011; Salker et al., 2012b). It is interesting to note that the levels of *ANXA7* transcripts in HESCs remain low during the initial pro-inflammatory decidual phase but rise sharply upon the emergence of specialist decidual cells around day 6 of the time-course, suggesting a role in limiting decidual inflammation and closure of the putative implantation window. This conjecture is in keeping with the mouse data. Together, these data point to a role of ANXA7 in participating in closing of the implantation window (**Supplementary Figure S6**).

Studies from our group and others demonstrated that RPL is associated with an impaired decidual response and mount a prolonged and highly disordered pro-inflammatory response (Salker et al., 2012b; Lucas et al., 2016). Emerging evidence indicates that abnormal decidualization impairs the "embryo-selectivity-checkpoint," which renders the endometrium excessively permissive to implantation (super-fertile or super-receptive) but unable to sustain the conceptus, thus leading to pregnancy loss (Teklenburg et al., 2010a,b; Sandra et al., 2011; Brosens et al., 2014; Macklon and Brosens, 2014). The current data also supports that the loss of ANXA7 impairs the expression of key decidual genes as well as an increased level of inflammation (PGE_2). In mice, during implantation, COX2 transiently emerges in stromal cells supporting implantation (Sucurovic et al., 2020). Therefore, it is possible that in the endometrium, in the absence of ANXA7, increased levels of COX2 temporarily create microenvironment suitable for embryo implantation with the synchronized increase of pro-implantation genes. In our murine model, we show that the loss of AnxA7 is associated with an increase of key receptivity genes such as, *Bmp2, Hb-egf, Ihh,* and *Cox2* at 5 dpc. Levels of *Prl8a2* tended to be lower, indicating an impaired decidual response. These data point to a role of AnxA7 in mice model modulating the window of implantation (Wang and Dey, 2006; Salker et al., 2012b).

Prostaglandin E_2 has a profound effect on embryo development, hatching, and embryo implantation (Niringiyumukiza et al., 2018). ANXA7 is at least partially effective by downregulation of COX2 and inhibition of PGE_2 formation. In mouse models, deficiencies of cytosolic PLA_2, COX2, or the use of prostaglandin inhibitors lead to several implantation defects (Pakrasi and Dey, 1982; Psychoyos et al., 1995; Salleh, 2014). Consistent with this hypothesis, re-addition of PGE_2 or the use of prostaglandin receptor agonists restores or increases implantation rates (Lim et al., 1999; Niringiyumukiza et al., 2018). In our results, we have shown that loss of AnxA7 resulted in a significant increase in the number of implantation sites and numbers of pups born. Furthermore, reduced or impaired endometrial prostaglandin synthesis is associated with dysregulated endometrial receptivity in women with repeated IVF failure (Achache et al., 2010). In keeping with this, we show that at transcript and protein levels ANXA7 was higher in subfertile patients, at least when compared to RPL subjects.

Insulin resistance associated with obesity or polycystic ovary syndrome affects endometrial receptivity, resulting in subfertility (Schulte et al., 2015). *In vivo*, AnxA7 deficiency was shown to decrease the insulin sensitivity of cellular glucose uptake and decreases Sodium/glucose cotransporter 1 (Sglt1) activity in the jejunum lowering glucose absorption and these effects were virtually abrogated by inhibition of COX2 with aspirin (Luo et al., 2015). Thus, the use of aspirin in treating implantation failure in those with insulin resistance is promising. However, clinical trials in the use of aspirin for the use implantation failure remain controversial (Fatemi and Popovic-Todorovic, 2013).

Annexin A7 is required for the stimulation of gastric acid secretion by glucocorticoids and thus contributes to the upregulation of gastric acid secretion by stressful situations (Pasham et al., 2013). It is tempting to speculate that ANXA7 is similarly required for the down-regulation of endometrial prostaglandin synthesis by glucocorticoids (Bazer et al., 2010) and thus, in co-operation with glucocorticoids, closes the receptive window during physical and psychological stressful situations. Glucocorticoid excess following stressful situations is a well-known cause of infertility (Rooney and Domar, 2016), which is, however, at least in part due to derangement of gonadotropin release (Iwasa et al., 2017). Further experiments are required to validate this hypothesis.

Several implantation events, including blastocyst-endometrium adhesion, growth factor and transcription factor signaling are regulated by Ca^{2+} signaling. When intracellular Ca^{2+} levels rise ANXA7 can redistribute itself to the plasma membrane and interact with PLA_2 (Herr et al., 2003). Further, ANXA7 is involved in the regulation of intracellular Ca^{2+} homeostasis in several cell types (Voelkl et al., 2014). In endometrial cells, impaired intracellular calcium signaling can decrease the expression of Ca^{2+}-responsive implantation genes such as COX2, WNT4, and BMP2 resulting in implantation failure (Salker et al., 2018). Moreover, in calcium-dependent-exocytotic secretory processes, ANXA7 -GTPase activity is increased (Herr et al., 2001; Watson et al., 2004). The joint contribution of GTP and Ca^{2+} increases membrane fusion and secretion (Herr et al., 2001; Watson et al., 2004). It is tempting to speculate that ANXA7 may interact with its Ca^{2+}-binding partners and is able to fine-tune intracellular Ca^{2+} levels, thus regulating various Ca^{2+}-dependent cellular processes essential for implantation. Deregulation of this balance may lead to reproductive failure. Clearly further studies are required to decipher these pathways.

Our results also reveal that loss of ANXA7 impaired decidualization, but promoted an environment conducive for implantation presumably in part by augmenting COX2 levels

and thus PGE_2 formation. Altered endometrial COX2 activity has been implicated in compromised fertility following several disorders (Vilella et al., 2013). It is noteworthy to point out that the 129/SVJ/*AnxA7*$^{+/+}$ (WT) mice are known to be poor breeders (Festing and Blackmore, 1971); and that the loss of *AnxA7* increases pup numbers significantly. The present observations, however, do not rule out the contribution of other mechanisms resulting up-regulation of COX2 and enhanced PGE_2 formation on receptivity in *AnxA7* deficient mice.

In conclusion, ANXA7 is expressed in human endometrium and upregulated during decidualization. Genetic knockout of *AnxA7* leads to increased litter size in mice potentially by extending the window of implantation. Low *ANXA7* transcript levels are observed in women suffering from RPL. Thus, ANXA7 participates in the orchestration of endometrial receptivity and its deranged expression compromises female reproduction.

MATERIALS AND METHODS

Cell Culture

Human endometrial stromal cells from Applied Biological Materials Inc (#T0533, Abm, Canada) (Krikun et al., 2004), were maintained at 37°C in a humidified 5% CO_2 atmosphere in DMEM/F-12 medium (Gibco, United Kingdom) containing 10% (v/v) dextran coated charcoal striped (Sigma, United States) fetal bovine serum (Gibco, Germany), 1% (v/v) antibiotic-antimycotic solution (Gibco, United States), and 1% (v/v) L-glutamine (Gibco, United Kingdom). Approximately, 200,000 cells were plated in 6-well plates and allowed to grown to (80–85%) confluency for 48 h. Before treatment or transfection, the culture medium was changed to 2% (v/v) dextran coated charcoal striped fetal bovine serum, 1% (v/v) antibiotic-antimycotic solution and 1% (v/v) L-glutamine. The cells were then decidualized with 0.5 μM 8-Bromo-cAMP (8-Bromo-cAMP, Tocris, United Kingdom) and 1 μM Medroxyprogesterone 17-acetate (CM; decidualizing stimulus) (MPA, Sigma, Germany) as indicated. Media was replaced every 48 h with fresh CM-media.

Transfection Experiments

In confluent cultures, *ANXA7* was silenced by using ON-TARGET plus SMARTpool small interference RNA (siANXA7, L-010760-00-0005; Dharmacon, United States). The siRNA was used at a final concentration of 2.75 nM with VIROMER GREEN Transfection Reagent (Lipocalyx GmbH, Germany) following the manufacturer's instructions. After incubating the cells with the transfection complex for 6 h, the medium was then discarded and replaced with treatment medium (CM; as described above) and the cells were then cultured for another 6 days.

Real-Time Quantitative PCR (qRT-PCR)

Total RNA was isolated from cells or (80 mg) tissues using a TRIzol reagent (Thermo Fischer) and transferred to a microcentrifuge tube. After 5 min incubation at room temperature 100 μl chloroform (Roth, Germany) was added and vortexed. The mixture was subsequently centrifuged at 13,000 rpm for 30 min at 4°C. The upper clear part was transferred to a new (RNASE free) microcentrifuge tube. 200 μl 2-propanol (Sigma) was added and mixed by vortexing. After 10 min incubation at room temperature, the sample was centrifuged at 13,000 rpm for 15 min at 4°C. The supernatant was discarded and the pellet air dried for 5 min. To this 50 μl of DEPC treated water (Sigma) was added to dissolve the pellet. The RNA concentration was measured by the nanoplate method (Thermo Scientific). The complementary DNA was synthesized by using Maxima H Minus cDNA synthesis kit (Thermo Scientific). qRT-PCR was then performed with PowerUp SYBR Green Master Mix (Thermo Scientific) using gene-specific primers purchased from Sigma. Primers were designed using primer blast (NCBI; **Supplementary Method 1**). For housekeeping controls; *L19* was used for human sample and *Cyclo* for mice samples. The expression levels of the samples are provided as arbitrary units defined by the $^{\Delta\Delta}$Ct method. All measurements were performed in duplicate. Melting curve analysis and agarose gel electrophoresis confirmed amplification specificity.

Human primers used for qRT-PCR:

Gene	Primer sequence (5′–3′)
L19	F: GCGGAAGGGTACAGCCAAT R: GCAGCCGGCGAAA
AnxA7	F: CTGCTGGGTCAGAATGTCATA R: AGGAGGATATCCAGGGAAAGGT
COX2	F: GCTCAAACATGATGATGTTTGCATTC R: GCTGGCCCTCGCTTATGA

Murine primers used for qRT-PCR:

Gene	Primer sequence (5′–3′)
Cyclo	F: TGGAGAGCACCAAGACAGACA R: TGCCGGAGTCGACAATGAT
Pr	F: GGTGGGCCTTCCTAACGAG R: GACCACATCAGGCTCAATGCT
Ihh	F: GCTTCGACTGG\]GTGTATTACG R: GCTCGCGGTCCAGGAAAAT
Hb-egf	F: CTTGCGGCTACTTGAACACA R: GAAAGCAGGATCGAGTGAGC
Bmp2	F: GGGACCCGCTGTCTTCTAGT R: TCAACTCAAATTCGCTGAGGAC
Prl8a2	F: TGCTCAGATCCCCTTGTGAT R: AGCTGGTGGGTTTGTGACAT

Western Blotting

Total protein samples were prepared by lysing the adherently cultured cells in a lysis buffer containing 0.5 M Tris hydrochloride (Roth) pH 6.8, 20% Sodium dodecyl sulfate (SDS, Sigma), 0.1% Bromophenol blue (Serva),1% beta mercaptoethanol (Sigma), and 20% glycerol (Roth). Tissue samples were extracted using the previous method (Salker et al., 2011). Equal amount

of total protein was separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene fluoride membranes (PVDF, GE Healthcare Life Sciences, Germany) as previously described (Salker et al., 2011). After blocking in 5% non-fat milk (Roth, Germany) in a Tris buffered saline (TBS-T) containing 0.1% Tween-20 (Sigma) for 1 h at room temperature. The membranes were then incubated overnight with primary antibodies AnxA7 (#3666; Cell Signaling, Netherlands), COX2 (#aa570-598; Cayman Chemical Company), GAPDH (#2218L; Cell Signaling) in the blocking buffer at 4°C overnight. The following day the primary antibody was removed and the membrane was washed four times with TBS-T each for 10 min. The membranes were then incubated with appropriate secondary antibodies in the blocking buffer at room temperature for 1 h followed by four washes with TBS-T. Chemiluminescent detection kit (WesternBright^{TM} ECL, Advansta, United States) used for the visualization of the protein complexes. The fluorescence signals were scanned with an iBright CL1000 (Thermo Scientific), and the intensities were assessed by a densitometry analysis to measure the relative expression of the target proteins using GAPDH as a control by ImageJ software. All samples are normalized with the control (Taylor and Posch, 2014).

Immunofluorescence

Human endometrial stromal cells (1000 cells) were plated on glass chamber slide and grown for 48 h. Treatment with CM was performed as described above for transfected and non transfected samples. Post treatment the cells were fixed for 15 min with 4% paraformaldehyde, washed with PBS, and permeabilized for 10 min in 0.1% Triton X-100/PBS. The slides were blocked with 5% BSA in 0.1% TritonX-100/PBS for 1 h at room temperature. Cells were stained for actin with eflour660-phalloidin (1:1000, #50655905) for 1 h at room temperature. The slides were mounted with ProLong Gold antifade reagent with DAPI (#P36931, Invitrogen). Microscopy was performed with an EVOS M7000 cell imaging system (Thermo Fischer) with an Apochromat 0.4 NA cover slip corrected × 10 objective. Scale bar was 100 μm.

ELISA

After treatment of the HESCs as stated above culture media were harvested and stored at −80°C. ELISA performed by using Human Prostaglandin E_2 ELISA Kit (Invitrogen) and Human Prolactin/PRL ELISA Kit (Abcam) following the manufacturer's instructions.

Animal Experiments

Experiments were performed in gene-targeted 129/SVJ mice lacking *AnxA7* (*AnxA7*$^{-/-}$) and in corresponding wild type mice (*AnxA7*$^{+/+}$). Generation, properties and genotyping of *AnxA7*$^{-/-}$ mice were described earlier (Herr et al., 2003). All animal experiments were conducted according to the German law for the care and use of laboratory animals and were approved by the local ethics committee. The mice (age 8–12 weeks) were fed a normal diet and had access to drinking water *ad libitum*. The mice were kept under constant humidity (55 ± 10%), temperature (22 ± 2°C) and 12 h light-dark cycle conditions. The, *n*, number was calculated using a power calculation. Implantation sites were counted as previously described (Salker et al., 2012a).

Patient Selection and Sample Collection

The study was approved by the National Health Service National Research Ethics–Hammersmith and Queen Charlotte's & Chelsea Research Ethics Committee (1997/5065). Subjects were recruited from the Implantation Clinic, a dedicated research clinic at University Hospitals Coventry and Warwickshire National Health Service Trust. Written informed consent was obtained from all participants in accordance with the guidelines in the Declaration of Helsinki 2000. Samples were collected using a Wallach Endocell sampler (Wallach) under ultrasound guidance as previously described (Salker et al., 2017). Endometrial biopsies were timed between 6 and 10 days after the preovulatory Luteinizing Hormone (LH) surge. Biopsies were collected in ovulatory cycles. None of the subjects were using hormonal treatments for at least 3 months prior to sample collection. The sub-fertility group consisted of women with a history of conception delay due to endometriosis, male factor, tubal factor, PCOS or unexplained infertility. RPL was defined as three or more consecutive pregnancy losses before 24 weeks gestation. Demographic and clinical characteristics are presented in **Supplementary Tables S1, S2**.

We confirm that all methods performed, including obtaining of consent, were performed in accordance with the relevant guidelines and regulations as approved by the ethics committee.

Statistical Analysis

Values are presented as means ± SEM. Data were analyzed using the Students *t*-test or 1 way-ANOVA for significance using the Graphpad Prism software (GraphPad software Inc., San Diego, CA, United States). Number of replicates (n). Power calculation for sample size was performed using the G*Power program. Values of $P \leq 0.05$ were considered significant. Figures presented were made using Graphpad Prism.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors upon reasonable request, to any qualified researcher.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the National Health Service National Research Ethics–Hammersmith and Queen Charlotte's & Chelsea Research Ethics Committee (1997/5065). The patients/participants provided their written informed consent to participate in this study. The animal study was reviewed and approved by the Regierungspräsidium Tübingen, Baden-Württemberg, Germany (35/9185.82-2 F01/19M).

AUTHOR CONTRIBUTIONS

MS and FL: conceptualization. MA, MS, AU, AW, JR, TO, and YS: methodology, formal analysis, and data curation. SB, DW, JB, and FL: resources or patient recruitment. MS: supervision and project administration. MS, SB, DW, and JB: funding acquisition. MA, MS, AU, JR, TO, YS, AW, SB, DW, JB, and FL: writing – original draft and writing – review and editing. All authors reviewed the manuscript and have approved its submission and publication.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcell.2020.00770/full#supplementary-material

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2.2. LEFTY2/endometrial bleeding-associated factor up-regulates Na^+ Coupled Glucose Transporter SGLT1 expression and Glycogen Accumulation in Endometrial Cancer Cells, Ni Zeng, Toshiyuki Okumura, Md Alauddin, Shayan Khozooei, Janet Rajaxavier, Shaqiu Zhang, Yogesh Singh, Bing Shi, Sara Y. Brucker, Diethelm Wallwiener, Satoru Takeda, Florian Lang, Madhuri S. Salker, PLoS One.

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RESEARCH ARTICLE

LEFTY2/endometrial bleeding-associated factor up-regulates Na^+ Coupled Glucose Transporter SGLT1 expression and Glycogen Accumulation in Endometrial Cancer Cells

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Abstract

LEFTY2 (endometrial bleeding associated factor; EBAF or LEFTYA), a cytokine released shortly before menstrual bleeding, is a negative regulator of cell proliferation and tumour growth. LEFTY2 down-regulates Na^+/H^+ exchanger activity with subsequent inhibition of glycolytic flux and lactate production in endometrial cancer cells. Glucose can be utilized not only for glycolysis but also for glycogen formation. Both glycolysis and glycogen formation require cellular glucose uptake which could be accomplished by the Na^+ coupled glucose transporter-1 (SGLT1; SLC5A1). The present study therefore explored whether LEFTY2 modifies endometrial SGLT1 expression and activity as well as glycogen formation. Ishikawa and HEC1a cells were exposed to LEFTY2, *SGLT1* and *glycogen synthase* (*GYS1*) transcript levels determined by qRT-PCR. SGLT1, GYS1 and phospho-GYS1 protein abundance was quantified by western blotting, cellular glucose uptake from 2-(*N*-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl)Amino)-2-Deoxyglucose (2-NBDG) uptake, and cellular glycogen content utilizing an enzymatic assay and subsequent colorimetry. As a result, a 48-hour treatment with LEFTY2 significantly increased *SGLT1* and *GYS1* transcript levels as well as SGLT1 and GYS1 protein abundance in both Ishikawa and HEC1a cells. 2-NBDG uptake and cellular glycogen content were upregulated significantly in Ishikawa (type 1) but not in type 2 endometrial HEC1a cells, although there was a tendency of increased 2-NBDG uptake. Further, none of the effects were seen in human benign endometrial cells (HESCs). Interestingly, in both Ishikawa and HEC1a cells, a co-treatment with TGF-β reduced SGLT1, GYS and phospho-GYS protein levels, and thus reduced glycogen levels and again HEC1a cells had no significant change. In conclusion, LEFTY2 up-regulates expression and

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activity of the Na^+ coupled glucose transporter SGLT1 and glycogen synthase GYS1 in a cell line specific manner. We further show the treatment with LEFTY2 fosters cellular glucose uptake and glycogen formation and TGF-β can negate this effect in endometrial cancer cells.

Introduction

LEFTY2 (endometrial bleeding associated factor; EBAF or LEFTYA) is a member of the transforming growth factor beta (TGF-β) superfamily. LEFTY2 is produced as a precursor protein that is cleaved, leading to release of the C-terminus monomeric active proteins [1]. Unlike other TGF-β family members, LEFTY2 does not function *via* receptor-mediated SMAD-dependent signaling, but rather by antagonizing the signaling of TGF- β and Nodal [2]. In brief, activin, belonging to TGF-β superfamily, binds to type II ActRII receptor, causing the phosphorylation and activation of the type I activin-like kinase 4 (ALK4; TGFR) receptor [3]. Activated ALK4 phosphorylates in turn SMAD proteins (SMAD2 and SMAD3) [4] forming complexes with SMAD4. The activated complexes translocate into the nucleus and affect TGF-β specific genes [3]. LEFTY2 can antagonize the signaling pathway by interacting with ActRII, thus blocking phosphorylation of SMAD and inhibiting downstream factors [3]. It is now well established that tumorigenesis is associated with development of resistance to TGF-β signaling, and for this reason, it is thought that TGF-β signaling acts as a potent tumor suppressor [5]. Since the normal function of the TGF-β signaling pathway is suppression of cellular proliferation and transformation, it could be proposed that the action of LEFTY2 could be a potential oncoprotein by counteracting TGF-β-mediated signaling. Further, LEFTY2 is highly enriched in embryonic stem cells and participates in the regulation of 'stemness' and embryonic differentiation [6–9]. This expression has been shown to re-appear in cancers, such as breast and melanoma [10].

Tumors reprogram nutrient pathways to meet the high bio-energetic demands of malignant cells [11, 12]. These reprogrammed activities are now acknowledged as the hallmarks of cancer [12, 13]. The reprogrammed metabolic pathway in cancer is known as aerobic glycolysis, a phenomenon known as the "Warburg effect" [11]. In the 1920s, Nobel Laureate Otto Warburg described that tumor slices and malignant ascites (presence of malignant cells in the peritoneal cavity) constitutively take up glucose and produce lactate irrespective of oxygen availability [14]. Glycolysis is a physiological response to hypoxia in normal tissues. Glycolysis fuels a substantial portion of ATP production in cancer cells [15–21] and is decisive for energy production particularly during ischemia [22]. Previously, LEFTY2 was shown to be an inhibitor of cell proliferation and is capable of stimulating apoptosis [23–26], thereby counteracting tumor growth [27–30]. LEFTY2 is partially effective by down-regulating Na^+/H^+ exchanger 1 (NHE-1), leading to a decrease of glycolytic flux (the rate at which molecules proceed through the glycolytic pathway) in endometrial cancer cells [31]. Glycolytic flux requires the maintenance of alkaline cytosolic pH since the rate-limiting enzymes of glycolysis are highly pH-sensitive and inhibited by cytosolic acidification [32]. In tumor cells, an alkaline cytosolic pH is accomplished by several transporters including the Na^+/H^+ exchangers (NHE1-9) [15, 33], Na^+ coupled bicarbonate co-transporters [33] and lactate or mono-carboxylate transporters [15, 33] extruding both, lactate and H^+ ions [34].

Maintenance of glycolytic flux critically depends on the supply of glucose. Normally, the delivery of glucose is partially accomplished by the passive glucose carriers of the GLUT family

[35, 36]. Glucose may in addition, be taken up by the Na^+-Glucose co-transporter (SGLT) family. The SGLTs mediate secondary active transport driven by the Na^+ gradient across the cell membrane [37, 38]. The two members of this protein family, SGLT1 and SGLT2, accomplish the concentrative cellular uptake of glucose across the apical cell membrane of epithelial cells [37].

SGLT1 expression is, however, not limited to healthy epithelial tissues, but has been identified in several tumor cells, which have an increased substrate demand and utilize mainly glucose for energy production [37, 39, 40]. SGLT1 allows cellular glucose uptake against a glucose concentration gradient and is particularly important for cell survival during ischemia and excessive glucose utilization [41–43]. Recently, it has been shown that SGLT1 is present in the endometrium and plays a decisive role in pregnancy outcome in both humans and mice [44]. We therefore sought to investigate the relationship of LEFTY2 and SGLT1 in endometrial cancer cells.

The present study investigated whether LEFTY2 affects the Na^+ coupled glucose transporter SGLT1 in human endometrial (Ishikawa and HEC1a) cancer cells. Surprisingly, LEFTY2 stimulated SGLT1 expression and activity. Further studies revealed that LEFTY2 fosters the incorporation of accumulated glucose into glycogen. None of these effects were seen in healthy endometrial cells. Interestingly, co-treatment with TGF-β negated the LEFTY induced effect.

Materials and methods

Cell culture

Ishikawa cells, a well differentiated endometrial carcinoma cell line (#ECACC 99040201) [45], Hec1A (type 2 endometrial adenocarcinoma; #HTB-112 purchased from ATCC) and benign human endometrial cells (#T0533; HESCs purchased from Applied Biological Materials Inc., Richmond, Canada) were routinely grown in monolayers using 75 cm^2 culture flasks maintained at 37˚C in a humid atmosphere containing 5% (v/v) carbon dioxide (CO_2) using a Heracell incubator. Cells were maintained in DMEM: F12 phenol free supplemented with 10% (v/v) FBS, 1% antibiotic/antimycotic solution and 1% L-Glutamine (Invitrogen, Germany), which was changed every other day and passaged when near confluent (1–2 times per week depending on growth rate). All work was carried out in a Class I safety cabinet. Cells were treated with LEFTY2 (25 ng/ml; 746-LF-025/CF; recombinant human: R&D Systems, Germany) or with TGF-β (10 ng/ml,14-8342-80, ebioscience, USA) in DMEM: F12 phenol free supplemented with 2% (v/v) Fetal Bovine Serum, 1% antibiotic/antimycotic solution and 1% L-Glutamine for 48 hours (all from Invitrogen, Germany). No Institutional review board approval was required for this study.

Messenger RNA (mRNA) extraction and quantitative real-time PCR (qRT-PCR)

RNase ZAP, RNase-free plastic-ware and DEPC-treated water were used to minimize degradation of mRNA. Total mRNA was extracted from cells cultured in 6-well plates by direct lysis using the miRNeasy® Mini Kit (Qiagen, Germany) according to the manufacturer's protocol. The mRNA concentration was determined using a Nanodrop (Eppendorf μCuvette® G1.0, Microvolume measuring cell for Eppendorf BioPhotometer® and BioSpectrometer®, Eppendorf, Germany) and the A260/ A280 ratio of 1.9–2.1 was used as a threshold. Samples with lower ratios indicating contamination were discarded. Samples were diluted to 1 μg/ μl with DEPC- water and stored at -80˚C.

One μg RNA was reverse transcribed by using the ThermoScientific Maxima^{TM} H Minus cDNA Synthesis Master Mix with dsDNase (Invitrogen). The resulting cDNA was diluted and used as a template for subsequent PCR reactions. Primers were designed using the NCBI primer blast software. Ribosomal L19 (housekeeping) was used to normalize for discrepancies in input cDNA. Gene expression was quantified using the PowerUp^{TM} SYBER® Green Master Mix (Invitrogen) and performed using the QuantStudio 3 Real-Time PCR System (Invitrogen). In each PCR reaction a non-template control (NTC) reaction (where cDNA is substituted with DNase/RNase free water) and reverse transcriptase (-RT) controls were included. The PCR products were not detected in NTC or RT control reactions. Primer sequences: *hSGLT1* forward (5'-3'): AGAGGGGAACAGACAACACA & reverse (5'-3'): ACCAAAACCAGGG CATTCCA, *hGYS1* forward (5'-3'): AGGGCTGCAAGGTGTATTTC & reverse (5'-3'): ACTCCG ATGTTGCAGGTATC, housekeeping *hL19* forward (5'-3'): GCAGCCGGCGCAAA & reverse (5'-3'): GCGGAAGGGTACAGCCAAT. Expression levels were calculated using the $^{\Delta\Delta}C_T$ method. The values are provided as arbitrary values (a.u.). All measurements were performed in triplicate. Melting curve analysis and agarose gel electrophoresis was also performed to confirm amplification specificity.

Western blotting

Whole cell protein extracts were harvested from 6-well plates using Laemmli buffer following a PBS wash (1ml/well). Cell scrapers were used to collect the lysates which were then pipetted into 1.0 ml tubes and heated for 10 min at 95˚C [46]. Proteins were resolved on 10% sodium dodecyl sulfate–polyacrylamide (SDS) gels using the Invitrogen XCell SureLock® Mini-Cell apparatus. 5 μl protein ladder (Biozym, Germany) was added to the first lane. Gels were run at 125 V for up to 2 hours until the dye front had migrated to the base of the gel, at which time the cassettes were opened and the gels used for transfer. The gels were transferred onto a PVDF membrane (Amersham Biosciences, Germany), activated in methanol, using a wet-transfer blotting method. The transfer was performed at 230 mA for 2 hours in a box of ice. The PVDF membrane was then air-dried and reactivated in methanol before being incubated with 5% non-fat dry milk in Tris-buffered saline with 1% Tween (TBS-T) (TBS; 130 mmol/L NaCl, 20 mmol/L Tris, pH 7.6 and 1% Tween) for 1h (RTP). This procedure aimed to prevent nonspecific binding. The membrane was then washed (once in TBS-T for 5 minutes and three times in TBS-T for 15 minutes) before incubation with the primary antibody. SGLT1, phosphorylated GYS1 (Ser641), and GYS1 were identified by primary antibodies against human SGLT1 (1:1000, #5042, Cell Signaling, Netherlands), human phosphorylated GYS1 (Ser641) (1:1000, #47043, Cell Signaling, Netherland), and human GYS1 (1:1000, #3886, Cell Signaling, Netherland) respectively. Equal loading was quantified using an antibody against GAPDH (1:1000, Cell Signaling). The TBS-T wash step was repeated before incubation with the HRP-conjugated antibody (raised against the primary antibody), for 1h at RTP (1:2000, Cell Signaling). Protein bands were visualized using a chemiluminescent detection kit (Advansta, Biozym, Germany) using iBright^{TM} Imaging System (Invitrogen). All experiments were performed in 3 or more cell cultures. Bands were quantified with ImageJ Software.

Cellular glucose uptake

The fluorescent glucose analogue 2-(N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)-2-deoxyglucose (2-NBD-glucose; Invitrogen, Darmstadt, Germany) was used to measure the relative uptake of glucose by flow cytometry. In each condition, cells were incubated with 2-NBD-glucose (30 μM) for 1 hour at 37 ˚C, subsequently washed twice in cold PBS and subjected to flow

cytometry (BD Biosciences, Heidelberg, Germany) in fluorescence channel FL1. Data were analyzed using the FlowJo Software.

Cellular glycogen content

At the end of the experiment, glycogen concentration was measured using a Glycogen Assay Kit (MAK016, Sigma, Germany), according to the manufacturer's protocol. Samples were measured using the Sunrise ELISA plate reader (Tecan, Germany)

Statistics and data availability

The data are given as arithmetic means ± SEM, *n* denotes the number of independent biological experiments. The data were analyzed for significance using unpaired Student's *t*-test using GraphPad Prism Software (CA, USA). Statistical significance was considered when $p < 0.05$. All relevant data are within the manuscript and its supporting information files.

Results

The present study addressed the effect of LEFTY2 on the Na^+ coupled glucose transporter SGLT1 and glucose utilization in Ishikawa and HEC1a cells. In the first series of experiments the expression of SGLT1 was determined. Quantitative real-time PCR was utilized to determine the *SGLT1* transcript levels. Ishikawa cells and HEC1a cells remained untreated or were treated with LEFTY2 (25 ng/ml) for 48 hours. As shown in **Fig 1A**, the expression of *SGLT1* transcript levels in Ishikawa cells was significantly enhanced following LEFTY2 treatment. As illustrated in **Fig 1B**, according to Western blotting, treatment of Ishikawa cells with 25 ng/ml of LEFTY2 for 48 hours was followed by a marked and significant increase of SGLT1 protein abundance. These effects were also seen in parallel in HEC1a cancer cells (**Fig 1C & 1D**). However, LEFTY2 was unable to increase SGLT1 levels in benign endometrial cells. (**S1A Fig**).

In the next set of experiments, we sought to quantify glucose transport. Ishikawa cells and HEC1a cells were treated with or without LEFTY2 (25 ng/ml) for 48 hours. One hour prior to the end of the experiment the cells were treated with a fluorescent glucose analog, 2-[*N*-(7-nitrobenz-2-oxa-1,3-diaxol-4-yl)amino]-2-deoxyglucose (2-NBDG). The uptake of the fluorescent substrate 2-NBDG was quantified using flow cytometry. As illustrated in **Fig 2**, treatment of Ishikawa cells and HEC1a cells with 25 ng/ml of LEFTY2 for 48 hours was followed by an increase of cellular 2-NBDG (glucose) uptake. However, it did not reach significance in HEC1a cells.

Next, in order to define the use of increased glucose uptake in endometrial cancer cells treated with LEFTY2, quantitative real-time PCR was utilized to determine *GYS1* transcript levels. Ishikawa cells or HEC1a cells remained untreated or were treated with LEFTY2 (25 ng/ml) for 48 hours. As shown in **S2A & S2B Fig**, the transcription of *GYS1* was significantly enhanced following LEFTY2 treatment in both cell lines.

Glycogen, a polysaccharide of glucose, serves as energy storage. GYS1 catalyzes the rate-limiting step of glycogen biosynthesis. GYS1 is inactivated by phosphorylation at several sites, and activation occurs by dephosphorylation [47]. To investigate GYS1 activity at protein level, Ishikawa cells or HEC1a cells were treated with 25 ng/ml of LEFTY2 for 48 hours and whole cell lysates were subjected to Western blotting. As illustrated in **Fig 3**, treatment with LEFTY2 resulted in an increase of total GYS1 protein abundance in both Ishikawa (**Fig 3A & 3B**) and HEC1a (**Fig 3C & 3D**) cells. Further, treatment with LEFTY2 resulted in a decrease of phosphorylated GYS1 (Ser641)/ GYS1 ratio when compared with the control suggesting active glycogen synthesis in Ishikawa cells. HEC1a cells showed no apparent change. No effect was seen on benign endometrial cells (**S1B Fig**).

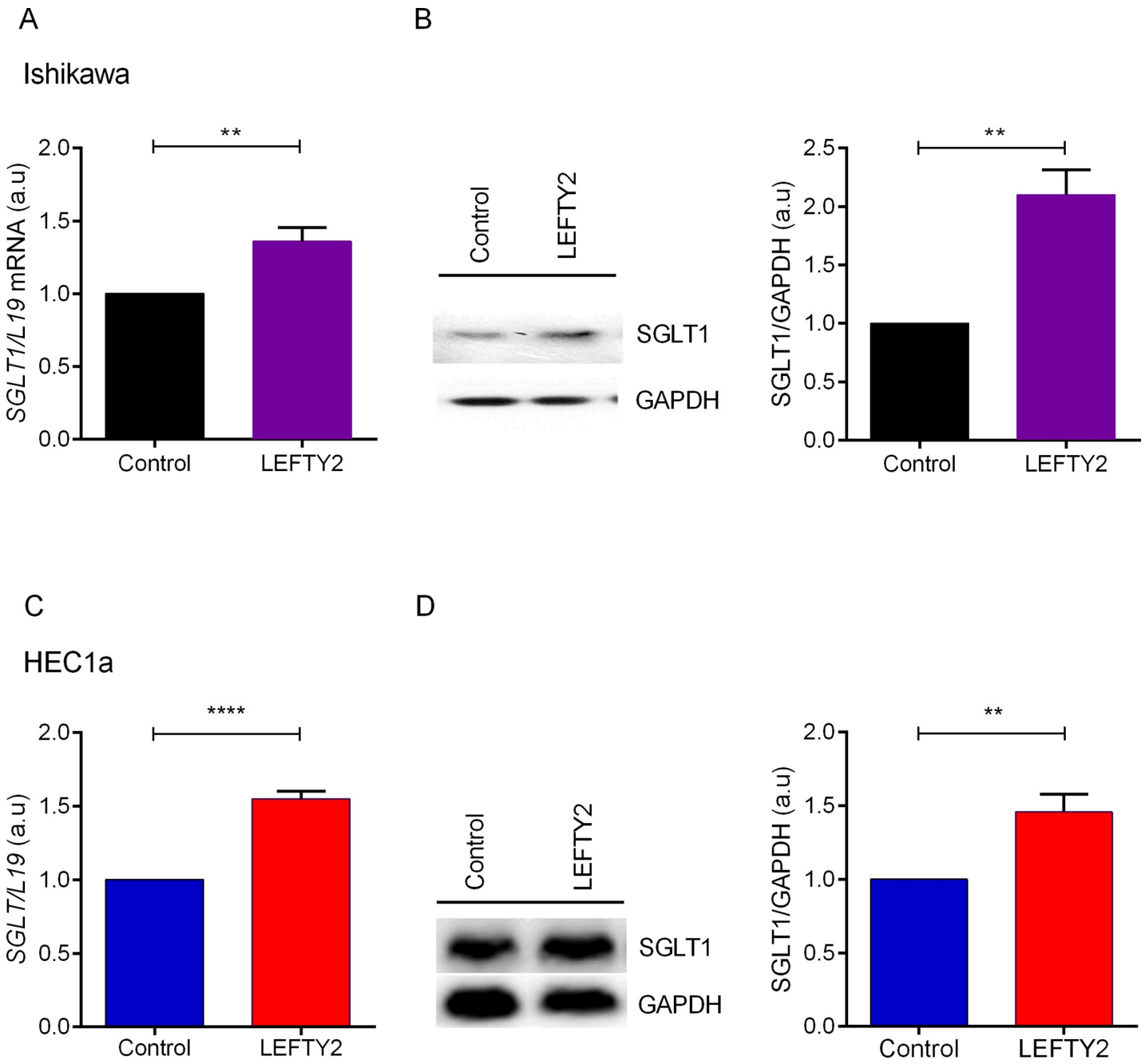

Fig 1. Effect of LEFTY2 on *SGLT1* transcript levels and protein abundance. A. Arithmetic means ± SEM (n = 4) of *SGLT1* transcript levels from Ishikawa cells without (black) or with treatment (purple) with 25 ng/ml LEFTY2 for 48 hours. *L19* was used as a housekeeping control. **B.** An original western blot of SGLT1 and GAPDH protein in whole cell lysates from Ishikawa cells without or with a 48 hours treatment with LEFTY2 (25 ng/ml). GAPDH was used as a loading control. Arithmetic means ± SEM (n = 4) of the SGLT1/GAPDH protein abundance ratios in cell lysate from Ishikawa cells without or following treatment with 25 ng/ml LEFTY2 (right side). **C.** Arithmetic means ± SEM (n = 5) of *SGLT1* transcript levels from HEC1a cells without (blue) or with treatment (red) with 25 ng/ml LEFTY2 for 48 hours. *L19* was used as a housekeeping control. **D.** An original western blot of SGLT1 and GAPDH protein in whole cell lysates from HEC1a cells without or with a 48 hours treatment with LEFTY2 (25 ng/ml). GAPDH was used as a loading control. Arithmetic means ± SEM (n = 7) of the SGLT1/GAPDH protein abundance ratios in cell lysate from HEC1a cells without or following treatment with 25 ng/ml LEFTY2 (right side). $^{**}(p<0.01)$ indicates statistically significant difference from untreated cells.

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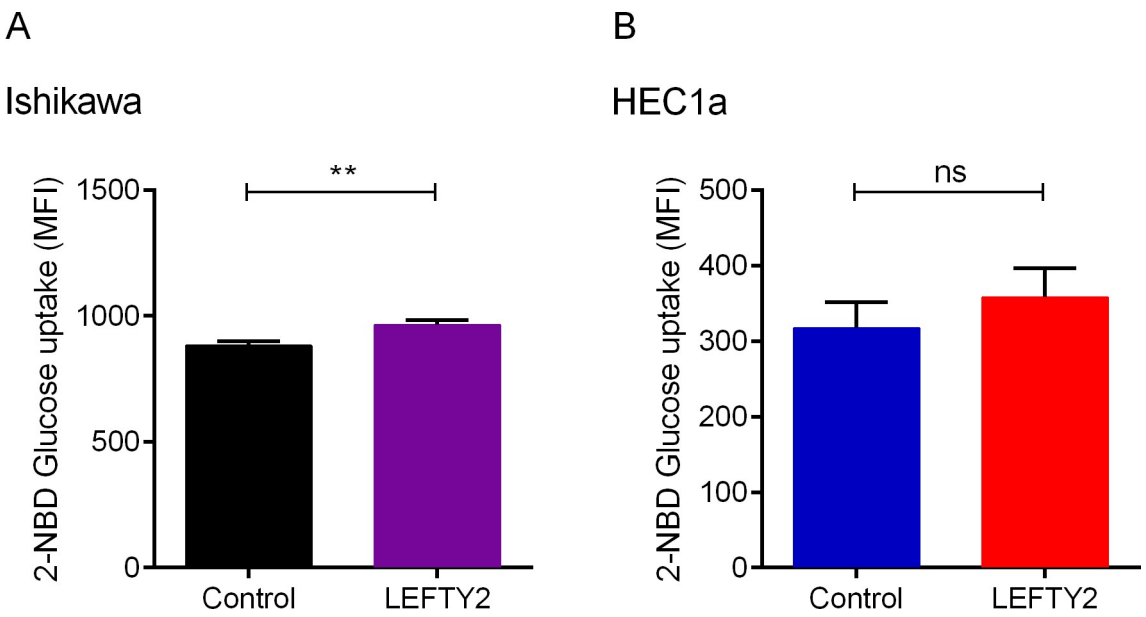

Fig 2. Effect of LEFTY2 on glucose uptake. Arithmetic means ± SEM of glucose uptake in (**A**) Ishikawa cells (n = 9) or (B) HEC1a cells (n = 6) untreated (control) or treated with 25ng/ml LEFTY2 for 48 hours and incubated with 2-NBDG (a fluorescent glucose analog) for 1 hour and analysis by flow cytometry. ** ($p<0.01$) indicates statistically significant difference from untreated cells) using Student's t-test.

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LEFTY2 has been described as a TGF-β antagonist and as a tumor supressor. We investigated whether TGF-β can inhibit glycogen synthesis induced by LEFTY2. We treated Ishikawa and HEC1a cells without or with LEFTY2 (25 ng/ml) or with TGF-β (10 ng/ml) for 48 hours. To test any antagonistic effects the cells were pre-treated with TGF-β (10 ng/ml) for 24 hours and then treated with LEFTY2 and TGF-β for a further 48 hours. Here, we show that treatment with TGF-β or in combination LEFTY2 decreased SGLT1, GYS and pGYS1 (Ser641) levels in both Ishikawa (**Fig 3A & 3B**; **S2A Fig**) and HEC1a cells (**Fig 3C & 3D**; **S2A Fig**).

To investigate if indeed LEFTY2 stimulates glycogen accumulation, we treated Ishikawa and HEC1a cells with or without LEFTY2 (25 ng/ml) for 48 hours and measured glycogen content using an ELISA based method. Here, we show that a 48 hours treatment with LEFTY2 significantly increased cellular glycogen content only in Ishikawa cells (**Fig 4A & 4B**). Intresetingly, treatment with TGF-β (10 ng/ml) alone or in combination negated the LEFTY2-driven glycogen accumulation in Ishikawa cells. No effect was seen on HEC1a cells.

Discussion

The present observations reveal a novel function of LEFTY2 in the regulation of SGLT1. According to our previous observations [31], LEFTY2 is a potent inhibitor of glycolytic flux in Ishikawa cells. Further, LEFTY2 decreased lactate concentration in the supernatant of Ishikawa cells [31]. This negative effect of LEFTY2 on glycolysis was explained by a marked cytosolic acidification, which was in part due to an inhibitory effect of LEFTY2 on Na^+/H^+ exchanger activity [31]. Glycolytic flux is highly sensitive to cytosolic pH and is disrupted by cytosolic acidification [32].

The present observations confirm that LEFTY2 increased transcript levels and protein abundance of *SGLT*1. Moreover, LEFTY2 increased the cellular uptake of the glucose carrier substrate 2-NBDG, an observation again pointing to enhanced carrier activity. It is tempting

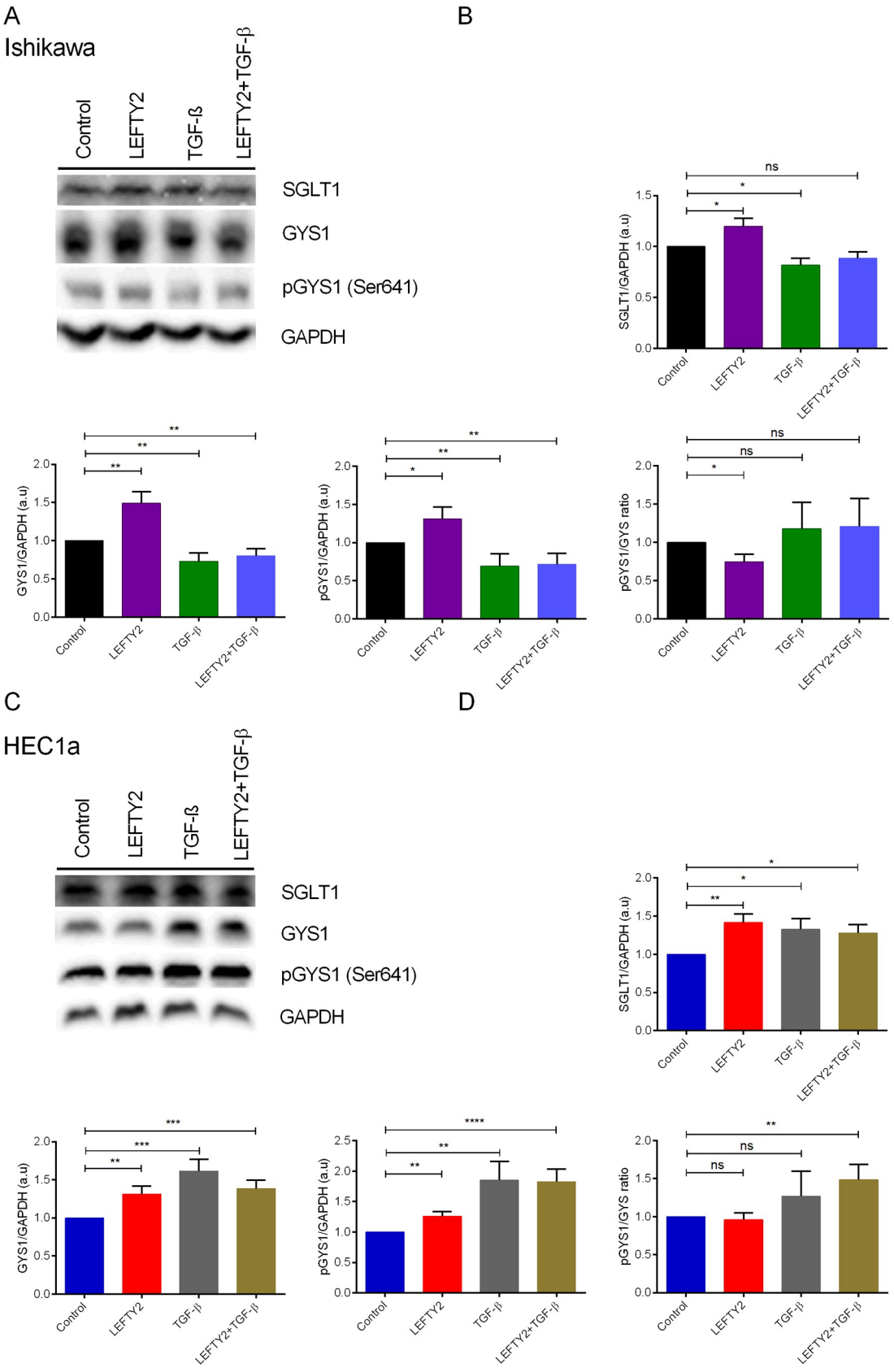
A
Ishikawa
Control
LEFTY2
TGF-ß
LEFTY2+TGF-β
SGLT1
GYS1
pGYS1 (Ser641)
GAPDH
B
SGLT1/GAPDH (a.u)
GYS1/GAPDH (a.u)
pGYS1/GAPDH (a.u)
pGYS1/GYS ratio
ns
Control
LEFTY2
TGF-β
LEFTY2+TGF-β
C
HEC1a
D

Fig 3. Effect of TGF-β on SGLT, GYS1 and on pGYS1 (Ser641) protein levels. A. Original western blots of SGLT1, GYS1, pGYS1 (Ser641), and GAPDH protein in whole cell lysates from Ishikawa cells without or with a 48 hours treatment with LEFTY2 (25 ng/ml) or with TGF-β (10 ng/ml) or in combination. GAPDH was used as a loading control. **B.** Arithmetic means ± SEM (n = 10) of SGLT1/GAPDH, GYS1/GAPDH (n = 9) and pGYS1 (Ser641)/GAPDH protein (n = 6) abundance ratios in cell lysates. **C.** Original western blots of SGLT1, GYS1, pGYS1 (Ser641), and GAPDH protein in whole cell lysates from HEC1a cells without or with a 48 hours treatment with LEFTY2 (25 ng/ml) or with TGF-β (ng/ml) or together. GAPDH was used as a loading control. **D.** Arithmetic means ± SEM (n = 10) of SGLT1/GAPDH, GYS1/GAPDH (n = 5) and pGYS1 (Ser641)/GAPDH protein (n = 4) abundance ratios in cell lysates. $^{*}(p<0.05)$, $^{**}(p<0.01)$ indicates statistically significant difference from untreated cells.

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to speculate that the simultaneous increase of cellular glucose uptake (by SGLT1 transporter) and inhibition of glycolysis served to foster the cellular formation of glycogen, an endometrial cancer-specific function. Notably, glycogen which is stored in the endometrium and considered to serve as an important energy source in early pregnancy [48]. Recently it has been shown that loss of SGLT1 impairs adequate endometrial glycogen stores for pregnancy and disruption of this histotrophic pathway leads to adverse pregnancy outcome and miscarriage in both humans and mice [44]. GYS1 activation occurs by dephosphorylation, thus increasing the cells capacity for glycogen storage [47]. Our data reveal that LEFTY2 indeed increased cellular glycogen abundance via GYS1 activation. Our additional experiments have showed no role of LEFTY2 on building glycogen stores and thus energy reserves in benign endometrium. Moreover, using HEC1a cells we also did not see an increase in glycogen accumulation this maybe in part due to a cell-line specific effect, further experiments are warranted to dicepher this. These observations reveal completely novel insight into the effect of LEFTY2 on glycolysis

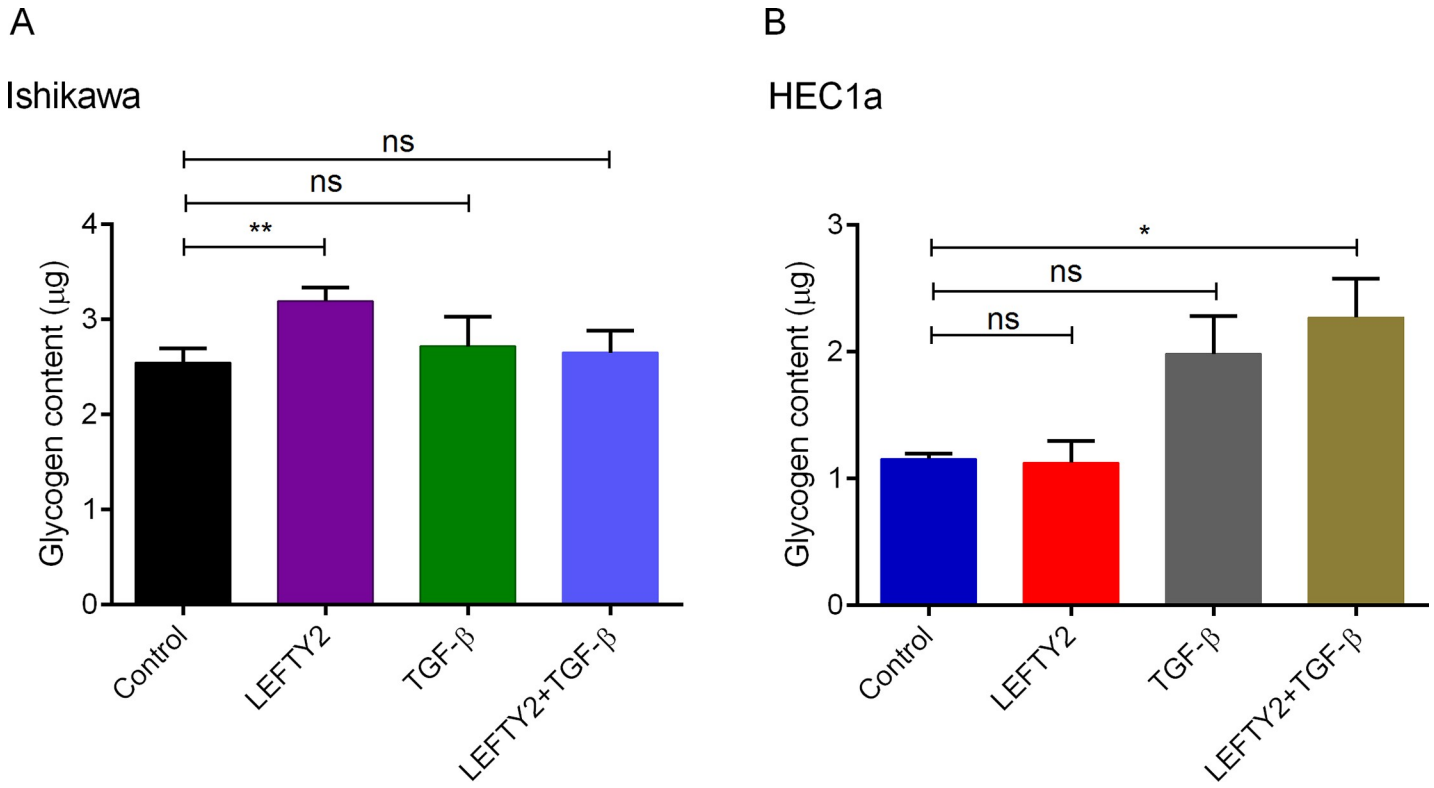

Fig 4. Effect of LEFTY2 on glycogen levels. Arithmetic means ± SEM glycogen content in (A) Ishikawa cells (n = 7) or (B) HEC1a cells (n = 4). Untreated (control) or treated with 25ng/ml LEFTY2 for 48 hours or with TGF-β (10 ng/ml) alone or in combination. $^{*}(p<0.05)$, ** $(p<0.01)$ indicates statistically significant difference from untreated cells (control).

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in endometrial cancer cells. We did not find that LEFTY2 can antagnosise TGF-β signaling. The combined stimulation of cellular glucose uptake and inhibition of glycolysis may serve to boost the cellular formation of glycogen. In this respect, the utilization of SGLT1 may be particularly valuable, as it is capable of cellular glucose uptake against a steep chemical gradient and is able to accomplish cellular glucose uptake at low extracellular and high intracellular glucose concentrations [49]. Further, our data supports the hypothesis that TGF-β could act as a tumor suppressor potentially by reducing glycogen accumulation in endometrial cancer cells. Further work is needed to confirm this conjecture.

Tumor cells are dependent on delivery of glucose to cover their excessive demand for this substrate [50]. Even in the presence of glucose, tumor cells degrade glucose to lactate and thus utilize only a small fraction of the energy, which could be generated by oxidative degradation of glucose [31, 51]. At least, in some tumor cells, the uptake of glucose with facilitative glucose carriers alone presumably fails to supply sufficient amounts of glucose. SGLT1 couples the uphill transport of glucose to Na^+ entry down its electrochemical potential gradient across the plasma membrane and is thus able to accomplish cellular glucose uptake when extracellular glucose concentrations are below those within the cell [17, 38].

Growing evidence suggests that those with type-2 diabetes are at elevated risk for cancer [52, 53]. Several carcinogenic risk factors involving the pathophysiology of type-2 diabetes, such as *obesity*, play a significant role in increasing endometrial cancer risk [52]. SGLT inhibitor-drugs developed for diabetes may be beneficial in treating cancers, either alone or in combination with other anti-cancer treatments. It is well established that increased glucose uptake and aerobic glycolysis are landmark signals of cancer cells (both are targets for cancer therapy) [11]. It is therefore, a huge clinical challenge to selectively inhibit glucose uptake in tumors, without disturbing the normal physiology of non-affected organs (heart, muscle, brain, etc.). However, in those tumors where glucose uptake occurs through SGLTs, it may be possible to significantly reduce glucose uptake and cell growth by inhibiting SGLT activity, though further studies are required to support this hypothesis [39, 40]. Moreover, additional experiments may uncover the coincidence of stimulated cellular glucose uptake and inhibition of glycolysis as a strategy to boost glycogen formation in other cell types.

In conclusion, the present study demonstrates a novel role of LEFTY2 in up-regulating transcript levels and protein abundance of the Na^+ coupled glucose transporter SGLT1, thus stimulating cellular glucose uptake and cellular formation of glycogen in endometrial cancer cells.

Supporting information

S1 Fig. No effect of LEFTY2 on benign endometrial cells. A. Arithmetic means ± SEM (n = 4) of *SGLT1* and (n = 5) *GYS1* transcript levels from benign endometrial cells without or with treatment with 25 ng/ml LEFTY2 for 48 hours. *L19* was used as a housekeeping control. **B.** An original Western blot of SGLT1, GYS1, pGYS1 (Ser641), and GAPDH protein in whole cell lysates from benign endometrial cells without or with a 48 hours treatment with LEFTY2 (25 ng/ml). GAPDH was used as a loading control. Arithmetic means ± SEM of SGLT1/GAPDH (n = 6), GYS1/GAPDH (n = 7) and pGYS1 (Ser641)/GAPDH (n = 7) protein abundance ratios in cell lysate without or following treatment with 25 ng/ml LEFTY2.
(TIF)

S2 Fig. Co treatment with TGF-β reduces SGLT1 and GYS1 transcript levels in Ishikawa and HEC1a cells. A. Ishikawa cells or **B.** HEC1a cells were treated 48 hours treatment with LEFTY2 (25 ng/ml) or with TGF-β (10 ng/ml) or in combination. Control cells remained untreated. Arithmetic means ± SEM (n = 5) of *SGLT1* and *GYS1* transcript. *L19* was used as a

housekeeping control. *($p<0.05$) and **($p<0.01$) indicates statistically significant difference from untreated cells (control).
(TIF)

S1 Data.
(XLSX)

S2 Data.
(XLSX)

Author Contributions

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Writing – review & editing: Florian Lang, Madhuri S. Salker.

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2.3. Gut Bacterial Metabolite Urolithin A Decreases Actin Polymerization and Migration in Cancer Cells, Md Alauddin, Toshiyuki Okumura, Janet Rajaxavier, Shayan Khozooei, Simone Pöschel, Satoru Takeda, Yogesh Singh, Sara Y Brucker, Diethelm Wallwiener, André Koch, Madhuri S Salker, Molecular nutrition & food research.

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Gut Bacterial Metabolite Urolithin A Decreases Actin Polymerization and Migration in Cancer Cells

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Scope: Urolithin A (UA) is a gut-derived bacterial metabolite from ellagic acid found in pomegranates, berries, and nuts can downregulate cell proliferation and migration. Cell proliferation and cell motility require actin reorganization, which is under control of ras-related C3 botulinum toxin substrate 1 (Rac1) and p21 protein-activated kinase 1 (PAK1). The present study explores whether UA can modify actin cytoskeleton in cancer cells.

Methods: The effect of UA on globular over filamentous actin ratio is determined utilizing Western blotting, immunofluorescence, and flow cytometry. Rac1 and PAK1 levels are measured by quantitative RT-PCR and immunoblotting. As a result, a 24 h treatment with UA (20 μM) significantly decreased Rac1 and PAK1 transcript levels and activity, depolymerized actin and wound healing. The effect of UA on actin polymerization is mimicked by pharmacological inhibition of Rac1 and PAK1. The effect is also mirrored by knock down using siRNA.

Conclusion: UA leads to disruption of Rac1 and Pak1 activity with subsequent actin depolymerization and migration. Thus, use of dietary UA in cancer prevention or as adjuvant therapy is promising.

1. Introduction

Urolithins are metabolites derived from ellagic acid and ellagitannins by gut microbiota after consumption of foods such as berries (strawberries, raspberries, blackberries), pomegranates, tropical fruits, nuts (walnuts, chestnuts, almonds, pistachios), oak barrel aged wines and spirits, and tea.[1–3] In the intestinal lumen, ellagic acid is extensively metabolized by gut microbes producing a series of metabolites called urolithins. Among the Urolithin metabolites, Urolithin A is the major metabolite observed in humans.[2,3] The health effects attributed to urolithins are numerous and diverse, ranging from antimalarial properties, free radical scavenger activity, and anti-proliferative effects in various types of cancer in vivo and in vitro.[1,4–19]

Ellagic acid has a potent negative effect on Na^+/H^+ exchanger 1 (NHE1) activity,[20] which is thought to induce cell death in tumor cells.[21–23] NHE1 activity is regulated by the small G protein ras-related C3 botulinum toxin substrate 1 (GTPase Rac1),[24] a member of the Rho GTPases family. Rac1 is a major regulator of actin cell cytoskeleton[25] that promotes the formation of lamellipodia[26] essential for cell motility.[27] For the motility of cancer cells, the regulatory proteins of the actin cytoskeleton play a pivotal role and contribute to cancer progression.[28,29] The capability of cancer cells to migrate and invade requires reorganization of the actin cytoskeleton.[30] Rac1 was found to be overexpressed in many cancers and conversely, loss of Rac1 activity suppresses tumor growth.[31–33] Rac1 was accordingly recognized as a potential therapeutic target for cancer.[34,35] The actin cytoskeleton determines its mechanical properties,[36,37] thus modification of actin polymerization is expected to modify cell stiffness thus migratory potential.[38,39]

Here, we report that treatment of Ishikawa cells (a well differentiated human endometrial carcinoma) with Urolithin A leads to dynamic change in mechanical cellular properties in tumor cells. We provide further evidence that in Ishikawa cells, Urolithin A decreases Rac1 activity, p21 protein-activated kinase 1 (PAK1) phosphorylation, actin polymerization and migration.

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2. Results

2.1. Effect of Urolithin A on Actin Polymerization in Ishikawa Cells

We have shown that ellagic acid can modulate the expression and attenuate the activity of the Na^+/H^+ exchanger NHE1.[20] NHE1 contributes further to the stabilization and localization of actin. We hypothesized that NHE1 inhibition could alter the cytoskeleton necessary for maintaining cell structure. We explored whether Urolithin A treatment affected actin polymerization dynamics. Strikingly, as apparent from both Western blotting analysis (**Figure** 1a,b; $^{***}p = 0.0006$) and flow cytometry (Figure 1c,d; $^{**}p = 0.0013$), a 24 h treatment of Ishikawa cells with 20 µM Urolithin A was sufficient to significantly increase the amount of soluble globular actin (G-actin) over filamentous F-actin ratio, an observation reflecting depolymerization of the actin filaments. Immunofluorescent images (Figure 1e, 75 µm) of F-actin organization shows reorganization of the actin cytoskeleton with Urolithin A treatment (Figure 1e, bar graph, $^{*}p = 0.01$). The effect of Urolithin A was compared to that of the cytoskeletal disruptor, latrunculin A, which also induces rapid actin depolymerization (Figure S1, Supporting Information). Using multispectral imaging flow cytometry (ImageStream, AMNIS), we visualized how the actin was distributed within the cell. Uniform actin was defined as equal distribution of actin throughout the cell (purple). Spotty actin was defined as actin that is distributed in a discontinuous manner ("spotty" or "patchy"; green). Polarized actin was defined as actin found in one location (yellow). We show that uniform actin decreases (Figure 1f; $^{*}p = 0.0315$) significantly in treatment with a parallel increase in spotty actin (Figure f; $^{*}p = 0.045$) and the effect on polarized actin had no significant change. We also show that there is a decrease in cell proliferation (Figure 1g; $^{****}p \leq 0.0001$). To test whether the effect of Urolithin A is limited just to Ishikawa cells, we treated HESC (benign endometrial stromal cells), HEC1a (type 2 endometrial cancer), and RPE (wild type and p53 mutant cells) with 20 µM Urolithin A for 24 h. The significant effect of actin depolymerization by Urolithin A was only seen in HEC1a and RPE p53 mutant. Interestingly, the benign cell lines (HESCs and RPE Wt.) had no difference (Figure S2, Supporting information). All further experiments were focused on Ishikawa cells, a model of well differentiated endometrial carcinoma.

2.2. Effect of Urolithin A on Rac1 Transcript Levels and Activity in Ishikawa Cells

In search for a cellular mechanism accounting for the reorganization of the actin filaments, the effect of Urolithin A on Rac1 expression and activity was tested. As shown in **Figure** 2a, Urolithin A treatment (20 µM for 24 h) was followed by a significant decline of Rac1 transcript levels normalized to L19 transcript levels ($^{***}p = 0.0009$). Moreover, Urolithin A treatment was followed by a significant decline of total and phospho-Rac1 protein levels (Figure 2b–d; Figure 2c; $^{*}p = 0.0245$; Figure 2d; $^{*}p = 0.0424$). To prove unequivocally that Urolithin A is a direct target of Rac1, Ishikawa cultures were first transfected with NT, Rac1, or PAK1-siRNA or in combination and then treated with Urolithin A (20 µM for 24 h). Rac1 knockdown was highly efficient and co-treatment with Urolithin A further suppressed total and phospho Rac1 protein expression (Figure 2e–g). Additionally, we tested whether the effect of Urolithin A could block EGF activity (a known activator of Rac1-PAK1 signaling). We show that co-treatment with Urolithin A (UA) can block EGF activated signaling (Figure S3, Supporting information)

2.3. Effect of Urolithin A on Actin Polymerization in Ishikawa Cells in Absence or Presence of Rac1 Inhibitor

A further series of experiments tested whether the effect of Urolithin A on Rac1 indeed contributed to the depolymerization following treatment with Urolithin A (20 µM for 24 h). As illustrated in **Figure** 3a,b, application of the Rac1 inhibitor NSC23766 trihydrochloride (100 µM) for 24 h was followed by a significant increase of soluble G-actin over filamentous F-actin in Ishikawa cells (Figure 3b; NSC, $^{**}p = 0.0052$), an effect thus mirroring the effect of Urolithin A treatment (Figure 3b, UA, $^{*}p = 0.0169$). The presence of the Rac1 inhibitor and Urolithin A (20 µM) leads to a significant further increase of the soluble G-actin over filamentous F-actin ratio in Ishikawa cells (Figure 3b, $^{*}p = 0.0175$). These results were also confirmed by FACS (Figure 3c,d; UA $^{*}p = 0.0321$, NSC $^{***}p < 0.0001$, UA NSC $^{*}p = 0.0432$)

2.4. Effect of Urolithin A on PAK1 Transcript Levels and Activity in Ishikawa Cells

Regulation of the actin network occurs when Rac1 activates PAK1 by phosphorylation.[40,41] As shown in Figure S4a–d, Supporting Information, Urolithin A (20 µM for 24 h) decreased both mRNA and proteins levels of total and phospho PAK1 significantly. To prove indisputably that Urolithin A modulates the Rac1-PAK1 signaling pathway, Ishikawa cultures were first transfected with NT, RAC or PAK1 siRNA or in combination and then treated the cells with Urolithin A treatment (20 µM for 24 h). PAK1 knockdown was highly efficient and co-treatment with Urolithin A further suppressed PAK1 protein expression (Figure S4e–g, Supporting Information).

Inhibition of PAK1 with IPA-3 (50 µM for 24 h) again paralleled the effect of Rac1 inhibition (**Figure** 4). Similarly, inhibition of PAK1 was followed by a significant increase of soluble G-actin over filamentous F-actin (Figure 4b; IPA, $^{*}p = 0.0292$ and Figure 4d; $^{*}p = 0.02$), mirroring the effect of Urolithin A treatment (Figure 4b; UA $^{***}p = 0.0006$ and Figure 4d; $^{*}p = 0.0206$). In the presence of the PAK1 inhibitor and Urolithin A, there was a further significant increase of soluble G-actin over filamentous F-actin ratio (Figure 4b; UA IPA $^{*}p = 0.0214$ and Figure 4d; $^{*}p = 0.0211$).

2.5. Effect of Urolithin A on Migration

Modification of actin polymerization is required for cell motility. To investigate whether Urolithin A affects wound healing, a scratch assay was performed. Once cells reached 100%

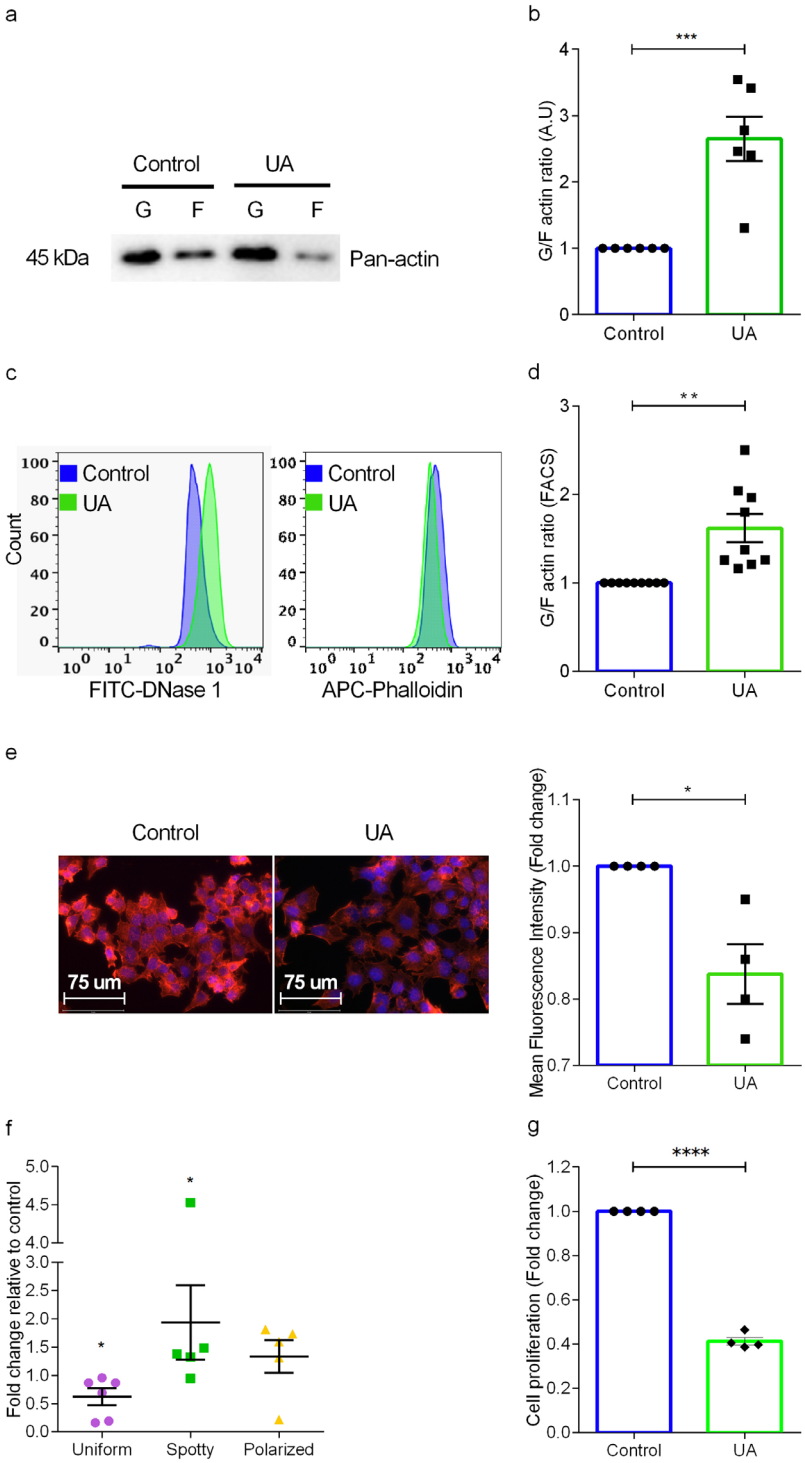


Figure 1. Effect of Urolithin A (UA) on actin polymerization in Ishikawa cells. a) Representative original Western blot of soluble G-actin over filamentous F-actin in human endometrial cancer Ishikawa cells after a 24 h treatment without and with Urolithin A (20 μM). b) Arithmetic means ± SEM (n = 6; arbitrary units) of soluble G-actin over filamentous F-actin ratio in Ishikawa cells after a 24 h treatment with and without Urolithin A (20 μM). c) Representative original histogram of DNAse1 (G-actin; Left) and Phalloidin (F-actin; Right) binding in Ishikawa cells after a 24 h treatment without and with Urolithin A (20 μM). d) Arithmetic means ± SEM (n = 5; arbitrary units) of G-actin over F-actin ratio in Ishikawa cells after a 24 h treatment without and with Urolithin A (20 μM). e) Original immunofluorescence images of eflour660-phalloidin binding to F-actin (red) and DAPI for nuclei (blue) in Ishikawa cells treated with or without Urolithin A and arithmetic means ± SEM (n = 6, 25 individual cells were measured) of actin mean fluorescence intensity in Ishikawa cells with and without Urolithin A treatment. f) Percentage (%) of uniform actin (purple), spotty (green), and polarized (yellow) actin measured using the Image Stream ISx (n = 5–6). g) Cell proliferation was decreased by 24 h Urolithin A (20 μM) treatment (n = 4). Error bars denote SEM of arithmetic means. *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001 using Student's t-test. Samples were compared to the control.

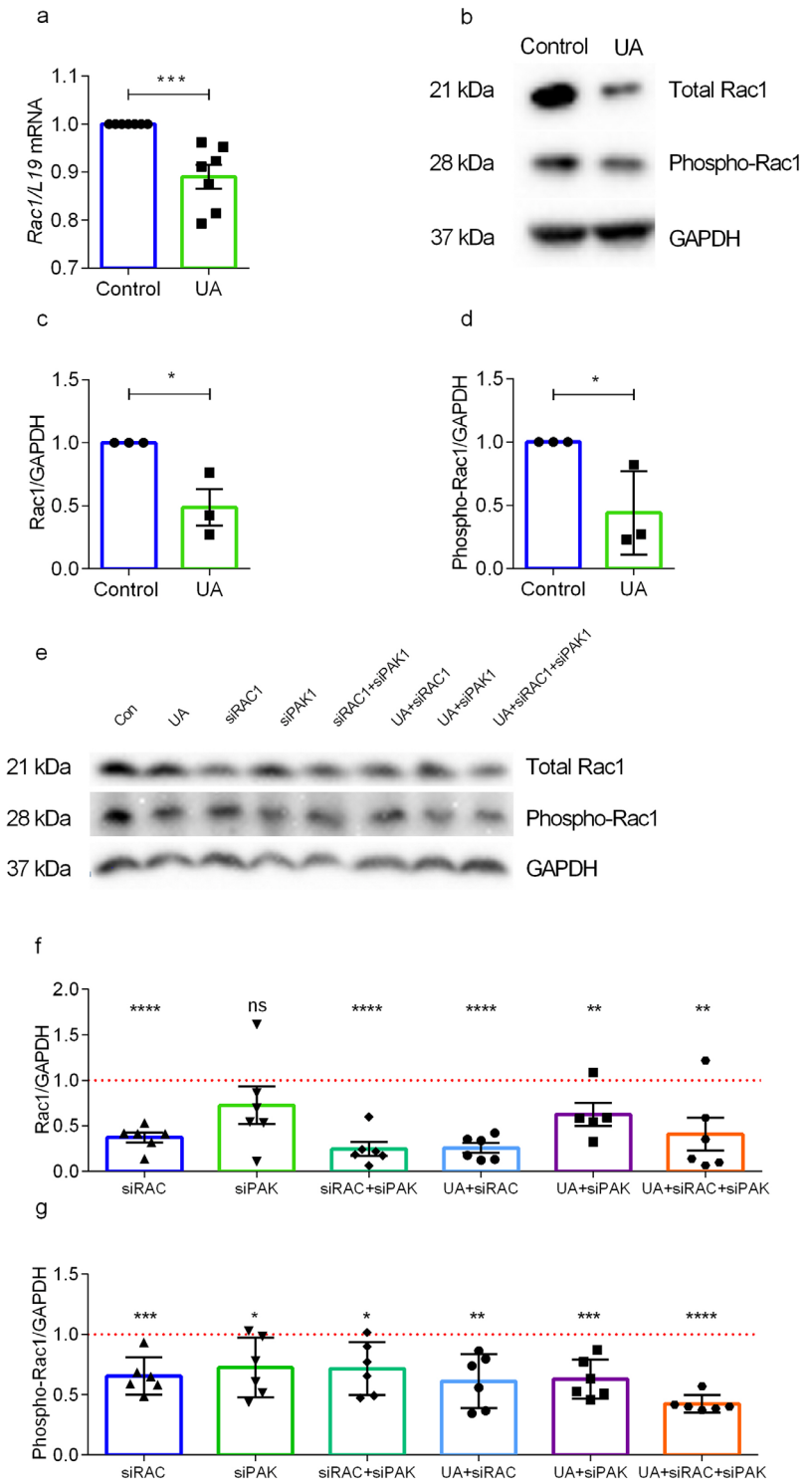


Figure 2. Effect of Urolithin A (UA) on Rac1 transcript and protein levels in Ishikawa cells. a) Arithmetic means ± SEM ($n = 7$) of Rac1 normalized to L19 transcript levels in human endometrial cancer Ishikawa cells following treatment with Urolithin A (20 μM) for 24 h. Data are depicted as fold induction relative to transcript levels of untreated samples. b) Representative original Western blots showing total Rac1 and phospho Rac1 protein abundance in Ishikawa cells after 24 h culture in the absence or presence of Urolithin A (20 μM). c) Arithmetic means ± SEM ($n = 3$, arbitrary units) of Rac1 protein ratio normalized to GAPDH. d) Arithmetic means ± SEM ($n = 3$, arbitrary units) of phospho-Rac1 protein ratio normalized to GAPDH. e) Ishikawa cells were transfected with siRNA targeting Rac1or PAK1 or in combination in the absence or presence of Urolithin A (20 μM). Representative original Western blots of total Rac1 and phospho-Rac1 protein abundance. f and g) Arithmetic means ± SEM ($n = 6$, arbitrary units) of total and phospho-Rac1 protein ratio normalized to GAPDH. Dotted line indicates control. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$ using Student's t-test. Samples were compared to the control.

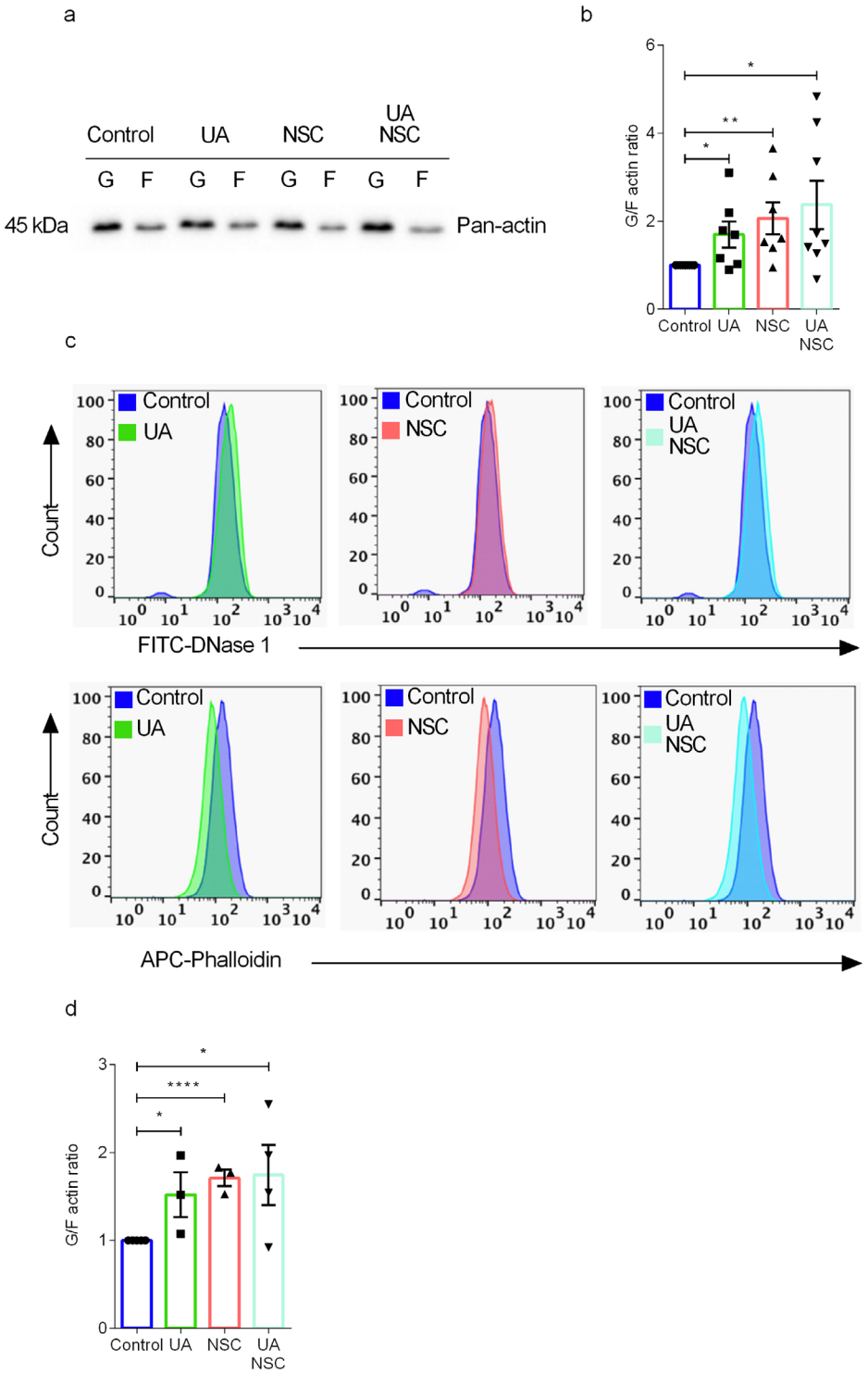


Figure 3. Effect of Urolithin A (UA) on actin polymerization in Ishikawa cells in absence or presence of Rac1 inhibitor. a) Representative Western blot of soluble G-actin over filamentous F-actin in human endometrial cancer Ishikawa cells after a 24 h treatment without and with Urolithin A (20 μM) in the absence and presence of the Rac1 inhibitor NSC23766 trihydrochloride (100 μM). b) Arithmetic means ± SEM ($n = 9$; arbitrary units) of soluble G-actin over filamentous F-actin ratio in Ishikawa cells after a 24 h treatment without and with Urolithin A (20 μM) in the absence and presence of the Rac1 inhibitor NSC23766 trihydrochloride (100 μM). c) Representative original histogram of DNAse1 (G-actin; Upper) and Phalloidin (F-actin; Lower) binding in Ishikawa cells after a 24 h treatment without and with Urolithin A (20 μM) in the absence and presence of the Rac1 inhibitor NSC23766 trihydrochloride (100 μM). d) Arithmetic means ± SEM ($n = 5$ arbitrary units) of G-actin over F-actin ratio in Ishikawa cells after a 24 h treatment without and with Urolithin A (20 μM) in the absence and presence of the Rac1 inhibitor NSC23766 trihydrochloride (100 μM). *$p < 0.05$; **$p < 0.01$; ****$p < 0.0001$ using Student's t-test. Samples were compared to the control.

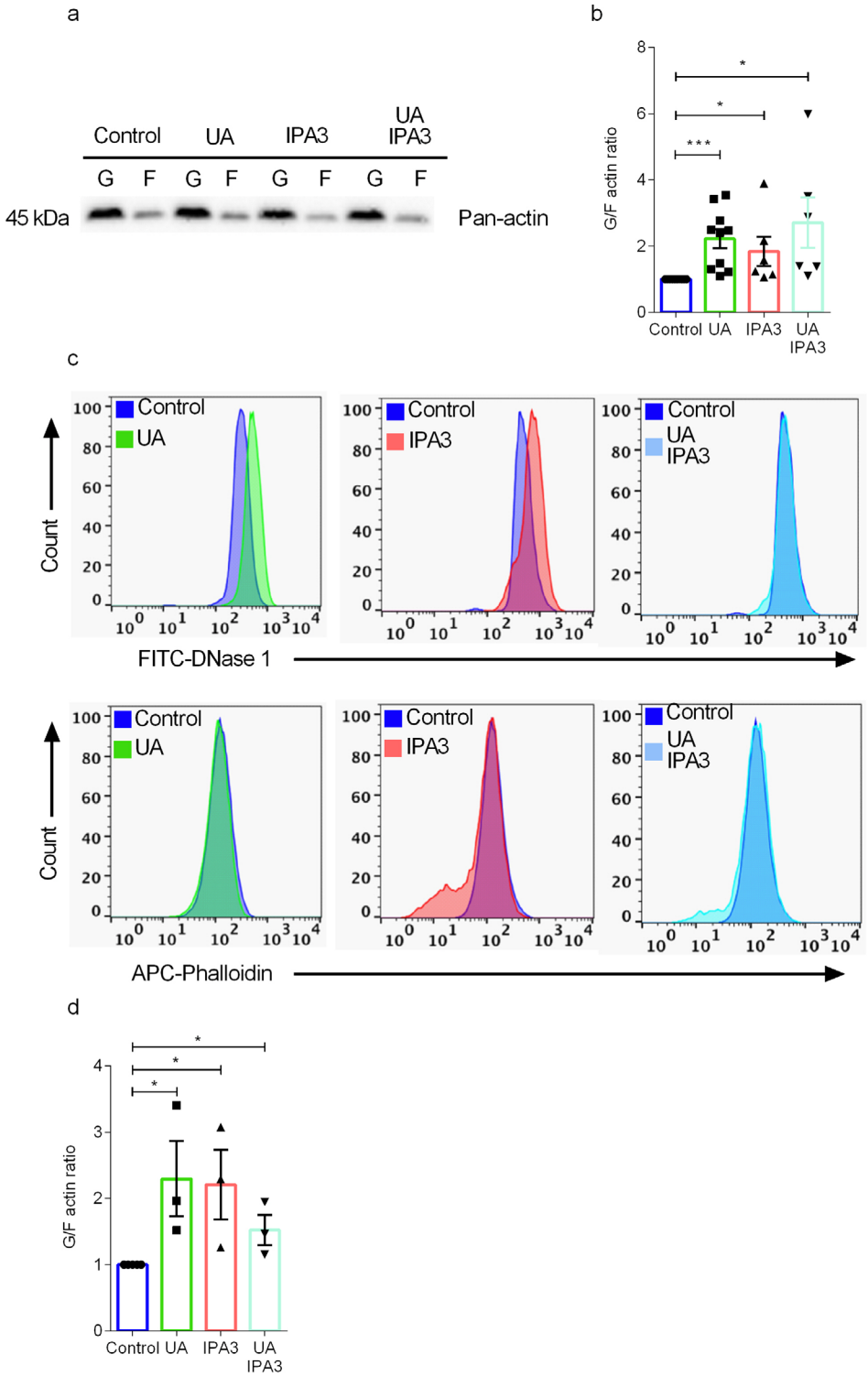


Figure 4. Effect of Urolithin A (UA) on actin polymerization in Ishikawa cells in absence or presence of PAK1 inhibitor. a) Representative Western blot of soluble G-actin over filamentous F-actin in Ishikawa cells after a 24 h treatment without and with Urolithin A (20 μM) in the absence and presence of the PAK1 inhibitor IPA-3 (50 μM). b) Arithmetic means ± SEM ($n = 9$; arbitrary units) of soluble G-actin over filamentous F-actin ratio in Ishikawa cells after a 24 h treatment without and with Urolithin A (20 μM) in the absence and presence of the PAK1 inhibitor IPA-3 (50 μM). c) Representative original histogram of DNAse1 (G-actin; upper) and Phalloidin (F-actin; lower) binding in Ishikawa cells after a 24 h treatment without and with Urolithin A (20 μM) in the absence and presence of the PAK1 inhibitor IPA-3 (50 μM). d) Arithmetic means ± SEM ($n = 5$; arbitrary units) of G-actin over F-actin ratio in Ishikawa cells after a 24 h treatment without and with Urolithin A (20 μM) in the absence and presence of the PAK1 inhibitor IPA-3 (50 μM). *$p < 0.05$; ***$p < 0.001$ using Student's t-test. Samples were compared to the control.

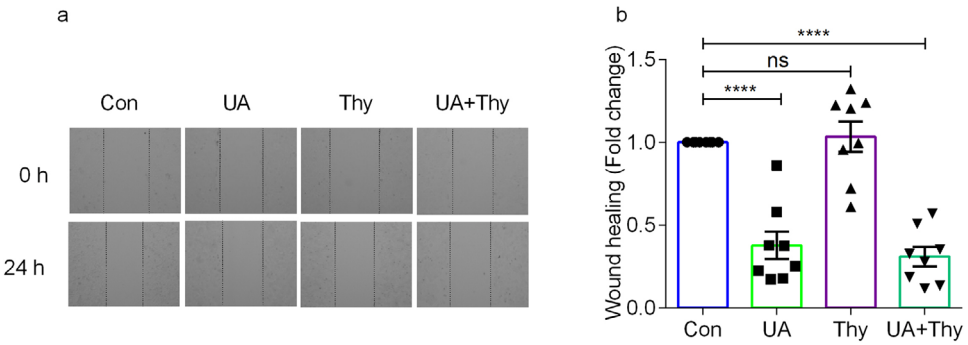


Figure 5. Effect of Urolithin A (UA) on cell migration in Ishikawa cells. a) Representative images of Ishikawa cells treated without and with Urolithin A (20 μM) and thymidine (2 mM) at 0 h (upper panels) and 24 h (lower panels) after scratching. b) Arithmetic means ± SEM ($n = 8$) of fold change of treated compared with treated cells. ****$p < 0.0001$ using Student's t-test. Samples were compared to the control.

confluency, cells were scratched mechanically (200 μL tip) and subsequently treated without or with Urolithin A (20 μM for) or Thymidine (2 mM, inhibitor of proliferation for 42 h), or a combination. The scratch was measured immediately (0 h) and after 24 h. As shown in **Figure** 5a,b, Urolithin A (20 μM) decreased wound healing (Figure 5b; ****$p \leq 0.0001$) significantly and in combination with thymidine (****$p \leq 0.0001$). These results indicate that the reduction of wound healing is due to a reduction in migration rather than proliferation. There was no effect on invasion when using transwell assays (Figure S5, Supporting Information)

3. Discussion

The gut microbiota is an important metabolic and immunological organ that plays a key role in homeostasis.[42] It is now well established that microbiota affects immune signaling, neuronal function, nutrient metabolism, and other physiological functions.[43–46] Since its recognition as an important player in human metabolism, the impact of gut microbiota on ingested natural products has become a subject of extensive studies.[45,46] Plant polyphenols, such as Urolithin A, are being comprehensively investigated as adjuvants for their ability to sensitize drug-resistant cancer cells to the chemotherapy as well as their ability to protect nontarget tissues from damage by their antioxidant and anti-inflammatory properties. The beneficial effects reported are due to the reduction of oxidative stress and inhibition of pro-inflammatory and pro-apoptotic pathways. The present study describes a completely novel function of Urolithin A in downregulating the small G-protein Rac1 and PAK1 with subsequent actin depolymerization as well as decrease of cell proliferation and migration in cancer cells.

Rac1 is known to be a crucial regulator of actin cytoskeleton organization.[47] The downregulation of Rac1 expression and activity following Urolithin A treatment contributes to or even accounts for the observed effect on actin reorganization, which in turn is expected to be followed by a reduction in migration. Rac1 activation is identified to be the main regulator of actin polymerization interacting with the Arp2/3-mediated actin nucleation pathway rather than with the formin-mediated F-actin polymerization.[48–50]

In our study, we observed a Urolithin A-induced decrease of Rac1 (and PAK1) phosphorylation, indicating disruption of these signaling molecules. In keeping, we observed reorganization of the actin filaments toward depolymerization of F-actin filaments. Modification of actin dynamics through depolymerization has been reported to account for filaments of lower mechanical stability,[51,52] our results on cell migration (wound healing) in response to Urolithin A treatment may directly be associated to Urolithin A-induced actin depolymerization and this hypothesis is in line with previous observations.[40,41] PAK1 phosphorylates actin, an effect resulting in actin depolymerization and redistribution of microfilaments.[53] In our observations, we reveal that treatment with Urolithin A reduces both RAC1- and PAK1-activity in even in the presence of an (EGF) activator. Inhibition of these two key molecules is further expected to decrease of lamellipodia formation,[26] which could explain the decrease of migration.

Regulators of actin polymerization include the focal adhesion kinase (FAK)], which activates several signaling molecules including Rac1.[54–58] Particularly, the signaling cascade activated by FAK[59,60] or Rac1[24] further influences NHE1 activity. Thus, the current findings of Rac1 inhibition supports the former observed inhibition of the Na^+/H^+ exchanger by ellagic acid.[20] However, further studies are needed to understand the intricacies and interplay of the molecular mechanism proposed in this research work. Additional experiments are required to rule out an effect of Urolithin A on autophagy and senescence as both these physiological processes influence actin polymerization.

Increasing the efficacy of cytotoxic drugs without compromising nontarget tissue is a critical issue for clinicians. However, the severe side effect (e.g., nephrotoxicity) of cytotoxic drugs and resistance to the treatment limits clinical success. Previous in vitro studies explored the effect of the combination of 5-fluorouracil (5-FU) and Urolithin A. These studies revealed that the co-treatment of 5-FU with Urolithin A resulted in a greater inhibition of cell growth and induction of apoptosis. Further, an in vivo study supported these in vitro findings by showing that Urolithin A could mitigate cisplatin-induced nephrotoxicity in rats by modulating the inflammatory cascade, and by inhibiting the pro-apoptotic pathway in nontarget tissues. It is noteworthy to point out, that chemotherapy can result in (severe) gastrointestinal toxicity, which is associated, at least partly, with the gut microbial imbalance (dysbiosis), perhaps albeit in theory, these changes in the gut microbiota composition could disrupt the ellagic acid bioconversion capacity to Urolithin A. Intriguingly, the possible effects

of chemotherapeutic agents on gut microbiota could be critical in the effectiveness of Urolithins during chemotherapy, further studies are required to support this hypothesis. Thus taken together, a new strategy to enhance the sensitivity of cancer cells to chemotherapy agents by combining them with naturally occurring compounds such as dietary polyphenols that could cause fewer side effects is worth pursuing.

In conclusion, the present study demonstrates that Urolithin A disrupts the expression and function of the small G-protein Rac1 and PAK1 with a consequent effect of depolymerization on the actin filaments and cell migration. Thus, this new role of Urolithin A may provide new avenues to pursue the development of novel cancer therapeutics.

4. Experimental Section

Cell Culture: Ishikawa cells, a well differentiated type 1 endometrial carcinoma cell line (ECACC #99 040 201, Sigma–Aldrich, Germany), Retinal pigment epithelial cells RPE-1 (WT and P53 knock-down, a kind gift from Rene H. Medema, NKI Amsterdam, the Netherlands), Hec1A (type 2 endometrial adenocarcinoma; -#HTB-112 purchased from ATCC), and benign human endometrial stromal cells, HESCs (#T0533 purchased from Applied Biological Materials Inc., Richmond, Canada) were cultured in DMEM/F12 without phenol red media, containing 10% fetal bovine serum (FBS), 1% antibiotic/antimycotic solution, and 0.25% L-glutamine (Invitrogen, Karlsruhe, Germany). Cells were treated for 24 h with Urolithin A (20 µM; SML1791; Sigma–Aldrich), RAC1 inhibitor (100 µM; NSC23766 trihydrochloride; Sigma–Aldrich), PAK1 inhibitor IPA 3 (50 µM; Tocris Bioscience, Germany), with the actin cytoskeleton-disrupting agent latrunculin A (5 µM for 1 h; L5163, Sigma) or EGF (10 ng mL^{-1} for 6 h, #AF-100-15, PeproTech, USA) was used as a positive control.

Transfection with siRNA: Ishikawa cells were plated in six-well plates at a density of 200 000 cells per well in 2% FCS-containing media as described above. Ishikawa cells were transfected with siRNA targeting Rac1 (#L-003560-00-0005, Dharmacon, USA) or PAK1 (#L-003521-00-0005, Dharmacon, USA) or nontargeting (control) both used at 2.75 nM by using VIROMER green (#230 155, Biozym, Germany) according to the manufacturer's protocol. After 24 h, the transfection mix was removed and the cells were either treated with Urolithin A (20 µM) for 24 h. Control cells remained untreated. Cells were collected for downstream analysis.

Quantitative RT-PCR: Total RNA was extracted from Ishikawa cultures using Trizol (Invitrogen) based on a phenol–chloroform extraction protocol. Equal amounts of total RNA (1 µg) were reverse transcribed by using the ThermoScientific Maxima H Minus cDNA Synthesis Master Mix with dsDNase (Invitrogen) for RT-PCR and the resulting cDNA used as template (10 ng) in quantitative RT-PCR analysis. The gene-specific primer pairs were designed using the Primer blast (NCBI) software. L19 was used to normalize for variances in input cDNA. Detection of gene expression was performed with the PowerUp SYBER Green Master Mix (Invitrogen) and quantitative RT-PCR was performed on a QuantStudio 3 Real-Time PCR System (Thermofischer, Karlsruhe, Germany). The expression levels of the samples were expressed as arbitrary units as calculated by the $\Delta\Delta C_t$ method. All measurements were performed in triplicate. Melting curve analysis and agarose gel electrophoresis confirmed amplification specificity. Primer sequences used were as follows: *Rac1* forward (5′-3′): TGCAGACACTTGCTCTCCTATGTAG; reverse (5′-3′): GAGTTCAATGGCAACGCTTCA, *PAK1* forward (5′-3′): GGGAGTTTACGGGAATGCCA, reverse (5′-3′): CCTGCGGGTTTTTCTTCTGC, *L19* forward (5′-3′): GCAGCCGGCGCAAA; reverse (5′-3′): GCGGAAGGGTACAGCCAAT.

Measurement of the G/F Actin Ratio by Triton X-100 Fractionation: To quantify actin polymerization in Ishikawa cells, cells were incubated in 100 µL of Triton X-100 extraction buffer containing 0.3% Triton X-100 (3051, Carl Roth, Germany), 5 mM Tris (T1503, Sigma–Aldrich; pH 7.4), 2 mM EGTA (E3889, Sigma–Aldrich), 300 mM sucrose (4621, Carl Roth), 2 µM phalloidin (P2141, Sigma–Aldrich), 1 mM PMSF, 10 µg mL^{-1} leupeptin (L8511, Sigma–Aldrich), 20 µg mL^{-1} aprotinin (A6279, Sigma–Aldrich), 1 mM sodium orthovanadate (S-6508, Sigma–Aldrich), and 50 mM NaF (S-1504, Sigma–Aldrich) for 5 min on ice. The supernatant containing the soluble proteins was removed by aspiration. The Triton X-insoluble pellet was scraped from the plate directly into 100 µL of RIPA buffer. Any remaining insoluble material was removed by centrifugation. Equal volumes of each fraction were boiled in Laemlli Buffer protein loading buffer at 95 °C for 5 min (as described previously). Proteins were separated on 12% SDS-polyacrylamide gels and transferred to PVDF membranes. Nonspecific binding sites were blocked by 1 h incubation with 5% nonfat dry milk in Tris-buffered saline with 1% Tween (TBS-T; 130 mmol L^{-1} NaCl, 20 mmol L^{-1} Tris [pH 7.6], and 1% Tween). The membranes were incubated overnight at 4 °C with monoclonal anti-pan actin antibody (HRP conjugated; 1:1000, #12 748, Cell Signalling, Frankfurt, Germany). Antibody binding was detected with the ECL Chemiluminescent Substrate Reagent Kit (WesternBright ECL, Advansta, USA) and bands were visualized with the iBright Imaging System (Thermofischer) and then quantified using ImageJ software.

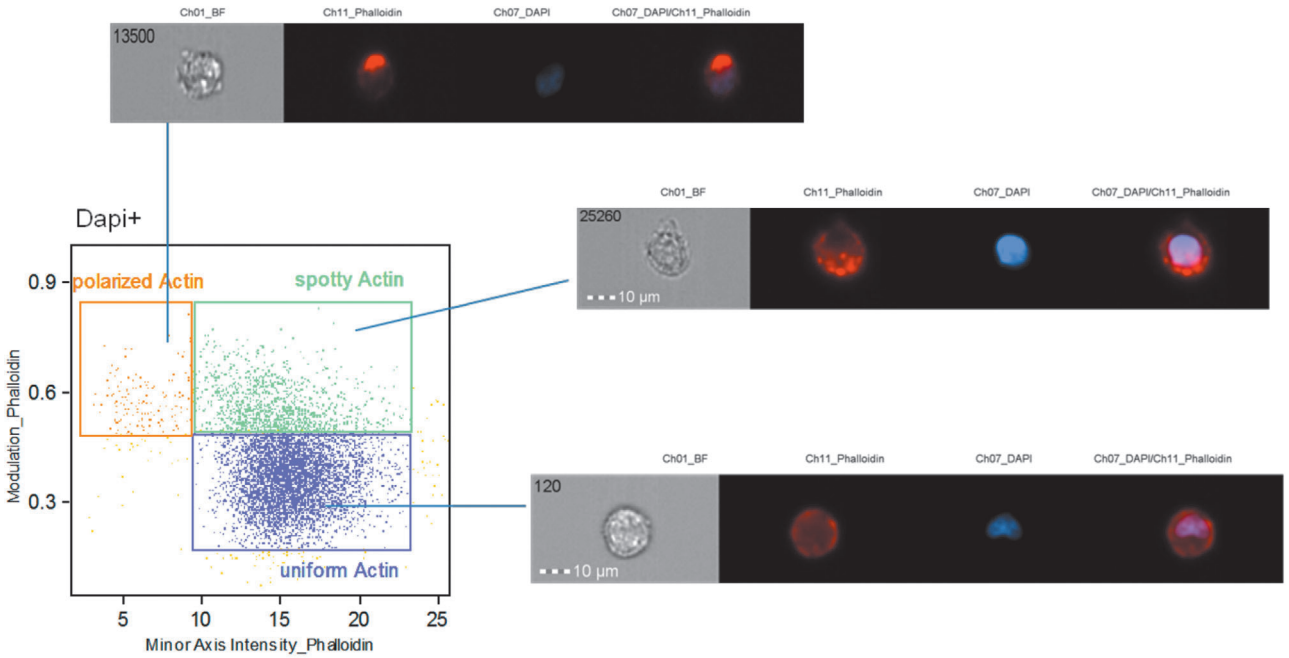
Western Blotting: Whole cell protein extracts were prepared by lysing cells in Laemmli buffer. Equal amounts of protein were separated by 10% SDS-PAGE before wet-transfer onto PVDF (Amersham Biosciences, UK). Nonspecific binding sites were blocked for 1 h at room temperature in with 5% nonfat dry milk in TBS-T. Membranes were probed overnight at 4 °C with an antibody against RAC1 #2465p, phospho RAC1 #2461p, PAK1 #2694, phospho PAK1 #2601s, and GAPDH #2118L (all antibodies were purchased from Cell Signalling, Leiden, The Netherlands). All primary antibodies were used at 1:1000, washed three times with TBS-T, followed by incubation with HRP-conjugated anti-rabbit secondary antibody (1:2000, 7074s, Cell Signalling). Protein complexes were visualized with a chemiluminescent detection kit (WesternBright ECL), and bands were visualized with the iBright Imaging System (Thermofischer) and then quantified using ImageJ software.

G/F Actin Ratio by Flow Cytometry: Ishikawa cells (1.0×10^5 cells) were fixed with 4% paraformaldehyde (PFA) and then permeabilized with 1× permeabilization buffer (eBioscience, Frankfurt, Germany) and subsequently stained with 1.0 µL of fluorescent DNAse1-Alexaflour-488 (50 mg mL^{-1}, #D12371) for detection of G-actin, 1.0 µL of fluorescent Phalloidin-eFluor® 660 (1×, #50 655 905) (eBioscience, Thermofischer, Germany) for detection of F-actin and 1.0 µg mL^{-1} DAPI (Invitrogen) for detection of nuclei. The abundance of the respective labels was measured using green (FL-1) and red channel (FL-4) on a FACSCalibur (BD Biosciences, Heidelberg, Germany).Analysis was performed using Flowjo software (Flowjo LLC, Oregon, USA). G- and F-actin geometric mean values were determined from the respective fluorescence and the ratio of G/F calculated from the geometric mean values.

Multispectral Imaging Flow Cytometry: Briefly, Ishikawa cells (1.0×10^5 cells) were fixed with 4% paraformaldehyde (PFA) and then permeabilized with 1× permeabilization buffer (eBioscience, Frankfurt, Germany) and subsequently stained with 1.0 µL of fluorescent DNAse1-Alexaflour-488 (50 mg mL^{-1}) for detection of G-actin, 1.0 µL of fluorescent Phalloidin-eFluor 660 (1×; eBioscience, Frankfurt, Germany) for detection of F-actin and 1.0 µg mL^{-1} DAPI (Invitrogen) for detection of nuclei. The abundance of the respective labels was measured using blue (DAPI; Nuclei; FL-1) and red channel (Phallodin; F-actin; FL-4) and bright field images (FL-1). Cells were acquired on an ImageStreamX imaging flow cytometer (Amnis) using a 488 nm laser set at 80 mW. A minimum of 2000 cells were acquired per sample at 60× magnification at a flow rate ranging between 25 and 50 cells s^{-1}. At this magnification, lateral resolution was ≈250 nm, whereas the depth of the optical slice was ≈4 mm.[61] Analysis was performed using the IDEAS software (6.0; Amnis). A compensation table was generated using the compensation macro built in the software and applied to the single staining controls. Proper compensation was then verified by visualizing samples in bivariate fluorescence intensity plots. A template analysis file to gate for single optimally focused cells was prepared and applied to the experimental samples in order to export this population to a new compensated image file to allow merging all experimental samples within a single file for direct sample analysis. The

peak mask was used to detect individual spots by setting a S/N ratio of 8. Then, the number of intensities was calculated using the feature spot count on the previously computed mask using the INSPIRE Instrument controller software. Uniform actin was defined as equal distribution of actin throughout the cell (purple). Spotty actin was defined as actin that was distributed in a discontinuous manner ("spotty" or "patchy") (green). Polarized actin was defined as actin found in one location (yellow). For data analysis, IDEAS software, version 6.2 was used.

Subsequently, the cells were trypsinsised and then seeded (1×10^5) on to six-well–transwell (8 µm transwell migration chamber, #3428, Costar, USA) using serum free media. The transwell inserts were then transferred to a six-well plate containing medium with thymidine and UA+thymidine in (2 mL) 10% FBS DMEM/F12. Cell invasion was allowed to proceed for 24 h at 37 °C in a tissue culture incubator. Post incubation cells were removed from the upper surface of the transwell with a cotton swab and cells that migrated to the lower surface were washed with methanol and



Whole cell: Channel 1_Bright field (Grey; Ch01_BF)
Actin: Channel 11_Phallodin (Red;Ch11_Phalloidin)
Nuclei: Channel 07_DAPI (Blue;Ch01_DAPI)
Overlay: Channel 07_DAPI_Ch11_Phallodin

Immunofluorescence: Ishikawa cells grown on chamber slides were fixed for 15 min with 4% paraformaldehyde, washed with PBS, and permeabilized for 10 min in 0.1% Triton X-100/PBS. The slides were blocked with 5% BSA in 0.1% Triton X-100/PBS for 1 h at RT. Actin was stained with eflour660-phalloidin (1:1000,# 50 655 905) for 1 h at room temperature. The slides were mounted with ProLong Gold antifade reagent with DAPI (#P36931,Invitrogen). Microscopy was performed with an EVOS M7000 cell imaging system (Thermofischer) with an Apochromat 0.4 NA cover slip corrected 40× objective. The mean fluorescence intensity from six related cells of each picture was quantified by ImageJ. Scale bar was 75 µm.

Wound Healing Assay: Ishikawa cells were seeded in six-well plate (200×10^3 cells per well). After reaching 100% confluency, the wells were scratched with a 200 µL tip. Cells were treated with either Urolithin A (20 µM) for 24 h or thymidine (2 mM; #T1895, Sigma) for 42 h or remained untreated. Pictures were then taken immediately (0 h) using the EVOS M7000 cell imaging system with a 2× objective (Olympus, Hamburg, Germany). Pictures were taken after 24 h. Assessment of wound healing was quantified using the ImageJ software and presented as fold change.

Cell Proliferation assay: Ishikawa cells were plated in 96-well plate (2000 cells per well). Cells were treated with Urolithin A (20 µM) or remained untreated. After 24 h cells were incubated with 5 µL of CellTiter 96 Aqueous One Solution (#G3580, Promega, Mannheim, Germany) reagent for 1 h at 37 °C. Absorbance at 492 nm for each well was measured on a Tecan Sunrise Microplate Reader Remote (TECAN, Germany). The OD values were normalized to the control. Data are respresented as fold change.

Transwell Assay (Invasion Assay): Ishikawa cells treated with thymidine (2 mM) and UA (20 µM) + thymidine or left untreated as described above. air dried. The dried membrane was stained with 0.5% crystal violet for 1 h and images were taken using the EVOSM7000 cell imaging system with a 2× objective (Olympus, Hamburg, Germany).

Statistics: Data are provided as means ± SEM, *n* represents the number of independent experiments. All data were tested for significance using Student's unpaired two-tailed *t*-test. Samples were compared to the control. Results with $p < 0.05$ were considered statistically significant. Figures and statistical analysis were made in Graphpad Prism (CA, USA).

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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Conflict of Interest

The authors declare no conflict of interest.

Author Contributions

M.A. and T.O. contributed equally to this work and thus share first authorship. M.S.S. was associated with conception and design of experiments. M.A, T.O, S.K, J.R, S.P, Y.S, and A.K performed experiments, acquisition of data, or analysis/interpretation of data, and provided resources. S.Y.B, D.W, S.T, A.K., and M.S.S. provided resources and funding acquisition. M.S.S. wrote the original draft. All authors have reviewed the manuscript and have approved of its submission.

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3. Results and Discussion

3.1. Study 1. “Annexin A7 Regulates Endometrial Receptivity (Alauddin et al., 2020b)”

Early pregnancy complications, including implantation failure are common and cause significant physical and psychological morbidity to couples (Lucas et al., 2016; Quenby et al., 2021). The endometrial glands, stroma, and luminal epithelium express ANXA7, according to the Human Protein Atlas (Uhlén et al., 2005). To estimate the role of ANXA7 herein, the cycle-dependence of *ANXA7* mRNA abundance in human endometrium was assessed by querying the GEO Profiles database (ID: 24464767, https://www.ncbi.nlm.nih.gov/geoprofiles/24464767, date of the query: 09.01.2018). Interestingly, endometrial *ANXA7* transcript levels increased from the proliferative to the early secretory phase of the cycle (Fig. 1A in (Alauddin et al., 2020b)), suggesting a role of ANXA7 in decidualization.

To investigate the role of ANAX7 in decidualization, primary human endometrial stromal cells (HESCs) were decidualized with 0.5 M 8-bromo-cAMP and 1 M medroxyprogesterone 17-acetate (MPA) for a total of 8 days and the expression of ANXA7 was studied at both the mRNA and protein levels. ANXA7 mRNA and protein levels remained low during the early pro-inflammatory decidual phase before rising around days 6–8, coinciding with the development of specialized decidual cells, according to independent primary cultures (Fig. 1B, C and Suppl. Fig. S1 in (Alauddin et al., 2020b)).

The classical marker genes prolactin (*PRL*) and insulin-like growth factor binding protein 1 (*IGFBP1*) are commonly utilized to determine the quality of the HESCs' decidual response (Saleh et al., 2011). To see if ANXA7 affects the expression of these decidual marker genes, primary HESCs were transfected with non-targeting (NT) or siRNA targeting *ANXA7*, then decidualized for 6 days with cAMP and MPA (CM). As observed in Fig. 2A, B and Suppl. Fig. S2, in

(Alauddin et al., 2020b), siRNA-mediated knockdown lowered ANXA7 expression at both mRNA, and protein level by 85% percent and 87%, respectively. In decidualizing cultures, *ANXA7* knockdown reduced *PRL* and *IGFBP1* mRNA levels as well as secreted PRL levels (Fig. 2C-E in (Alauddin et al., 2020b)). In cells targeted with *ANXA7* siRNA, the actin cytoskeleton alterations associated with decidualization were also reduced in phalloidin staining of filamentous actin (F-actin) as compared to NT cultures (Fig. 2F in (Alauddin et al., 2020b)).

Altered endometrial COX2 activity has been associated with decreased fertility following several disorders (Vilella et al., 2013a). Embryo development, hatching, and implantation are significantly influenced by PGE_2 (Niringiyumukiza et al., 2018). Many implantation abnormalities are observed in mouse models due to deficiencies in cytosolic PLA_2, COX2, or the use of prostaglandin inhibitors (Psychoyos et al., 1995; Salleh, 2014). To further study the effect of *ANXA7* knockdown on COX2 and PGE_2, cultured HESCs were transfected with non-targeting (NT) or *ANXA7*-targeting siRNA and then decidualized for 6 days with CM. qRT-PCR analysis revealed that silencing of *ANXA7* raised *COX2* mRNA abundance (Fig. 3A in (Alauddin et al., 2020b)) when compared to the non-targeting control. In parallel, the deletion of *ANXA7* resulted in a significant increase in COX2 protein levels, according to western blot data (Fig. 3B and Suppl. Fig. S3 in (Alauddin et al., 2020b)). Moreover, PGE_2 levels increased dramatically when ANXA7 was downregulated as determined in cell culture supernatants by ELISA (Fig. 3C in (Alauddin et al., 2020b)).

Due to its role in prostaglandin formation, ANXA7 could also be a potential regulator of endometrial receptivity. As a matter of fact, publicly accessible microarray data profiling gene expression in the mouse uterine luminal epithelium during implantation (GEO ID: GSE44451), suggested that *ANXA7* levels in mice significantly increased as they transitioned from the receptive to the refractory phase (Suppl. Fig. S4 in (Alauddin et al., 2020b)).

Some literature has already reported that progesterone receptor (*Pr*), indian hedgehog (*Ihh*), bone morphogenetic protein 2 (*Bmp2*) and heparin binding epidermal growth factor (*Hb-egf*) play a role in uterine receptivity and implantation (Wang and Dey, 2006). To assess this issue, pseudopregnancy was provoked in *AnxA7*$^{-/-}$ and WT (*AnxA7*$^{+/+}$) female mice to define the extent of modulating endometrial receptivity by ANAX7. To this end, total RNA was isolated from uterine horns obtained 5.0 days following coitus. The number of oocytes in both groups was the same, excluding "hyper-ovulation". Several murine endometrial receptivity genes (*Bmp2, Hb-egf, Ihh,* and *Cox2*) were up-regulated in the *AnxA7*$^{-/-}$ female mice, as shown in Fig. 4A (Alauddin et al., 2020b). Although the difference was not statistically significant, the decidualization marker prolactin family 8 subfamily a member 2 (Prl8a2) was found to be lower in *AnxA7*$^{-/-}$ female as compared to the *AnxA7*$^{+/+}$ mice. These findings suggest a functional significance of AnxA7 in regulating the implantation window in a mouse model of pregnancy.

We further assessed how the loss of AnxA7 affects the number of implantation events and the size of the litter. Knockout animals had significantly more implantation sites (Fig. 4B) and larger litter numbers than WT mice (Fig. 4C in (Alauddin et al., 2020b)).

To estimate to which extent these *in vitro* and preclinical *in vivo* data might be translated into the clinical situation, we compared midluteal endometrial RNA-sequencing and clinical data (GEO Profiles ID: GSE65102) from 10 subfertile patients and 10 women with a history of recurrent pregnancy loss (RPL). In particular, *ANXA7* mRNA abundance and endometrial receptivity were analysed. The data suggested that RPL is linked to a longer implantation window, out-of-phase implantation, and increased receptivity (super-fertility), which is defined by a quick time to pregnancy (Lucas et al., 2020; Salker et al., 2012; Teklenburg et al., 2010; Ticconi et al., 2020). Moreover, endometrial *ANXA7* transcript levels were higher in subfertile patients as compared to RPL participants, as seen in Fig. 4D and Suppl. Tab. S1 in (Alauddin et al., 2020b).

Western blot analysis confirmed the difference in endometrial ANXA7 protein expression between both patient groups (Fig. 4E, Suppl. Fig. S5, and Suppl. Tab. S2 in (Alauddin et al., 2020b)).

In studies conducted by our laboratory and by others (Gellersen and Brosens, 2014; Salker et al., 2012), ANXA7-repressed prolactin expression has been linked to a defective decidual as well as to a protracted and highly disorganized pro-inflammatory response. According to new data, aberrant decidualization disrupts the "embryoselectivity-checkpoint," rendering the endometrium very favourable to implantation (super-fertile or super-receptive) but is unable to retain the conceptus, resulting in pregnancy loss (Brosens et al., 2014; Macklon and Brosens, 2014; Sandra et al., 2011; Teklenburg et al., 2010). On the other hand, ANXA7 has been identified in the present study to downregulate the COX2-PGE_2 axis. Since COX2 appears transiently in stromal cells supporting implantation in mice during implantation, it is tempting to speculate that ANXA7 absence is associated with higher COX2 levels in the endometrium that generate a milieu conducive to embryo implantation through a coordinated increase in pro-implantation genes. Combined, our findings suggest that ANXA7 deficiency impairs decidualization but promotes an implantation-friendly environment. Since, RPL sufferers are more fertile than healthy women, meaning they are more receptive to the implanting embryo (Ticconi et al., 2020). ANXA7 activity might be a new molecular determinant of endometrial receptivity.

To translate our findings into the treatment of RPL sufferers, the molecular mechanisms and the involved cell types of ANXA7-regulated endometrial receptivity and/or decidualization must be analysed in future studies. To this end, uterine PGE_2 concentration, as well as immunohistochemistry and transcriptome analysis of the endometrium might be compared between *AnxA7*$^{-/-}$ and wildtype mice during endometrial decidualization and embryo implantation. Moreover, pharmacological targeting of PLA2 or COX2 in this mouse model might bring first hints about the “druggability” of the putative

ANXA7/COX2/PGE_2 pathway and further COX2/PGE_2-regulating pathways in the endometrium.

Like ANXA7, the endometrial bleeding-associated factor LEFTY2, a member of the transforming growth factor-beta family has been shown to be differentially expressed during the menstrual cycle and to regulate endometrial receptivity (Gellersen and Brosens, 2014; Tabibzadeh, 2005). In particular, LEFTY2 inhibits, in normal endometrial stromal cells expression of receptivity genes such as COX2. Upregulation of LEFTY2 in decidualizing endometrial stromal cells during the menstrual cycle reportedly occurs in the late luteal phase where proposed to contribute to closure of the implantation window via inhibition of store-operated Ca^{2+} release and subsequent downregulation of Ca^{2+}-regulated receptivity genes (Salker et al., 2018).

On the other hand, LEFTY2 factor has been demonstrated to control epithelial-to-mesenchymal transition (Gao et al., 2018) and migration (Alowayed et al., 2016; Gao et al., 2018; Salker et al., 2016) of endometrial cancer cells. The next study addressed a potential further oncogenic function of LEFTY2.

3.2. Study 2. "LEFTY2/endometrial bleeding-associated factor up-regulates Na^+ Coupled Glucose Transporter SGLT1 expression and Glycogen Accumulation in Endometrial Cancer Cells (Zeng et al., 2020)"

Here, we tested the effect of LEFTY2 on glucose fuelling of human endometrial adenocarcinoma cell lines (Ishikawa and HEC1a) *in vitro*. The Na^+ coupled glucose transporter-1 (SGLT1) allows endometrial cancer cells to accumulate glucose even against a high chemical gradient. Therefore, the effect of LEFTY2 on SGLT1 and glucose uptake was investigated. To this end, Ishikawa cells and HEC1a cells were left untreated or stimulated with LEFTY2 (25 ng/ml) for 48 hours, and the expression of SGLT1 at the mRNA and protein levels were investigated. As seen in Fig. 1A, B in (Zeng et al., 2020) LEFTY2 dramatically

increased SGLT1 mRNA and protein abundances in Ishikawa cells and HEC1a cells ((Fig. 1C, D) in (Zeng et al., 2020)). LEFTY2, on the other hand, was unable to raise SGLT1 levels in benign endometrial cells (HESCs) (Suppl. Fig. S1A, B in (Zeng et al., 2020)).

Especially glycogen, which is stored in the endometrium and is considered to be a crucial source of energy in the first trimester (Dean et al., 2014) has been found to be affected by SGLT1 loss. As the endometrial glycogen stores are required for pregnancy and interference of this histotrophic pathway, SGLT1 loss results in an unexpected pregnancy outcome and miscarriage in both mice and humans (Salker et al., 2017). Another positive regulator of cellular glycogen storage is GYS1 (Dent et al., 1990).

In the next series of experiments, Ishikawa and HEC1a cells were treated as described above with or without LEFTY2 (25 ng/ml) for 48 hours, and one hour before the end of the experiment, the cells were incubated with the fluorescent glucose analogon 2-[N(7-nitrobenz-2-oxa-1,3-diaxol-4-yl)amino]-2-deoxyglucose (2-NBDG). Flow cytometry experiments suggested a LEFTY2-induced increase in cellular 2-NBDG (glucose) uptake in Ishikawa, but not in HEC1a cells, as shown in Fig. 2A, B in (Zeng et al., 2020).

Next, we measured glycogen synthase-1 (GYS1) transcript levels in both cell lines following stimulation with LEFTY2 (0 or 25 ng/ml for 48 hours). As a result, the abundance of *GYS1* mRNA was greatly elevated following LEFTY2 treatment in both cell lines, as demonstrated by Suppl. Fig. S2A, B in (Zeng et al., 2020).

Phosphorylation at numerous locations inactivates GYS1, while dephosphorylation activates it (Dent et al., 1990). Since the rate-limiting step in glycogen production is catalysed by GYS1, Ishikawa and HEC1a cells were treated with 0 or 25 ng/ml of LEFTY2 for 48 hours to evaluate GYS1 activity at the protein level. As a result, LEFTY2 induced an increase in total GYS1 protein

abundance in both, Ishikawa (Fig. 3A, B) and HEC1a cells (Fig. 3C, D) in (Zeng et al., 2020). Furthermore, when compared to the control, LEFTY2 treatment resulted in a decrease in the ratio between the phosphorylated-(Ser641)-GYS1 and the unphosphorylated GYS1 protein, indicating LEFTY2-associated GYS1 activity and glycogen synthesis in Ishikawa cells. This, in contrast, was neither observed in HEC1a (Fig. 3C, D) nor in benign endometrial cells (Suppl. Fig. S1B in (Zeng et al., 2020)). Accordingly, only in Ishikawa cells, a 48 hours treatment with LEFTY2 (0 or 25 ng/ml) resulted in a significant increase in cellular glycogen content (Fig. 4A, B in (Zeng et al., 2020)) as determined by ELISA. Surprisingly, TGF-β (10 ng/ml), prevented LEFTY2-induced glycogen accumulation in Ishikawa cells.

To further define TGF-β-induced suppression of LEFTY2-induced glycogen synthesis, Ishikawa and HEC1a cells were pre-treated with TGF-β (10 ng/ml) for 24 hours before being post-incubated with LEFTY2 and TGF-β for another 48 hours. In both Ishikawa (Fig. 3A, B and Suppl. Fig. S2A) and HEC1a cells (Fig. 3C, D and Suppl. Fig. S2B in (Zeng et al., 2020)), treatment with TGF-β or in combination with LEFTY2 decreased SGLT1, GYS1, and pGYS1 (Ser641) protein abundances.

Combined, our data suggest that LEFTY2 may foster glucose uptake and glycogen synthesis in endometrial cancer cells in a TGF-β-regulated manner. Future studies have to quantify the tumour-regulating capacity of LEFTY2. Initial experiments might analyse in several endometrial carcinoma cell lines the effect of LEFTY2 alone or in combination with ionizing radiation or chemotherapy on cell death, clonogenic survival, and proliferation of the cells. Knock-down of SGLT1 or GYS1 might define their roles herein. Moreover, as fuelling glycogen synthesis should lower ATP synthesis by anaerobic glycolysis, the effect of LEFTY2 on adaptation to tumour hypoxia might be determined. Such LEFTY2 effect might be clinically relevant since mal-perfusion and hypoxia of solid tumours are major obstacles of radiation- and chemotherapy.

Beyond remodelling of the metabolism which comprises among others a switch from oxidative phosphorylation to aerobic lactic acid fermentation, increase in glucose uptake and storage, carcinoma cells are characterized by upregulated migration and tissue invasion especially upon epithelial-to-mesenchymal transition. Since actin cytoskeleton dynamics accompanies cell migration, we analysed in the next study the effect of an actin destabilizing metabolite from gut bacteria on the migration of endometrial carcinoma cells.

3.3. Study 3. "Gut Bacterial Metabolite Urolithin A Decreases Actin Polymerization and Migration in Cancer Cells (Alauddin et al., 2020a)"

Reportedly, the Urolithin A precursor ellagic acid controls the activity and expression of the Na^+/H^+ exchanger NHE1 in the plasma membrane (Abdelazeem et al., 2017). NHE1, in turn, aids the localization of actin and stabilizes the cytoskeleton (Salker et al., 2016). We, therefore, hypothesized that NHE1 inhibition might interfere with the cytoskeleton required to preserve cell structure. To test for such a possible interference of Urolithin A with actin cytoskeleton, Ishikawa cells were treated with Urolithin A (0 or 20 µM for up to 24 hours) and kinetics of actin de-polymerization was analysed by western blotting and flow cytometry. As a result, Urolithin A increased the ratio between soluble globular actin (G-actin) and filamentous F-actin (Fig. 1A-D in (Alauddin et al., 2020a)) indicating actin depolymerization.

The action of Urolithin A (0 or 20 µM for 24 hours) on actin dynamics was further characterized by immunofluorescence microscopy using the cytoskeletal disruptor Latrunculin A (Suppl. Fig. S1 in (Alauddin et al., 2020a)) as a positive control. Like Latrunculin A, Urolithin A induced a decrease of F-actin as determined by eflour660-phalloidin binding (Fig. 1E in (Alauddin et al., 2020a)). In particular, multispectral imaging flow cytometry revealed that Urolithin A decreased in Ishikawa endometrial carcinoma cells uniformly distributed and

increased spotty or patchy distributed cellular actin while having no effect on polarized actin (Fig. 1F in (Alauddin et al., 2020a)).

To confirm the Urolithin A effect on actin depolymerization in further cell models, HESCs (benign endometrial stromal cells), HEC1a, and retinal pigment epithelial RPE-1 (wild-type and p53 mutant) cells were incubated with Urolithin A (0 or 20 µM for 24 hours) and the G actin/F actin ratio was determined by flow cytometry. As shown by Suppl. Fig. S2 in (Alauddin et al., 2020a), Urolithin A induced actin depolymerization in HEC1a and p53-mutant RPE but not in RPE p53-wildtype and HESCs. Since Ishikawa (https://web.expasy.org/cellosaurus/CVCL_2529) and HEC1a (https://web.expasy.org/cellosaurus/CVCL_0293) are both p53-mutated, one might speculate that p53-mediated signalling counteracts Urolithin A induced actin depolymerization.

Cell motility and cell division strongly depend on actin dynamics suggesting that Urolithin A interfere with basic cell biological processes. As a matter of fact, Urolithin A profoundly impaired cell proliferation of Ishikawa cells (Fig. 1G in (Alauddin et al., 2020a)). Lower mechanical stability of filaments has been attributed to depolymerization of actin, which has been reported to alter actin dynamics (Stournaras et al., 1996; Tsapara et al., 1999). Its inhibition has also been anticipated to reduce the formation of lamellipodia, (Nobes and Hall, 1995) which might suggest a Urolithin A effect on cell motility. To define the effect of Urolithin A-mediated actin depolymerization on cell migration, confluent Ishikawa cells pre-treated (0 or 2 mM for 42 hours) with the proliferation inhibitor thymidine were post-incubated (0 or 20 µM for 24 hours) with Urolithin A prior to mechanically scratching the monolayer (200 µL pipette tip) and analysing wound healing 0 and 24 hours after scratching. As depicted in Fig. 5A, B in (Alauddin et al., 2020a), Urolithin A significantly reduced wound healing in proliferation-locked Ishikawa cells indicative of a Urolithin A-mediated inhibition of cell motility. Transwell invasion assays performed in parallel, however, did

not show any Urolithin A effects in Ishikawa cells (Suppl. Fig. S5 in (Alauddin et al., 2020a)).

To decipher the signalling underlying the cellular Urolithin A effects on the actin depolymerization in Ishikawa cells, expression and activity of Rac1 was examined in dependence on Urolithin A treatment (0 or 20 µM for 24 hours). The data suggest that Urolithin A decreased L19-normalized *Rac1* transcript levels as well as total and phospho-Rac1 protein abundances (Fig. 2A-D in (Alauddin et al., 2020a)).

Rac1 reportedly activates PAK1 by phosphorylating it, which reportedly controls actin network (Salker et al., 2016; Zeng et al., 2016). Urolithin A (20 µM for 24 hours) significantly decreased the amounts of total and phospho PAK1 mRNA and protein (Suppl. Fig. S4A-D in (Alauddin et al., 2020a)). Most importantly, Rac1 inhibitor NSC23766 trihydrochloride (100 µM for 24 hours, Fig. 3 in (Alauddin et al., 2020a)) or PAK1 inhibitor IPA-3 (50 µM for 24 hours, Fig 4. in (Alauddin et al., 2020a)) mimicked the Urolithin A effect on actin depolymerization as demonstrated by western blotting and flow cytometry. Of note, combined treatment of Urolithin A and NSC23766 trihydrochloride or IPA-3 did not exert additive effects strongly suggesting that Urolithin A acts via the Rac1/PAK1 pathway on actin dynamics.

In the future, preclinical animal studies might confirm *in-vivo* a Urolithin A-induced impairment of metastasis in endometrial carcinoma. Since Urolithin A has been demonstrated to sensitize 5-fluorouracil (5FU)-resistant colon carcinoma cells to 5FU (Ghosh et al., 2022), further work might screen in various endometrial carcinoma models for Urolithin A effects that are additive or synergistic to radio- and/or chemotherapy protocols. Finally, the function of urolithins other than Urolithin A on the Rac1/PAK1 pathway and on actin dynamics might be investigated in endometrial carcinoma cells in order to find the most potent tumoricidal plant polyphenol.

Taken together, our *in vitro* data of the third study suggests that Urolithin A may impair proliferation and migration of endometrial carcinoma cells. This might hint to a potential endometrial cancer-inhibiting action of ellagic acid-rich diets warranting further research on this topic.

4. Summary

In adolescents and young adult women with endometrial cancer, maintaining fertility is a crucial aspect in preserving a high quality of life. This cumulative thesis summarizes three published studies on the biology of endometrial cancer and endometrial receptivity. A deeper understanding of both may facilitate the development of a fertility-sparing treatment of endometrial cancer.

The first study (Alauddin et al., 2020b) describes how annexin A7 (ANXA7), a phospholipid binding protein, affects fertility and embryo implantation. In this study, we performed *in silico* analysis of transcriptome data, *in vivo* experiments on *AnxA7*$^{-/-}$ and WT (*AnxA7*$^{+/+}$) mice as well as *in vitro* measurements using human endometrial stromal cells. As a result, endometrial tissue expressed ANXA7 that rose in a time-dependent manner following decidualization of human endometrial stromal cells (HESCs). Silencing of *ANXA7* reduces expression of canonical decidual marker genes, while increasing cyclooxygenase 2 and prostaglandin E_2 levels. Moreover, number of implantation sites and litter sizes significantly increased in mice with genetic knockout of *AnxA7*. Furthermore, the study of human endometrial biopsies revealed that ANXA7 mRNA and protein levels are lower in women with recurrent pregnancy loss (RPL) during the midluteal window of implantation compared to subfertile individuals. In summary, our data suggests critical roles of ANXA7 in the regulation of endometrial receptivity and implantation.

The second study (Zeng et al., 2020) investigated whether the endometrial bleeding related factor LEFTY2 physiologically involved in closure of the implantation window during menstrual cycle, may regulate the glucose metabolism of endometrial adenocarcinoma. Beyond fuelling glycolysis, glucose is used for glycogen synthesis for long-term storage. Reportedly, cancer cells may utilise Na^+ coupled glucose transporter-1 (SGLT1; *SLC5A1*) for cellular glucose uptake. Here, we analysed the effect of LEFTY2 on SGLT1 expression and activity, as well as on glycogen synthesis in endometrial Ishikawa and

HEC1a adenocarcinoma cells. LEFTY2 increased transcript and protein abundances of SGLT1 and glycogen synthase-1 in both cell lines. In LEFTY2-stimulated Ishikawa but not in HEC1a cells, uptake of the glucose analogue 2-NBDG and cellular glycogen levels were significantly increased as compared to unstimulated cells. Co-treatment with TGF-β blunted these LEFTY2 effects, suggesting that LEFTY2 may upregulate glycogen synthesis in endometrial adenocarcinoma in a TGF-β dependent manner.

Finally, the third published study to be included in this cumulative thesis (Alauddin et al., 2020a) analysed the possible effects of the ellagic acid metabolite Urolithin A on the progression of endometrial adenocarcinoma *in vitro*. To this end, the impact of Urolithin A on actin dynamics, proliferation and cell migration as well as the signalling underlying observed effects were determined. The data indicate that Urolithin A inhibited actin polymerization, cell proliferation and migration of endometrial adenocarcinoma cells likely by interfering with Rac1 and PAK1 expression and signalling suggesting a tumoristatic action of ellagic acid-rich diet.

5. Zusammenfassung

Diese kumulative Promotionsschrift fasst drei Originalpublikationen zur Biologie und Physiologie des humanen Endometriums zusammen. Die erste Studie (Alauddin et al., 2020b) analysierte mittels *in silico* Auswertung von Transkriptom-Daten, *in vivo*-Experimenten an *AnxA7*$^{-/-}$ und WT (*AnxA7*$^{+/+}$) Mäusen sowie *in vitro*-Untersuchungen an humanen Endometrium-Stroma-Zellen (HESCs) die Funktion von Annexin-A7 (ANXA7), einem Phospholipid-bindenden Protein, für Fertilität und Embryo-Implantation. Dabei stellte sich heraus, dass die Expression von ANXA7 im humanen Endometrium nach Dezidualisierung der HESCs ansteigt. Experimentelles Herunterregulieren von ANXA7 reduzierte einerseits die Expression von kanonischen Dezidualisierungsmarkern und führte andererseits zu höheren Cyclooxygenase-2- und Prostaglandin E_2-Leveln. Darüber hinaus resultierte aus dem genetischen *AnxA7*-Knockdown eine erhöhte Anzahl an Implantationen und eine höhere Wurfgröße. Zusätzlich waren in Biopsien von humanen Endometriumproben die ANXA7 mRNA- und Protein-Mengen während der mittleren Lutealphase des Implantationsfenster höher als diejenigen von subfertilen Frauen. Zusammenfassend zeigen die Daten, dass ANXA7 die Empfänglichkeit des Endometriums und die Implantations-wahrscheinlichkeit reguliert.

Die zweite Studie (Zeng et al., 2020) untersuchte *in vitro* inwieweit der Endometrial-Bleeding-Related-Factor LEFTY2, der unter physiologischen Bedingungen während des Menstruationszyklus das Implantations-Zeitfenster mit beendet, den Glukose-Stoffwechsel des Endometrium-Adenokarzinoms reguliert. Neben dem Abbau in der Glykolyse wird Glukose auch in Form von Glykogen gespeichert. Krebszellen verwenden auch Na^+-gekoppelte Glukose-Transporter-1 (SGLT1; *SLC5A1*) zur zellulären Glukose-Aufnahme. In dieser Arbeit analysierten wir die Auswirkungen einer LEFTY2-Stimulation sowohl auf die SGLT1-Expression und -Aktivität als auch auf die Glykogen-Synthese in Ishikawa- und HEC1a-Endometrium-Adenokarzinom-Zellen. LEFTY2 erhöhte

die mRNA- und Protein-Mengen von SGLT1- und Glykogen Synthase-1 in beiden Zelllinien. In LEFTY2-stimulierten Ishikawa- aber nicht in HEC1a-Zellen, war die Aufnahme des Glukose-Analogons 2-NBDG und die zellulären Glykogen-Level gegenüber den unstimulierten Zellen signifikant erhöht. Gleichzeitige Behandlung mit TGF-β schwächte diese LEFTY2 Effekte ab. Dies deutet auf ein LEFTY2-induziertes und TGF-β antagonisiertes Hochregulieren der Glykogen-Synthese im Endometrium-Adenokarzinom-Zellen hin.

Das dritte Projekt (Alauddin et al., 2020a) analysierte *in vitro* mögliche Effekte des Ellagsäure-Metabolits Urolithin A auf die Progression des Endometrium-Adenokarzinoms. Hierzu wurde die Interaktion von Urolithin A mit Aktin-Dynamik, Proliferation und Zellmigration sowie das zugrundeliegende Signalling untersucht. Die Daten zeigen dabei, dass Urolithin A Aktin-Polymerisierung, die Zellmigration und Proliferation der Endometrium-Adenokarzinom-Zellen vermutlich durch Beeinflussung von Rac1- und PAK1-Expression und -Signalling, hemmt. Dies könnte möglichweise auf eine tumorhemmende Wirkung einer Ellagsäure-reichen Diät hindeuten.

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7. Statements

7.1. Statement of Authorship

I hereby declare that the thesis, I submit for my doctorate with the title: “Biological and pathobiological processes of human endometrium: endometrial receptivity, glucose metabolism, and cell migration” is my own independent work, that I used only the sources and resources cited and have clearly indicated all content adopted either word-for-word or in substance. I solemnly swear that this information is true and that I have not concealed any relevant information. I am aware that making a false declaration is punishable by a fine or by a prison term of up to three years.

Tübingen, _____ _____

Date Signature

7.2. Statement on the contribution of others

The dissertation work was carried out at the Department für Frauengesundheit under the supervision of Prof. Dr. med. Sara Brucker. The study was designed by Dr. Madhuri S. Salker in collaboration with Prof. Dr. Florian Lang. After training by Dr. Madhuri S. Salker, I carried out the experiments in cooperation with Dr. André Koch. Anja Umbach has performed the *in vivo* experiments to measure the number of implantation sites and litter size. Ni Zeng, Toshiyuki Okumura, Shayan Khozooei and Janet Rajaxavier contributed to the qRT-PCR and western blotting experiments. Janet Rajaxavier also contributed to the immunofluorescence imaging experiment. Toshiyuki Okumura contributed to the measurement of G/F actin ratio by flow cytometry, immunofluorescence imaging and transwell assay experiment. Prof. Jan J. Brosens provided the RPL and Subfertile patient samples and data. Simone Pöschel performed the multi spectral imaging flow cytometry. Statistical analysis was carried out under the supervision of Dr. Madhuri S. Salker, in cooperation with Dr. Yogesh Singh by myself.

I confirm that I wrote the manuscript myself under the supervision of Prof. Dr. Stephan Huber and that any additional sources of information have been duly cited.

Manuscript 1:

Alauddin M, Salker MS, Umbach AT, et al. Annexin A7 Regulates Endometrial Receptivity. *Front Cell Dev Biol*. 2020;8:770. Published 2020 Aug 14.

The study was conceived and designed by Dr. Madhuri S. Salker and Prof. Dr. Florian Lang. In this study, the focus was to investigate the role of ANXA7, a phospholipid binding protein in the regulation of endometrial receptivity and implantation. Anja Umbach has performed the *in vivo* experiments to measure the number of implantation sites and litter size. Janet Rajaxavier contributed in the immunofluorescence imaging experiment. Prof. Jan J. Brosens provided the RPL and Subfertile patient samples and data. I performed all the cell culture, transfection experiments, qRT-PCR, western blotting, ELISA, and immunofluorescence imaging. I quantified and analyzed the data and constructed the figures under the supervision of Dr. Madhuri S. Salker. I wrote and revised the manuscript together with Dr. Madhuri S. Salker and Prof. Dr. Florian Lang.

Manuscript 2:

Zeng N, Okumura T, **Alauddin M**, et al. LEFTY2/endometrial bleeding-associated factor up-regulates Na^+ Coupled Glucose Transporter SGLT1 expression and Glycogen Accumulation in Endometrial Cancer Cells. *PLoS One*. 2020;15(4):e0230044. Published 2020 Apr 1.

I share first-authorship of this manuscript with Ni Zeng and Toshiyuki Okumura. The study was conceived and designed by Dr. Madhuri S. Salker and Prof. Dr. Florian Lang. Ni Zeng, Toshiyuki Okumura, Shayan Khozooei, and Janet Rajaxavier contributed in the qRT-PCR and western blotting experiments. I performed the cell culture, qRT-PCR, western blotting, cellular glucose uptake, and cellular glycogen content measurement. Dr. Yogesh Singh contributed to the analysis of flow cytometry data. I quantified and analyzed the data and constructed the figures under the supervision of Dr. Madhuri S. Salker. I wrote

and finalized the manuscript together with Dr. Madhuri S. Salker, Prof. Dr. Florian Lang, Ni Zeng and Toshiyuki Okumura.

Manuscript 3:

Alauddin M, Okumura T, Rajaxavier J, et al. Gut Bacterial Metabolite Urolithin A Decreases Actin Polymerization and Migration in Cancer Cells. *Mol Nutr Food Res*. 2020;64(7):e1900390. Published 2020 Feb 17.

I share first-authorship of this manuscript with Toshiyuki Okumura. The study was conceived and designed by Dr. Madhuri S. Salker. The study was performed to investigate the potential impact of the ellagic acid metabolite Urolithin A on the progression of endometrial adenocarcinoma. Toshiyuki Okumura contributed in the measurement of G/F actin ratio by flow cytometry, immunofluorescence and Transwell Assay experiment. Shayan Khozooei and Janet Rajaxavier contributed in the qRT-PCR, western blotting, and measurement of the G/F actin ratio by flow cytometry. Simone Pöschel performed the multi spectral imaging flow cytometry. Dr. Yogesh Singh contributed to the analysis of flow cytometry data. I performed the cell culture, transfection with siRNA, qRT-PCR, western blotting, cell proliferation assay, wound healing assay and immunofluorescence. I quantified and analyzed the data and constructed the figures along with Toshiyuki Okumura under the supervision of Dr. Madhuri S. Salker. Dr. Madhuri S. Salker wrote the original draft.

Tübingen, _____ _____

Date Signature

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