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Die Wirkung von LEFTY 2 auf Zellmigration und Proliferation in Endometriumkarzinom

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ERT	Estrogen Replacement Therapy
HRT	Hormone Replacement Therapy
DM	Diabetes mellitus
TAM	Tamoxifen
PCO	Poly cystic ovarian syndrome
COC	Combined oral contraceptive
TUV	Transvaginal ultrasound
MRI	Magnetic resonance imaging
RT	Radiation therapy
DUB	Dysfunctional uterine bleeding
LH	Luteinizing hormone
TGF-β	Transforming growth factor beta
Rac1	small G protein ras-related C3 botulinum toxin substrate1
PAK1	p21-aktivierte Kinase 1
FAK	Focal adhesion kinase
E-Cad	E-Cadherin
mRNA	Messenger RNA
miRNA	Micro RNA
ECM	Extracellular matrix
G-actin	Globular actin
F-actin	Filamentous actin
NHE1	Na+/H+ exchanger

PBS	Phosphate buffered saline
EMT	Epithelial mesenchymal transition
RIPA	Radio immuno precipitation assay
TBS	Tris buffered saline
gr	Gram
r.p.m	Revolution per minute
RT PCR	Real time PCR
AFM	Atomic force microscopy
FACS	Flow cytometry
V	voltage
min	minute
h	hour
PVDF	Polyvinylidene fluoride
µl	Micro Litre
µM	Micro Maller
PS	Phosphatidylserine
rcf	Relative centrifuge force
BMI	Body Mass Index
g	gravity

1. Introduction:

1.1 Endometrial carcinoma:

The carcinoma is the disease that refers to unregulated cell growth which could develop in any tissue type with the ability to invade neighboring organs and could metastasize to distant organs (Hanahan and Weinberg, 2011). Transition from normal cells to cancer cells requires gaining the six hall markers of the cancer which include: firstly, uncontrolled growth, secondly, insensitive to tumor suppressors, thirdly, resistance to programmed cell death, fourthly, immortality, fifthly, activation of angiogenesis and lastly, triggering metastasis which makes the difference between the malignant and benign (Hanahan and Weinberg, 2011). Endometrial carcinoma (EnCa) is an epithelial cancer which arises from the endometrium with a potential to invade the myometrium of the uterus or/ and the cervix (Torre et al., 2015).

1.1.1 Incidence and survival

Endometrial carcinoma, which arises from the endometrium, is a frequent reproductive carcinoma in females and accounts for around 95% of the uterine cancers (Torre et al., 2015). Uterine sarcoma, which arises from the myometrium surrounding the endometrium, is much rarer type and counts around 5% of the cases (Torre et al., 2015). The incidence changes based on the region and the country, for example its incidence rate in developing countries is several times less than in Caucasia (North America and Europe) where EnCa is considered as the most frequent carcinoma in women after mammary, non/small cell lung and bowel (colorectal) cancers (Parkin et al., 1999). Global Cancer Statistics in 2012 reported that yearly, endometrial carcinoma is diagnosed in 320,000 women worldwide and led to 76,000 deaths in that year (Fig1.1-1). In Canada & USA, EnCa is considered as the eighth most common reason of death and 9,000 women die in Europe each year from endometrial carcinoma (Amant et al., 2005). Most patients are diagnosed after

the onset of menopause (Amant et al., 2005). Often, the cases are diagnosed in the early-stage: Probably; that is because of the early appearance of the cancer symptoms. Despite the advanced techniques for early detection, mortality rate remains the same without increase of survival rates.

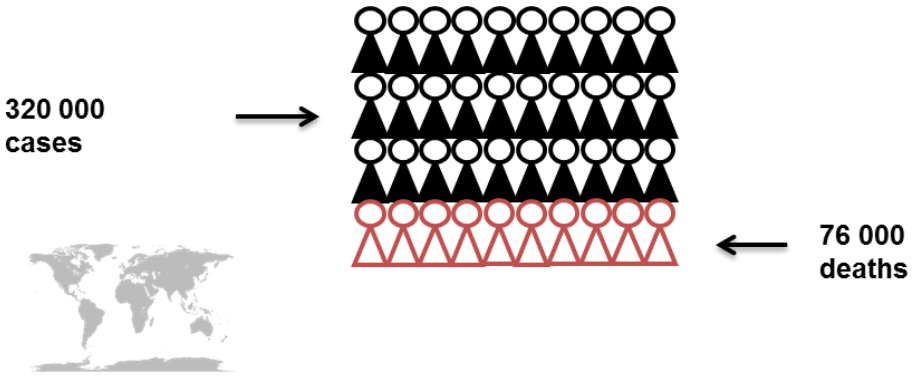

Figure1.1-1. **The yearly incidence of endometrial carcinoma worldwide.** Each year 320 000 cases are diagnosed and 76,000 will die from Endometrial cancer worldwide. This figure is drawn by me based on the following articels (Torre et al., 2015), (Parkin et al., 1999) and (Amant et al., 2005).

1.1.2 Types of the Endometrial cancer

80-90% of EnCa is differentiated based on histologic properties into endometrial carcinoma Type I (T1), which is also called endometrioid because of the histological similarity between this type and the endometrium, is usually detected in the early stage of the cancer (Duong et al., 2011). Women with EnCa Type II (T2; non-endometrioid), which counts 10-20% of the cases, are usually diagnosed with an older age compared to women with EnCa Type I (Duong et al., 2011). EnCa TI, is oestrogen dependant and occurs due to unopposed (stimulation of) oestrogen that leads to hyperplasia of the endometrium (Buchanan et al., 2009). EnCa TII is non-oestrogen dependent and is associated with endometrial atrophy (Amant et al., 2005; Duong et al.,

2011). The most invasive of subtype EnCa TII is uterine serous cancer (Hendrickson et al., 1982). The first type of EnCa TI is more likely in Caucasian females. However, EnCa TII is more likely in African females, according to the American College of Obstetricians and Gynecologist (Hendrickson et al., 1982). Fig 1.1-2 shows the types of EnCa.

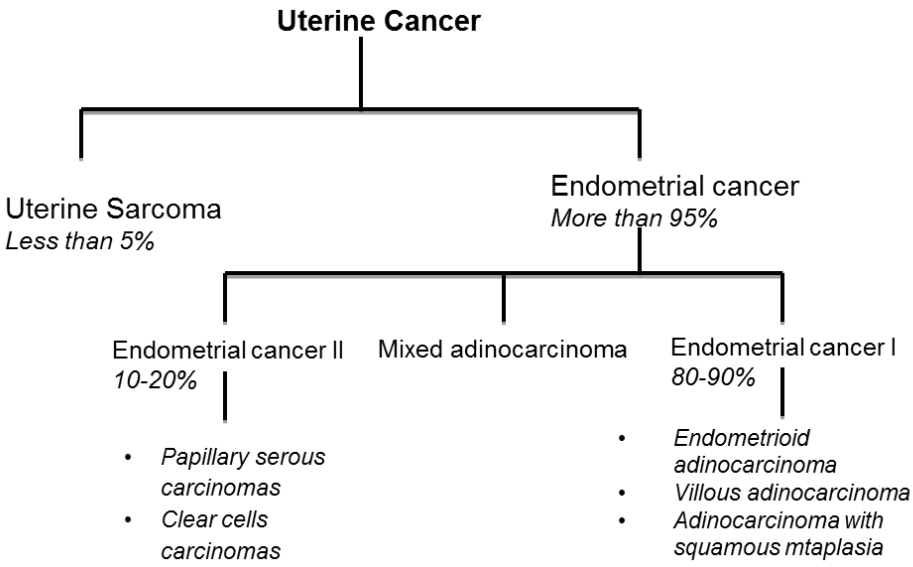

Figure 1.1-2 **Types of endometrial carcinoma.** This figure is drawn by me based on (Buchanan et al., 2009) and (Amant et al., 2005; Duong et al., 2011) and (Hendrickson et al., 1982).

1.1.3 Staging endometrial carcinoma

Staging of endometrial carcinoma can be detected only surgically because 20% of the cases that is staged clinically were incorrect (Savelli et al., 2008). International Federation of Gynecology & Obstetrics (FIGO; Table1.1) describes the clinical presentation of EnCa. FIGO staging should be preceded by preoperative evaluation included physical examination and chest radiology.

FIGO stage	Description
Stage IA	Tumor confined to the uterus, no invasion or invasion of less than one-half of the myometrial thickness
Stage IB	Tumor confined to the uterus with invasion of more than one-half of the myometrial thickness
Stage II	The tumor invades the cervical stroma, but does not extend beyond the uterus
Stage IIIA	The tumor invades the uterine serosa or adnexa
Stage IIIB	Vaginal and/or parametrial involvement
Stage IIIC	The tumor has spread to pelvic or para-aortic lymph nodes
Stage IIIC1	Pelvic lymph node involvement
Stage IIIC2	Para-aortic lymph node involvement (with or without pelvic nodes)
Stage IVA	Tumor invasion of the bladder and/or bowel mucosa
Stage IVB	Distant metastases including abdominal metastases and/or inguinal lymph nodes

***Table1.1-1* EnCa FIGO staging**, adapted from (figo, 2015)

1.1.4 Risk Factors:

Some of the risk factors can be changed such as smoking and obesity in some cases. Other risk factors can't be changed, like increasing age and family history (Ali, 2013). Not all females with these predisposing factors will get EnCa. On the other hand, some women with the EnCa don't have any known risk factors (Ali, 2013).

Risk factors of endometrial carcinoma are divided into two groups: factors increasing risk of endometrial carcinoma and factors decrease risk of endometrial carcinoma (Ali, 2013).

1.1.4.1 Factors increasing risk of endometrial carcinoma:

Long term of unopposed Estrogen:

In the reproductive age, estrogen is secreted primarily by the ovaries, and also from adipose tissue and adrenal glands (Hawkins and Matzuk, 2008). During proliferative phase in the normal menstrual cycle, estrogen levels begin to increase leading to increasing in thickness of the endometrial lining. The proliferative process associated with estrogen includes the endometrial stroma and the glands (Hawkins and Matzuk, 2008). In the post-ovulatory (progesterone dependant) phase of the (menstrual) cycle, progesterone levels rise leading to glycogen and mucus secretion. At the end of this phase, with the absence of pregnancy and with the accompanying drops of the both hormones, the endometrium will shrink and shed. Without the opposing factor of progesterone, surplus estrogen promotes angiogenesis and endometrial cell proliferation (Hawkins and Matzuk, 2008) that may cause cancer. Previously, Estrogen Replacement Therapy (ERT) was prescribed to oppose the symptoms associated with the postmenopausal age without progesterone. This long term treatment of estrogen increased the risk of developing endometrial carcinoma about 17% per year (Weiderpass et al., 1999). Adding progesterone to oppose estrogen, which is called menopausal hormone therapy (HRT), decreases the chance of developing EnCa when compared with ERT (Weiderpass et al., 1999) (Trabert et al., 2013). HRT could increase the risk of developing endometrial cancer only after 10 years or more of use (Pike et al., 1997).

Obesity and Diabetes:

Obesity is a high risk factor not only for many diseases like cardiovascular disease but also for number of cancers including colorectal, pancreatic, breast and endometrial carcinoma (Calle and Thun, 2004; Kulie et al., 2011; Schmandt et al., 2011). Adipose tissues are endocrine organs, that secretes Adiponectin,

which is an adipokine (cytokine secreted by the adipocytes)(Scherer et al., 1995). This hormone is an anti-inflammatory, anti-metabolic and increases insulin sensitivity in the healthy adipose tissue (Hada et al., 2007; Tishinsky et al., 2012). Obese adipose tissues are hypertrophic and associated with inflammation. Together, this can lead to very low expression of adiponectin, which in turn decreases the insulin sensitivity and the anti-inflammatory effect of adiponectin and could lead to carcinogenesis (Nagaraju et al., 2016). Body Mass Index (BMI) ≥ 25kg/m^2 have been shown in more than 50% of endometrial carcinoma patients (Amant et al., 2005; Reeves et al., 2007). The combination of high BMI and low Adiponectin levels increases the risk of endometrial carcinoma 6 fold (Kelesidis et al., 2006; Petridou et al., 2003). Insulin resistance reduces the activity of the liver and increases insulin-like-growth-factor (IGF) (Pollak, 2008). IGF decreases the cell apoptosis and increases the cell proliferation in the cancer (Gooch et al., 1999). Glucose can promote the production of the tumor cells as long as it is a source of energy (Vrachnis et al., 2016). Diabetes mellitus (DM) is a major risk factor of endometrial carcinoma (Vrachnis et al., 2016). Diabetic compared to non-diabetic women have a 2-3 fold increased risk factor to develop endometrial carcinoma (La Vecchia et al., 1994). Obesity is found in most cases of DM Type 2 and it has been shown that diabetes associated with obesity increases the risk factor of developing endometrial carcinoma to 6 fold (Friberg et al., 2007). This risk increases to 10 times when these patients never do physical exercise (Friberg et al., 2007).

Increasing age:

It is known that the endometrial carcinoma is the cancer of postmenopausal women (Amant et al., 2005). 90% of the cases were diagnosed after the age of 50 years with a peak in the age 60-70s (Amant et al., 2005; Schottenfeld, 1995). This peak could change from geographical region to another (Ali, 2013). Less than 14% of the cases are diagnosed in women under 40 years old (Fadhlaoui et al., 2010).

Early menarche and late menopause:

Many female cancers like breast (ductal or lobular), ovarian and endometrial carcinomas are associated to the young age of the first menstruation and advanced age of menopause (Rice, 2010) (Zucchetto et al., 2009) due to more exposure time of oestrogen due to more menstrual cycles (Brinton et al., 1992). Turkish women with early age of menarche (under fifteen) developed endometrial carcinoma 9 times more compared to women with menarche after the age fifteen (Reis and Beji, 2009). Researchers compared between the women who had menopause aged 50 -54 years and women whose menopause took place before 45 years of age. The first group had a 67% higher risk of EnCa. Women which developed menopause later (over 55 years old) had a 79% higher risk (Wolfman et al., 2010).

Familal history:

A familial history in EnCa in first blood female family members (mother, sister, and daughter) occurs only in 5% of the cases. (Ali, 2013; Parazzini et al., 1994). The familal history of EnCa, but not the breast or ovarian cancer, increases the risk 3 fold compared to women without family history of EnCa (Ali, 2013; Gruber and Thompson, 1996). Non-presence of EnCa in Turkish families was a decreased-risk factor of the same cancer (Reis and Beji, 2009).

History of breast cancer:

Breast cancers rarely metastasize to the endometrium (Huo et al., 2015) but a primary endometrial carcinoma can develop in women with breast cancer (Bland et al., 2009). Maybe it is because of the shared risk factors. Women with breast cancer have an increased risk of developing a serous endometrial cancer (Gehrig et al., 2004).

Tamoxifen:

Tamoxifen (TAM) specifically modulates the estrogen receptors, in the breast tissue it works as an antagonist, whilst in the endometrium it is an agonist (Gallo and Kaufman, 1997). Since 1970, TAM has been used as an anti-tumor adjuvant therapy against positive estrogen receptor breast cancer (Grilli, 2006). Long-term (≥ 36 months) use TAM for previous breast cancer increases the risk of developing different abnormalities in the endometrium, including endometrial carcinoma (Neven et al., 1998) but not during the first three years of TAM treatment. Raloxifene hydrochloride is also another modulator of estrogen receptor which is indicated for preventing and treating the osteoporosis following the post menopause. This modulator does not have the same stimulating effect on the endometrium that TAM has, and is safe to be prescribed for five years (Jolly et al., 2003).

Infertility and nulliparity:

Infertile females experience an increased risk to develop EnCa than fertile females (Brinton et al., 2005). The majority of young infertile women who suffer from chronic anovulation, leads to elevated levels of the serum estrogen that increases the risk to develop endometrial carcinoma (Ali, 2013). Poly cystic ovarian syndrome (PCO) is a hormonal syndrome which is mostly presented as; high levels of serum androgen, obesity and chronic anovulation - leads to the accumulation of unopposed oestrogen. This increases the risk of developing endometrial carcinoma (Spritzer, 2014). PCO occurs in 5–10% of females reproductive/child-bearing age. It is the most frequent cause of primary infertility, due to anovulation (Group, 2012). Almost 30% of the endometrial carcinoma patients have PCO in premenopausal age (Navaratnarajah et al., 2008).

Oligo- and nulliparity increase the risk factor of endometrial carcinoma up to four fold (Ali, 2013). As well, the age at the first live birth is another risk factor to develop endometrial carcinoma. Females who delivered their first baby at

middle age (≥ 30 years) were at 40% lower risk compared to teenagers (13-19 years) (Wernli et al., 2006). There is no significantly difference between females who have one live baby and multi- live births to develop endometrial carcinoma (Wernli et al., 2006).

Race:

Few studies have been reported the differences of risks among the ethnic groups. In America, it was shown that white women are at a higher risk to develop endometrial carcinoma type I compared to other races (Buchanan et al., 2009;Setiawan et al., 2007). The rate of endometrial cancer incidence between 1998 and 2002 in African-Americans was only 74% of in the American white women (Farley et al., 2007; Setiawan et al., 2007). Whilst, the mortality rate in the African women was 79% of whites. Maybe this increase of the deaths was associated with the endometrial carcinoma in black women is related to the late diagnose of this cancer and treatment (Farley et al., 2007).

1.1.4.2 Factors decreasing risk of endometrial carcinoma:

Combined-oral-contraceptive COC:

Recently, it has been recommended that the duration of progesterone that protects the endometrium is 12-14 days each month (Grady and Ernster, 1997). Combined oral contraceptive (COC) that contains estrogen and progesterone decreases the incidence of endometrial carcinoma (Buchanan et al., 2009; Deligeoroglou et al., 2003).

Smoking:

Many studies showed smoking as a protector factor against endometrial carcinoma (Buchanan et al., 2009; Viswanathan et al., 2005). In 2005, Zhou and colleagues analysed 34 studies and have concluded to decrease in the risk of endometrial carcinoma up to 30% associated to cigarette smoking (Zhou et al., 2008). The chance of developing EnCa was reduced in those who smoke ≥ 35 cigarettes a day and among women who smoke for ≥ 40 years (Viswanathan et al., 2005). This relation between smoking and decreased risk of endometrial carcinoma was reported only in postmenopausal women (Ali, 2013). Maybe the effect of smoking as an antiestrogen and reduction the age of menopause associated with smoking can explain the protective role of smoking against endometrial carcinoma (Ali, 2013; Baron et al., 1990).

Life style:

In 2011, Terry P and colleagues showed in their study that a good life style could decrease the risk of endometrial carcinoma: increasing physical exercise significantly decreased the risk of developing endometrial carcinoma independently of weight (Terry et al., 1999). Low intake of fruits and vegetables increased the risk while there was no difference associated to alcohol intake (Ali, 2013; Terry et al., 1999).

1.1.5 Symptoms of endometrial carcinoma

Abnormal uterine bleeding (AUB) is the most frequent manifestation in EnCa. Atypical vaginal and/or AUB occurs in 90% of cases & will present with this clinical symptom (Amant et al., 2005). All postmenopausal women who suffer from this symptom with predisposing factors should have further examination and diagnostic assessment (Amant et al., 2005). 5-10% of postmenopausal women with abnormal uterine bleeding could develop endometrial carcinoma.

This percentage increases in the presence of the endometrial carcinoma risk factors (Gredmark et al., 1995).

1.1.6 Diagnosis

Physical examination and History: When a postmenopausal woman come to the clinic with abnormal uterine bleeding, the medical history and the physical examination, is taken. Any abnormality in the uterus, ovaries, vagina or pelvis is included in the preoperative evaluation, as well the patient history should be taken to evaluate the risk factors (Committee on Practice, 2012).

Transvaginal Ultrasound (TVU) and magnetic resonance imaging (MRI): Transvaginal Ultrasound is not an invasive test and is considered as the first step in case of diagnosing the cause of postmenopausal bleeding (American College of and Gynecologists, 2009b) as it offers the chance to check the thickness of the endometrium. When TV sonography is not possible because of marked obesity for example, an alternative method is indicated (American College of and Gynecologists, 2009a). Depth of invasion and metastases into the cervix and lymph nodes play critical role in MRI staging (Meissnitzer and Forstner, 2016).

Endometrial Biopsy: Endometrial thickness less than 4mm has a negative chance of developing malignancy, according to a Nordic multicenter study, and does not require endometrial sampling (Karlsson et al., 1995). The sensitivity of the blind endometrial biopsy, specifically by Pippelle device, has been calculated to detect endometrial carcinoma 99% in postmenopausal women and up to 90% in premenopausal women with specificity up to 98% (Dijkhuizen et al., 2000). Blind biopsy is more useful in global lesions than in focal one (Goldstein, 2009). It is indicated to take 3mm to exclude the endometrial carcinoma in postmenopausal women (Timmermans et al., 2010). Discomfort, bleeding and infection could be caused by endometrial biopsy (2002).

Hysteroscop and Saline Infusion Sonography (SIS): Sterile saline (NaCl, 0.9%) is pumped into the uterus for better visualization of the cavity and its structures. It is indicated when the ultrasound suggests a focal lesion which could contain malignancy or when an endometrial biopsy showed normal or for a non-diagnostic sample (Buchanan et al., 2009; Clark et al., 2002; de Kroon et al., 2003). Saline Infusion sonography or hysteroscopy could still be a risk factor as there is a potential of peritoneal contamination by the cancer cells after the procedure.

Pap smears: Normal cervical smears do not exclude endometrial carcinoma. However, this method is not a sensitive procedure for EnCa, only when it shows abnormality, then it is associated with high grades of EnCa (Gu et al., 2001).

1.1.7 Treatment of endometrial carcinoma

Preoperative Staging: Preoperative staging is an important step before surgery to define the risk based on the invasion to the cervix and the myometrium, and the lymph node metastases and to define the right surgical assessment. Both TVU and MRI are used for preoperative staging. MRI is sometimes not available because of high cost, so when TVU does gives not high quality images, then MRI is offered (Savelli et al., 2008). To detect the lymph node metastases MRI, if not available the CT, are used (Morice et al., 2016).

Surgery: For endometrial carcinoma stage I, total hysterectomy with fallopian tubes and the ovaries is indicated and affective alone in 75% of the cases (Morice et al., 2016). Only 0.8% Women with low risks and tumor ≤ 2cm and myometrial invasion ≤ 50% could have lymph metastases. Lymphadenectomy has no effect on the survival or the cancer recurrence in the early stage of disease (Frost et al., 2015). Lymphadenectomy of the pelvic nodes and paraoortic nodes is indicated in the presence of any of the following features: serous, clear cell or high-grade histology, > 50% myometrial invasion, Tumor

size > 2 cm (Ytre-Hauge et al., 2015). Minimally invasive techniques (laparoscopy or robot-assisted surgery) are less invasive than laparotomy and need shorter stay in the hospital and causes fewer complications than laparotomy (Janda et al., 2010).

Adjuvant therapies:

Radiation: Radiation therapy (RT) for endometrial carcinoma significantly decreased the chances of prolapses and is indicated as a 6 weeks postoperative treatment (Ahmad et al., 1995). Women with noninvasive endometrial carcinoma are in a low risk of recurrent cancer and RT is not indicated to be included in their treatment (Keys et al., 2004). Primary RT is an option for medically inoperable patients with survival fewer than 16% (Podzielinski et al., 2012).

Chemotherapy: Women with high-risk endometrial carcinoma are indicated for chemotherapy with or without RT (Colombo et al., 2013).

1.2 Dysfunctional uterus bleeding (DUB) and LEFTY2

1.2.1 Dysfunctional uterus bleeding (DUB):

The endometrium is a unique tissue in the female body that it sheds monthly during menstruation. This process which leads to monthly menstrual bleeding is still not totally understood. Many factors affect menstrual bleeding. The most important implicators are the ovarian steroids (Oestrogen and Progesterone) (Papageorgiou et al., 2009; Tabibzadeh, 2011). In the case that implantation does not occur, serum levels of progesterone (in the late secretory phase) decreases leading to menstrual bleeding (Hawkins and Matzuk, 2008). The term *dysfunctional uterine bleeding* (DUB) is abnormal endometrial bleeding with the absence of systemic or structural disease (Bravender and Emans, 1999). Almost half of the women who suffer from DUB are 40 to 50 years old (March, 1998) and occurs often when ovulation does not happen (Bayer and DeCherney, 1993; March, 1998). DUB in adolescents is usually associated with the immaturity of the hypothalamic-pituitary-ovarian axis which in turn leads to lack of the luteinizing hormone (LH) then the secretory phase in the menstrual cycle does not occur (Bayer and DeCherney, 1993). Similarly DUB in peri-menopausal women is secondary to aging of the ovary which leads to decrease of the estrogen production, LH surge and the secretory phase do not develop so the endometrium is exposed to the estrogen without opposed progesterone which leads to DUB (Bravender and Emans, 1999; Hickey and Fraser, 2000). This DUB could occur during the secretory phase due to insufficiency of the luteal phase. DUB in most cases is associated with the anovulation and especially chronic anovulation which could lead in high risk of endometrial carcinoma (Bayer and DeCherney, 1993) and an endometrial biopsy is indicated in women over 35 years old with DUB (Bradley and Gueye, 2016). DUB can be treated medically with combination oral contraceptives or progestin alone (Bradley and Gueye, 2016; Hickey and Fraser, 2000). Surgical procedures including hysterectomy are indicated in case of medicaments failure, other symptoms such as pain occur and the patient’s request for surgery (Farrell, 2004).

1.2.2 LEFTY2, TGFβ4, the endometrial bleeding-associated factor (EBAF)

In 1983, Transforming Growth Factor-beta (TGF-β) was first described (Assoian et al., 1983). It is a multifunctional protein that controls proliferation, differentiation and is involved in other functions in many cell types (Assoian et al., 1983). LEFTY2 (TGFβ4; EBAF) is a member of TGF β superfamily (Meno et al., 1996; Tabibzadeh et al., 1997; Thisse and Thisse, 1999,Kothapalli et al., 1997). During the development of the vertebrae embryonic morphogenesis, the fetus body plan is associated with three axes: the dorsovenrtal, the anterioposterior and the Lefty-Right axes to achieve the normal developing of the embryo in three dimensional spaces (Mercola and Levin, 2001). It has been reported that mutations in the gene that encoded LEFTY2 have been associated with left-right axis malformations, specifically in the heart and the lungs. In both mice and humans, the gene of LEFTY2 is localized to chromosome 1. The locus contains two murine genes Lefty1 and Lefty2 with the same transcriptional orientation (Kosaki et al., 1999). Human LEFTY1 and LEFTY2 are close to each other. LEFTY2 shares 91% sequence identity and 331 amino acids with LEFTY2. Both of them block the Nodal signaling and prevent the assembly of an functioning receptor complex (Nodal-Activin) (Schier and Shen, 2000). Meno C and his colleagues in 1998 reported that the role of LEFTY2 is to restrict the expression of LEFTY2 and Nodal to the left side (Meno et al., 1998). Human LEFTY2 polypeptide is secreted as a 42-kDa precursor susceptible to proteolytic cleavage (Ulloa et al., 2001). This protein presents in normal endometrium and its levels increase about 100-fold at the perimenstrual phase and the menstrual endometria (Cornet et al., 2002;Tabibzadeh et al., 2000). Previously it has been reported, that in *ex vivo* increasing of LEFTY2 occurs with the absence of steroid hormones leading to increasing in MMP3 and MMP7 in an explant from proliferative endometria, while progesterone controls both the presence of LEFTY2 and its effect on MMP7. This may explain the dramatic increase of LEFTY2 at the perimenstrual phase is pointing that LEFTY2 is a key regulator for the endometrium breaking down and the

menstrual bleeding by stimulating the production of MMPs (Cornet et al., 2002). It has been shown that the levels of LEFTY2 decreases during the implantation window in the fertile subjects (Tabibzadeh, 2011; Tabibzadeh et al., 2000) while in the infertile women the levels of LEFTY2 were dysregulated in the window of implantation (Tabibzadeh et al., 2000). So LEFTY2 is not only associated with infertility but also with the abnormal uterine bleeding (Kothapalli et al., 1997;Salker et al., 2011; Tabibzadeh and Kothapalli, 1996; Tang et al., 2005).

1.2.3 LEFTY2 inhibits Activin A

Activins, which belong to TGF-β family, are involved in several biological processes such as; cell growth, cell differentiation and cell death (Guo and Wang, 2009). The human endometrium locally secretes the activins proteins to modulate the stromal decidualization, a critical step before implantation. The interaction between activins and other regulating processes leads to this effect (Florio et al., 2010). By interacting with serine-threonine receptor kinases, Activin A can signal to its targets. First, Activin A binds to ActRII receptor, causing the phosphorylation and thus stimulation of ALK4 receptor (Welt et al., 2002). Activated ALK4 phosphorylates Smad proteins (Smad2 and Smad3) (Derynck et al., 1998). Then, they form complexes with Smad4 and translocate into the nucleus and affect specific genes (Welt et al., 2002). LEFTY2 and Activin A share 65% structural similarity. LEFTY2 can interact with the ActRII blocking phosphorylation of Smads and thus impeding downstream factors or pathways (Welt et al., 2002). Fig 1.2-1 shows the negative role of Lefty2 on Activin A signaling.

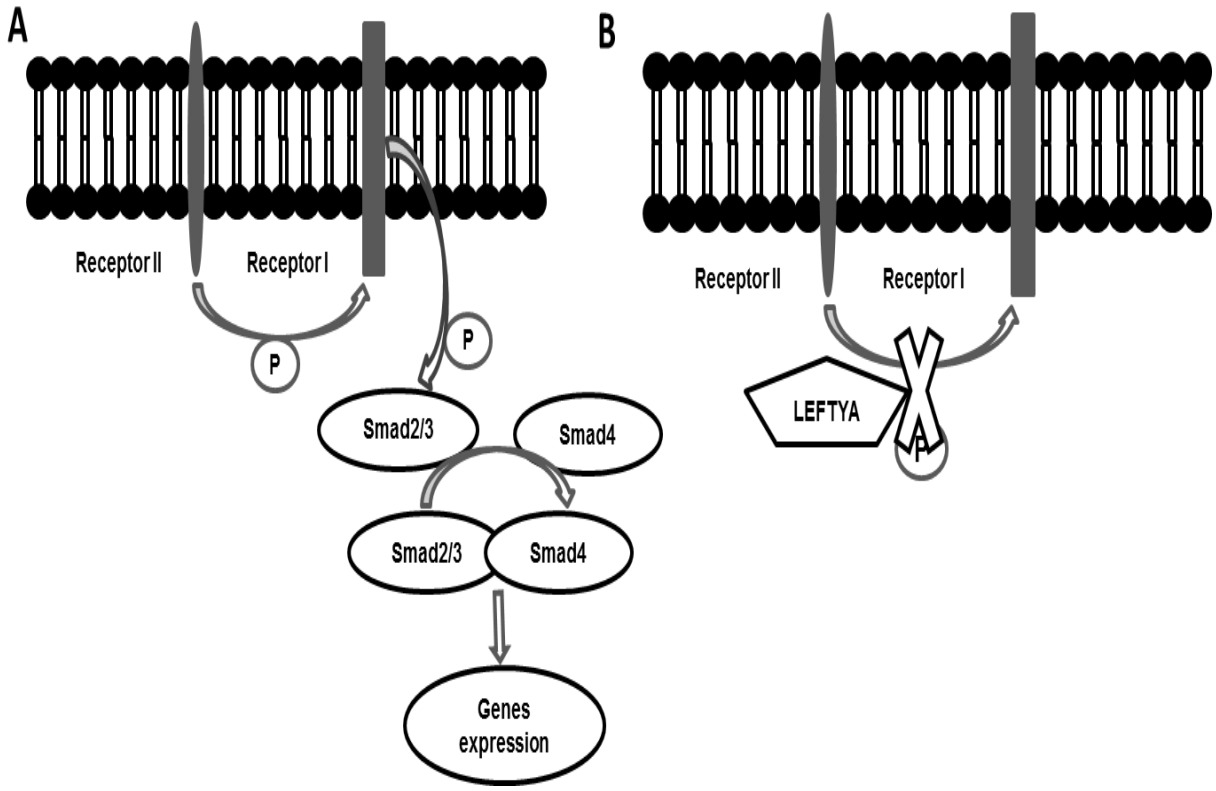

Figure 1.2-1 **LEFTY2 inhibits Activin A signaling. (A)** Dimerization of Activin firstly interacts with ActRII, which mediates and activates (phosphorylation) Alk4 leading to activation of Smads 2/3 forming a complex with Smad 4. affecting downstream genes. **(B)** LEFYT2 inhibits this pathway by binding to the receptor II and preventing the phosphorylation of Smad proteins. This figure is drawn by me based on (Guo and Wang, 2009)(Florio et al., 2010)(Welt et al., 2002)(Derynck et al., 1998)

1.3 LEFTY2 as a strong suppressor of tumor cells

In normal cells TGF-β inhibits the normal cell proliferation by arresting the cell in stage G1, so that TGF-β encourages cells to differentiate or to apoptosize (Ravitz and Wenner, 1997) and TGF-β is a powerful regulator of the production of extra cellular matrix (ECM) which affects the adhesive properties of the ECM. TGF-β increases the production of ECM proteins and cell-adhesion proteins, and decreases the production of ECM degradation enzymes (Massague, 1990).

In cancer cells, if a mutation occurs in the TGF-β signaling pathway, this can lead to residency in the cells, leading to uncontrolled proliferation. Increased secretion of TGF-β can lead to increased angiogenesis leading to aggressive cancer (Gordon and Blobe, 2008).

Nodal, a protein belonging to TGF β (Oshimori and Fuchs, 2012), is very important during embryo development. It acts as an organizing signal to start the axis formation and the nodal signaling is involved also in the L/R patterning (Baker et al., 2008). Nodal has been shown as a cancer marker in several tumors such as mammary carcinoma (Strizzi et al., 2012), and not only as a diagnostic marker but also as a therapeutic target in some cancers like melanoma (Strizzi et al., 2009). In prostate cancer cells, it has been shown that the reactivation of Nodal signaling promotes cellular proliferation of tumour cells (Lawrence et al., 2011). It has been further shown that the intensity of Nodal staining was upregulated in the advanced and invasive stages of BrCa comparing with the early stages and the treatment by blocking antibodies of the nodal signaling *in vitro* reduced the proliferation of the cells and increased apoptosis (Strizzi et al., 2012). LEFTY2 has been shown as an inhibitor of nodal signaling (Miyata et al., 2012; Sun et al., 2014). It has been shown that LEFTY2 was absent in normal tissues such as stomach, liver kidney, breast, ovaries and fallopian tubes while it presents in many tumor tissues such as adenocarcinomas in colonic and ovarian adenocarcinomas. This expression was absent in the adenocarcinomas of colon metastatic to the liver (Tabibzadeh et al., 1997). A previous study investigated the anti-cancer role of LEFTY2 in Human Adult Liver Stem Cells HLSC, and showed that the conditioned medium (CM) of HLSC inhibited the *in vitro* growth of the cells and promoted the apoptosis of the cells that expressed a deregulated Nodal pathway. These effects were associated to LEFTY2 (Cavallari et al., 2013). LEFTY2 mediates growth inhibition and suppressed cells proliferation in pancreatic cells (Miyata et al., 2012).

1.4 The cytoskeleton and its regulators:

1.4.1 Rac1 and PAK1, key regulators of actin reorganization:

The cytoskeleton is like the “backbone of the body”, it holds the organelles in the cell together, connects the cell to the external environment, maintains the cell shape and gives it the ability to move when it is necessary (Fletcher and Mullins, 2010). The cytoskeleton is not a fixed structure but it is a dynamic one whose components could polymerize or depolymerize according to the case (Fletcher and Mullins, 2010). The cytoskeleton consists of three different filaments: microtubules, actin and intermediate filaments. The reorganization of the actin and microtubules filaments occurs by the two different processes: polymerization and depolymerization- and gives the cytoskeleton the dynamic structure (Fletcher and Mullins, 2010). Microtubules are the stiffest filament in the cytoskeleton and play the most major role in the stabilization of extended cells, including nerve cells (Zheng et al., 1993). They polymerize vertically when cells become columnar as during neurulation (Burnside, 1971). Actin filaments are less stiff than the microtubules, but the concentrations of the crosslinking proteins that bind to the filaments change the stiffness (Pollard and Cooper, 2009). Assembly of actin proteins is responsible of the cell movement within the cell and the whole cell over the surface, for example during the embryo development the cells change their location. The immune cells also migrate searching for the pathogens. Even cancer cells migrate using the same mechanism (Pollard and Cooper, 2009). The crawling process of the cell involves four steps: 1) extension of the actin filaments which applies the force on the cell membrane leading to protrusion or lamellipodium, 2) new adhesion to the surface, 3) translocation and 4) deadhesion (Pollard and Borisy, 2003). Shaped like a double helix, actin filaments consist of polymers of globular monomeric sub-units G-actin which polymerize into polymeric state or filamentous actin F-actin (Carlier et al., 2015;Fletcher and Mullins, 2010). The movement is based on the actin exchanges between these two states. G-actin binds to ATP or ADP. ATP-actin polymerizes faster in the positive filament end

which is called barbed end than the negative one which is called the pointed end. This polymerization of actin leads to elongation of F-actin. The elongation continues as long as the rate of the elongation is bigger than the loss of ADP-actin from the pointed end (De La Cruz and Gardel, 2015;Pollard and Borisy, 2003). Fig 1.4-1 shows the Actin polymerization.

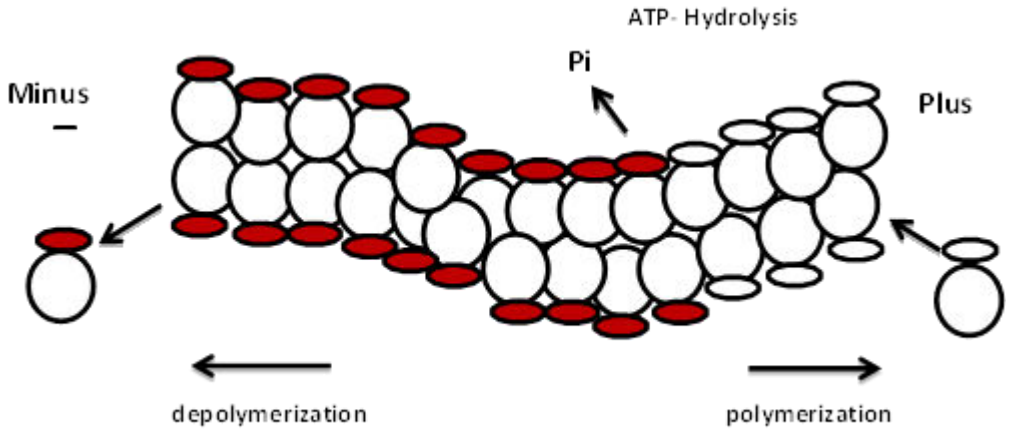

Figure 1.4-1 **Actin polymerization.** Monomer actin binds with ATP (white) to polymerize and be added to the plus end. ATP hydrolyzes by releasing Pi. Actin-ADP (red) depolymerizes from the minus end. This figure is drawn by me based on (Pollard and Cooper, 2009,Pollard and Borisy, 2003,Carlier et al., 2015; Fletcher and Mullins, 2010,De La Cruz and Gardel, 2015; Pollard and Borisy, 2003)

Cell shape maintenance, motility of the cells and the cell-cell adhesion are all processes requiring a dynamic reorganization in the cytoskeleton. This finding was investigated in different types of epithelial cells (Adams et al., 1998; Adams et al., 1996). The small G protein ras-related C3 botulinum toxin substrate 1 (GTPase Rac1) belongs to the Rho family of Guanosine Triphosphatase (GTPases), which is known as a regulator of various signaling pathways that control the reorganization of the cytoskeleton to produce the stress fibers and leads to the formation of the filopodia and lamellipodium which has a major role in the beginning of cellular motility process (Nobes & Hall, 1995; Small et al., 2002). In mammals, the Rho family contains 22 members. Rac1, Cdc42 and

RhoA are the most common members and are involved in most researches because of their crucial roles in the actin dynamic and cell migration and proliferation (Jaffe and Hall, 2005; Ridley, 2006). Table 1.4-1 shows the most common members of Rho Family and its effect on the actin filamenst.

Rho Family member	Effect on actin filaments
Rac1	Filopodia formation
Cdc42	Lamellipodia formation
RhoA	Stress fibers

Table 1.4-1 **The effect of the most common members of Rho family on the actin filaments.** This figure is drawn by me based on (Adams et al., 1998; Adams et al., 1996,Nobes & Hall, 1995; Small et al., 2002Jaffe & Hall, 2005; Ridley, 2006).

Rho family members work as molecular switches. They change their states from (off) to (on) and the opposite by two opposing enzyme groups: guanine exchange factor (GEFs), GTPase-activating proteins (GAPs). The capabilty of tumour cells to invade other organs depends on their ability to move and cross the barriers which indeed require a high organization of the cytoskeleton (Yamaguchi and Condeelis, 2007). Rac1 overexpression is found in different types of cancers includes melanoma progression and metastasis (Bauer et al., 2007) testicular cancer, gastric, squamous cell cancer and aggressive breast cancer tissues compared to normal tissues (Kamai et al., 2004; Parri and Chiarugi, 2010; Schnelzer et al., 2000). P21-activated kinase (PAK1) is an effector of Rac1. Membrane- bound Rac1-GTP inducts PAK by binding to their Cdc42-Rac1 interactive binding (CRIB) domain leading to auto-phosphorylation of PAK1(King et al., 2014). In normal endometrium, PAK1 level expression is higher in proliferative phase compared to secretory phase (Siu et al., 2015). PAK1 is significantly overexpressed in EnCa which suggests that PAK1 plays a role in the development of EnCa especially in post-menopausal women (Siu et

al., 2015). PAK1 has been shown as an important regulator of cytoskeleton dynamics, cells proliferation and apoptosis, and cells migration and motility (King et al., 2014; Ye and Field, 2012).

Therefore Rac1 and PAK are suggested as therapeutic targets against cancer angiogenesis and metastasis (Bid et al., 2013; Senapedis et al., 2015). The next figure (Fig.1.4-2) shows the Rac1 and PAK1 signaling.

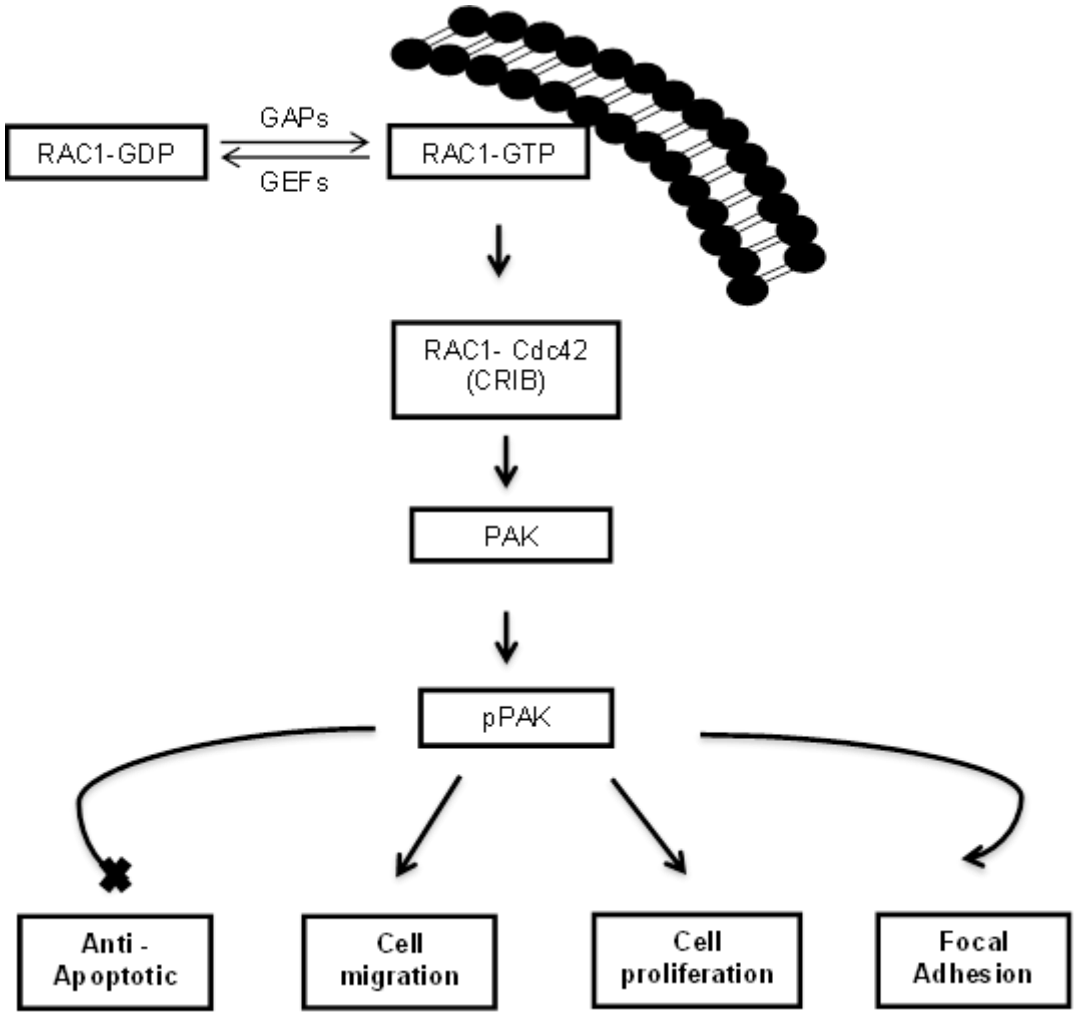

Figure 1.4-2 **Rac1 and PAK1 signaling.** PAK proteins are influenced by Rac1 and Cdc42. PAK1 takes part in cell migration, cell growth, focal adhesion and anti-apoptosis. This figure is drawn by me depending on (Yamaguchi and Condeelis, 2007,Bauer et al., 2007,Kamai et al., 2004; Parri and Chiarugi, 2010; Schnelzer et al., 2000 Siu et al., 2015 King et al., 2014; Ye and Field, 2012.Bid et al., 2013; Senapedis et al., 2015).

It has been shown that the acidosis in the microenvironment increases the metastatic capacity of the cancer cells (Cuvier et al., 1997). Na+/H+ exchanger 1 (NHE1) is known as an important regulator of pHi in the tumor microenvironment and also has a major role in the organization of the cytoskeleton (Boyer & Tannock, 1992; Denker et al., 2000). Activation of NHE1 increases in turn the capability of tumour cells to become highly invasive (Reshkin et al., 2000). Rac1 is a critcal regulator of NHE1 in BrCa. It has been shown that increasing levels of Rac1 in BrCa cells leaded to activation of NHE1, increasing in the cell motility and decreasing of F-actin (Paradiso et al., 2004). Applying a pharmacologic inhibitor of NHE1 on renal (epithelial) cells lead to downregulation of actin polymerization and migration of the cells (Klein et al., 2000). LEFTY2 has a powerful effect on NHE1. It has been shown that LEFTY2 downregulated the expression of NHE1 expression and the exchanger activity which leaded to downregulation of pHi in Ishikawa cells and cell volume (Salker et al., 2015).

1.5 E-Cadherin and cell-cell junctions:

Cells in multicellular organs connect to each other to maintain shape and structure (Bryant and Mostov, 2008). By the lateral surface, epithelial cells are connected to the neighbors by cell-cell junctions. Tight junctions act as a barrier to regulate the water and fluid movement (Aijaz et al., 2006). Desmosomes anchor the intermediate filaments to the plasma membrane giving mechanical strength to the tissues such as in epidermis and myocardia (Garrod and Chidgey, 2008; Johnson et al., 2014). Adherens junctions are formed basically from the classical adherens such as E-cadherin and bind directly to the actin cytoskeleton (Halbleib and Nelson, 2006; Tepass et al., 2001). E-cadherin is expressed from the early stages during the embryo development. E-cadherin deficient embryos failed to form the blastocyst cavity and died at implantation (Larue et al., 1994). In the tumor cells, this loss of intercellular adhesions is a result of reduction of intracellular E-cadherin function and leads to dedifferentiation, higher motility, invasiveness and distant metastasis (Birchmeier and Behrens, 1994). Some tumor cells once they are separated

from the primary tumor become unable to re-express E-cadherin protein, which confirms the role of E-cadherin in metastasis and cells migration (Bukholm et al., 2000). Forced expression of E-cadherin by E-cadherin cDNA transfection prevented the invasiveness of the tumor cells (Frixen et al., 1991). Fig1.5-1 shows the cell junctions.

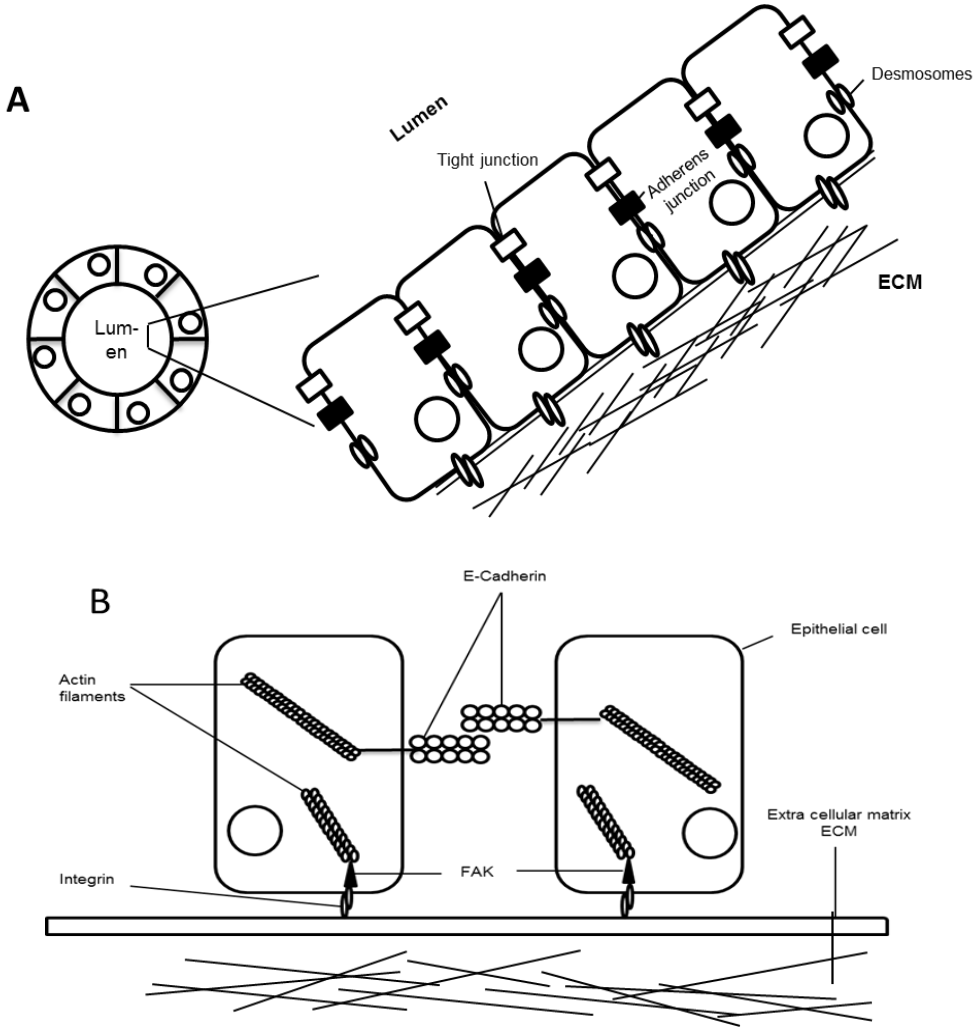

Figure 1.5-1 **Cell junctions.** **(A)** cells of the epithelium: are joined to each other by adherens, tight junctions, desmosomes and the basal surface to the ECM providing the luminal shape and to regulate the exchange of the materials and the signals. **(B)** The Adherens junction between the epithelial cells and the focal adhesion with the ECM connect directly with the cytoskeleton. This figure is drawn by me depending on (Bryant and Mostov, 2008)(Aijaz et al., 2006)(Garrod and Chidgey, 2008; Johnson et al., 2014)(Halbleib and Nelson, 2006; Tepass et al., 2001)(Larue et al., 1994)

The adhesions between the cells and the extra cellular matrix (ECM) are mediated through integrin receptors that bind to focal adhesion kinase FAK, a 120 KDa protein and was given its name based on its localization in focal adhesion (Schaller et al., 1992). β-Integrin facilitates FAK auto phosphorylation at Y397 and supplies the mechanical connection between the cytoskeleton and the Integrins (Shi and Boettiger, 2003; Toutant et al., 2002). FAK integrin-associated phosphorylation leads to FAK-Src complex which leads in turn to activate many other cellular pathways through protein phosphorylation, which are involved in many cellular functions such as such as cell growth, migration in normal and cancer cells, tumorigenesis, cell polarization and cell shape (Cohen and Guan, 2005; Guan et al., 1991; Mitra and Schlaepfer, 2006; Schlaepfer et al., 2004). High expression of FAK has been reported in many types of carcinomas such as squamous cell carcinoma, colonic, prostatic, BrCa and ovarian carcinoma (Aronsohn et al., 2003; Judson et al., 1999; Owens et al., 1995; Tremblay et al., 1996). The overexpression of FAK in oesophageal squamous-cell cancer was related with the invasiveness and metastases into the lymph nodes (Miyazaki et al., 2003). Inhibition of FAK phosphorylation lead to suppression in cell proliferation and tumor growth in pancreatic neuroendocrine tumors (Moen et al., 2015) and induced the cell apoptosis (Shanthi et al., 2014). This overexpression of FAK in many types of cancers made it as a potential cancer therapeutic target (Shanthi et al., 2014) and its inhibitors as treatments of cancer metastasis (Shanthi et al., 2014). The Figure1.5-2 shows the FAK signaling.

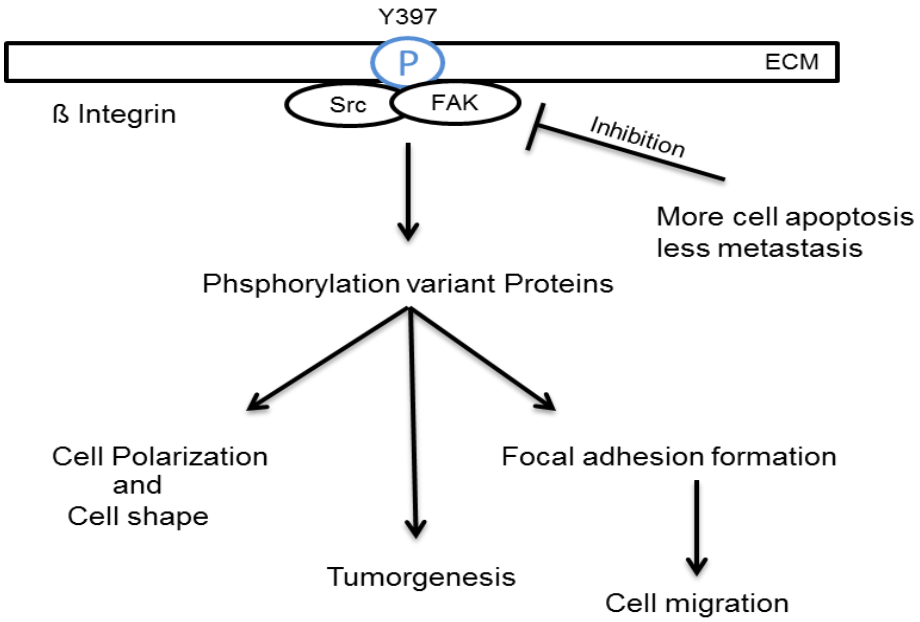

***Figure1.5-2* FAK signaling.** Phosphorylation at Y397 in one of FAK activation pathway and the complex FAK-Src formation which leads to many proteins phosphorylation that takes part ia a variety of biological functions. FAK inhibition increases cell apoptosis & decreases cancer metastasis. This figure is drawn by me based on (Shi and Boettiger, 2003; Toutant et al., 2002)(Cohen and Guan, 2005; Guan et al., 1991; Mitra and Schlaepfer, 2006; Schlaepfer et al., 2004). (Moen et al., 2015)(Shanthi et al., 2014).

1.6 MicroRNAs (miRNA)

Micro RNAs (miRNA) were first described in 1993 (Lee et al., 1993). It has been shown that LIN-4 gene encoded a protein that bound to 3' Untranslated Region (3´ UTR) of LIN-14 message RNA (mRNA) and inhibited the translation of LIN-14 (Lee et al., 1993). miRNAs are small encoding RNAs and 20-25 nucleotides long. They control multiple genes through binding to the 3´ UTR of mRNAs so they prevent the translation of this target RNA or directly they degrade the target. Single miRNA is not specific for one mRNA, it can be responsible for multiple messenger RNA (Christopher et al., 2016). MiRNAs are expressed in the most organisms including the viruses regulating the expressions of the genes which are involved in different biological processing in the organisms such as cell proliferation and motility (Christopher et al., 2016). Overexpression

and underexpression of miRNAs are associated with variety pathogenies including hepatitis C, inflammatory diseases and hepatocellular carcinoma. MiRNAs could be new therapeutic targets (Christopher et al., 2016). The figure 1.6-1 shows the effect of miRNA on protein synthesis.

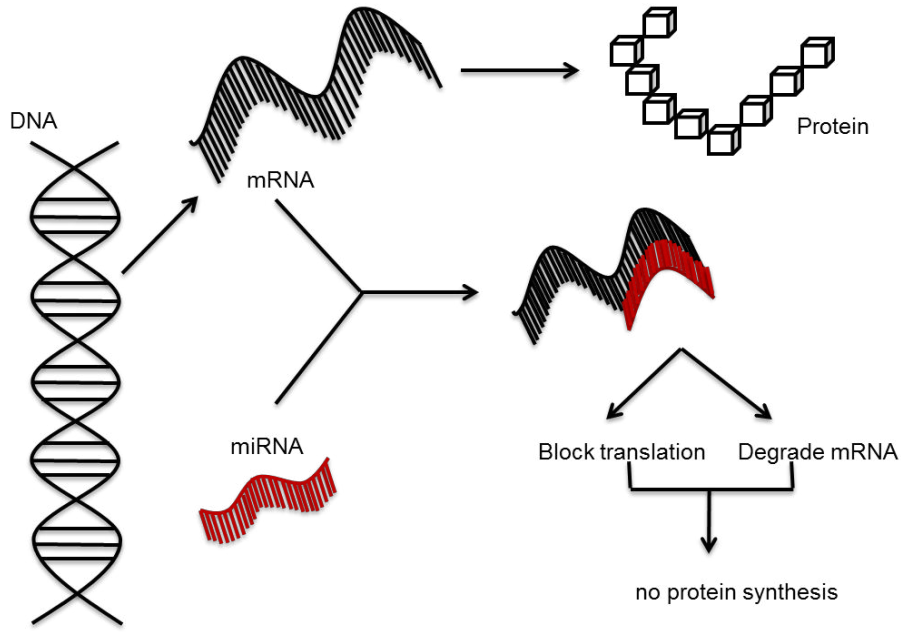

***Figure 1.6-1* miRNA prevents protein synthesis.** The binding between mRNA and miRNA prevent the protein synthesis by one of two mechanisms: blocking the mRNA translation or degrading the target mRNA. This figure is drawn by me based on (Lee et al., 1993, Christopher et al., 2016)

Many researchers have reported about the critical roles of miRNAs in the tumor metastasis and showed that miRNAs act as suppressors and/or sometimes promoters of cancer metastasis such as in BrCa (Asangani et al., 2008; Ma et al., 2007). Till now it is not obvious in which steps of metastasis process are these miRNAs are involved, but it has been shown that the member of the miR-200 lineage has a major role as suppressors for Epithelial Mesenchymal Transition (EMT) through upregulation of E-Cadherin and inhibition the migration in breast cancer and HeLa cells (Korpal et al., 2008). The miR-200 family has 5 members (miR-200a, -200b, -200c, -141, & -429) (Humphries and Yang, 2015). EMT is the mechanism that which the cells of epithelium gain the properties of mesenchymal cells by undergoing morphologic changes so it can

move and separate from the primary tumor and becomes more invasive (Han et al., 2013; Kong et al., 2011). This process includes actin cytoskeleton reorganization and loss of the cell-cell junctions characterized by down regulation of E-cadherin (Schmalhofer et al., 2009). Increasing evidences show the role of this process in the cancer metastasis (Hollier et al., 2009; Moustakas and Heldin, 2007). EMT is induced by many extracellular ligands including TGF-β which plays a critical role to initiate this process in development and cancer by stimulating SMAD and non-SMAD pathways (Derynck et al., 2014). Zinc-Finger E-box Binding Homeobox (ZEB) can activate EMT by pressing the epithelial phenotype and activating the mesenchymal phenotype proteins. ZEB suppresses E-cadherin and tight junction proteins. By being a suppressor of junction proteins between cells and enhancing mesenchymal properties, ZEB is known as modulator of EMT (Abba et al., 2016). It has been reported that miRNA-200 members are inhibitors of EMT by targeting ZEB (Chen et al., 2013).

Aim of the study:

My aims in this thesis is to explore whether:

- LEFTY2 has an effect on the actin polymerization of human endometrial carcinoma cells (Ishikawa) which could lead to dynamic changes in cell stiffness, area, volume and migration.
- LEFTY2 regulates RAC1 expression and activity and PAK1 phosphorylation in EnCa which play a critical role during the beginning of cell movement.
- LEFTY2 affects the proliferation in human EnCa cells (Ishikawa).
- LEFTY2 could affect focal adhesion kinase (FAK) phosphorylation.
- LEFTY2 could affect miR-200 and E-Cadherin expression in Ishikawa cells leading to reduce of migration.

- **Declaration of originality**

- I am declaring that the submitted dissertation is my original doctoral project. I also like to ensure that this thesis or any part of this thesis is not submitted directly or indirectly, anywhere in Germany or world as a separate Doctoral thesis or for funding purposes. To the best of my concern, this dissertation does not possess any data from other researchers without acknowledging them through proper citation or references or without taking written consent.

- The entire experimental work was conducted in the Institute of Physiology1, Eberhard Karls University of Tübingen, Germany.

- This dissertation is made by using the data from the following published articles-

1.*LEFTY2 Controls Migration of Human Endometrial Cancer Cells via Focal Adhesion Kinase Activity (FAK) and miRNA-200a.*

Alowayed N, Salker MS, Zeng N, Singh Y, Lang F.
Cell Physiol Biochem. 2016;39(3):815-26. doi: 10.1159/000447792. Epub 2016 Aug 9.

2.L*eftyA decreases Actin Polymerization and Stiffness in Human Endometrial Cancer Cells.*

Salker MS, Schierbaum N, Alowayed N, Singh Y, Mack AF, Stournaras C, Schäffer TE, Lang F.
Sci Rep. 2016 Jul 11;6:29370. doi: 10.1038/srep29370.

2. Materials and methods:

2.1 Materials

2.1.1 Treatments

Name	Concentration	Manufacture	Country
LEFTY2	25 ng/ml	R&D Systems	Oxford, UK
NSC 23766 (RAC inhibitor)	100 µM	Sigma	Hannover, Germany
IPA 3 (PAK inhibitor)	50 µM	TOCRIS bioscience	Wiesbaden-Nordenstadt, Germany
PF 573228 (FAK inhibitor)	50 µM	TOCRIS bioscience	Wiesbaden-Nordenstadt, Germany
Cytochalasin D	10 µM	Sigma	Hannover, Germany

2.1.2 Antibodies

Antibody	Dilution	Manufacture	Source	Molecular weight
E-Cadherin	1:1000	Cell Signaling	Rabbit	135 KD
Total FAK	1:1000	//	//	125 KD
pFAK	1:1000	//	//	125 KD
Total RAC1	1:1000	//	//	21 KD
pRAC1	1:1000	//	//	28 KD
Total PAK1	1:1000	//	//	68 KD
pPAK1	1:1000	//	//	68 KD
Pan-Actin	1:1000	//	//	45 KD
GAPDH	1:500	//	//	37 KD
Polyclonal Goat Anti-rabbit IgG-HRP(2°AB)	1:2000	Dako		

2.1.3 Cell line

Cells	Cells line
Ishikawa Cells	Epithelial Endometrial Carcinoma

2.1.4 Reagents

Name	Manufacture
30% (w/v) Acrylamide	National Diagnostic
2-Butanol	VWR
Chloroform	Sigma
Dimethylsulfoxid (DMSO)	Carl Roth GmbH
Isopropanol	Sigma
Methanol	VWR
Ethanol	VWR
TEMED	Sigma
Paraformaldehyde (PFA)	//
Sodium dodecyl sulphate (SDS)	//
Tris base	//
Triton X-100	//
Tween 20	//
Bradford	//
Glycine	//
Developer and Replenisher	Kodak
Fixer and Replenisher	Sigma
Propidium Iodide Staining Solution	eBIOSCIENCE
Annexin V conjugated	//

2.1.5 Technical equipment and stock materials

Name	Manufacture and country
Tissue culture plate 6, 96 well	Fisher Scientific, Dreieich, Germany
Eppendorf pipette 10-20, 200, 1000 µl	Eppendorf AG, Hamburg, Germany
Stripette® 5, 10, 25 ml	Corning Incorporated, Corning NY, US
Tissue Culture Flask 75	TPP from Sigma, Munich, Germany
Sterile Tube 15, 50 ml	Cell Star from Sigma, Munich, Germar
X-well Tissue Culture Chambers	Sarstedt, Nümbrecht, Germany
Vortex Genie	Scientific Industries, Bohemia NY, USA
Centrifuge RotiFix 32	Hettich Zentrifugen, Tuttlingen, Germa
Flow cytometry tubes	BD Bioscience, Heidelberg, Germany
Flow cytometry machine	//
miRNeasy Mini Kit	Qiagen, Dusseldorf, Germany
Real-Time PCR machine; BioRad iCycler iQTM	Bio-Rad Laboratories, München, Germ
Gel cassettes	Invitrogen, Karlsruhe, Germany
Western blot module	The Invitrogen XCell II II™
96 well qPCR plates	Peqlab, Erlangen, Germany
qPCR Sel	//
Kodak film	Sigma, Hannover, Germany
Densitometer Quantity One	BioRad, München, Germany
Fixer	//

2.1.6 Cell Culture chemicals

Name	Manufacture
Dulbecco's modified eagles medium (DMEM)	Invitrogen, Karlsruhe, Germany
L-Glutamin, 200 Mm (X100)	//
Penicillin /10,000 U/ml)- streptomycin (10,000 µg/ml) solution (X100)	Invitrogen,
Foetal bovine serum (FBS)	Invitrogen, Karlsruhe, Germany
Trypsin-EDTA solution (X1)	//
Phosphate buffered saline (PBS)	GIBCO, Carlsbad, Germany
Fetal bovine serum (FBS)	Sigma, Hannover, Germany

2.1.7 Software

Name	Manufacture
Microsoft office 2010 SP3	Microsoft corp. Redmond, USA.
CFX Manager software	Bio-Rad, München, Germany
Image J	US National Institutes of Health (NIH)
Flowjo LLC	Oregon, USA

2.1.8 Buffers and solutions

Phosphate-Buffered Saline (PBS) 140 mM NaCl 2.5 mM KCl 1.5 mM KH_2PO_4 10 mM Na_2HPO_4	***Tris-Buffered Saline (TBS)*** 130 mM NaCl 20 mM Tris, pH 7.6
TBS-Tween 20 (TBST) 0.1% Tween 20 in TBS	***4% PFA (stored in 4 °C)*** 4% PFA (w/v) in PBS Adjust to Ph 7.4 with NaOH
Radio Immuno Precipitation Assay (RIPA) Buffer (stored in -20°C) 1% NP-40 (v/v) 1% Triton X-100 150 mM NaCl 2 mM NaF 1% Sodium Deoxycholate (w/v) 0.1% SDS (w/v) 10 mM Tris, pH 8.8 1 Complete Protease Inhibitor Cocktail Tablet per 50 ml Buffer	***Protein Loading Buffer (Laemmli Buffer) (stored in -20°C)*** 50 mM Tris-HCl, pH 6.8 50 mM Imidazole, pH 6.8 1% (w/v) SDS 10% (v/v) Glycerol 2% (v/v) β -mercaptoethanol 0.002 (w/v) Bromophenol Blue
Western SDS Running Buffer 250 mM Tris Base 1.9 M Glycine 1% (w/v) SDS	***Western Transfer Buffer*** 250 mM Tris Base 192 mM Glycine, pH 8.3 20% (v/v) Ethanol
Western Blocking Buffer and Secondary Antibody Incubation Solution (stored in 4 °C) 5% (w/v) non-fat Milk in TBS-T	***Western Primary Antibody Incubatic Solution*** 3% (w/v) BSA in TBS-T

3.2 Methods:

The methods described below were performed either by the manufcturers' protocols or were described by the following 2 articles.

1.LEFTY2 Controls Migration of Human Endometrial Cancer Cells via Focal Adhesion Kinase Activity (FAK) and miRNA-200a. Alowayed N, Salker MS, Zeng N, Singh Y, Lang F. Cell Physiol Biochem. 2016;39(3):815-26. doi: 10.1159/000447792. Epub 2016 Aug 9.

2.LeftyA decreases Actin Polymerization and Stiffness in Human Endometrial Cancer Cells.Salker MS, Schierbaum N, Alowayed N, Singh Y, Mack AF, Stournaras C, Schäffer TE, Lang F. Sci Rep. 2016 Jul 11;6:29370. doi: 10.1038/srep29370.

3.2.1 Cell culture

The experiments of this study are based on Ishikawa Cell samples, a well differentiated endometrial carcinoma adherent cell model. Ishikawa cells were cultured in DMEM/F12 by Gibco ® without phenol red-free media, containing 10% fetal calf serum (FCS), 1% antibiotic/antimycotic solution and 0.25% L-Glutamine (Invitrogen, Karlsruhe, Germany).

For passaging: Cells were passaged with the use of 2ml of 0.25% Trypsin-EDTA (gibco®) 37 °C/ 5% CO_2, incubated for 5 min then the flask was tapped gently to detach the cells. 8ml of 10% media was added which quenched the detachment reaction. Cells were then centrifuged (1000 r.p.m / 5 minutes) and the pellet was re-suspended in 10% media.

Cells were placed in T75 flasks after counting the cells with the calculation:

Total cells = number of cells/16 qm X 10^4 X dilution X Volume Per 1million cells, 1 ml of 10% media was added. For cell culture, 1.5 ml of cells was added to 10 ml of 10% media & incubated in T75 flask. For experiments, 100 µl of cells per 2 ml of media per well & incubated for 48 hours before treating when necessary. Cells were incubated in Binder GmbH incubator: temperature 37 °C, CO_2 concentration 5% and Humidified. To inhibit growth of bacteria, viruses, algae,

fungi, and precipitation of inorganic salts, Aqua Clean by WAK - Chemie Medical GmbH was used (5ml/1liter Water Carl Roth GmbH) every 4 weeks.

3.2.2 RNA isolation

3.2.2.1 messenger RNA (mRNA) isolation

Total mRNA from Ishikawa was extracted by using ice-cold Trizol (Invitrogen) based on a phenol-chloroform extraction protocol: Ishikawa cells were scratched by using 400 µl ice-cold Trizol and then transferred to 2ml Eppendorf sterile tubes. 200 µl ice-cold chloroform was added and then vortexed for 30 seconds. The samples were kept at -80 °C for 20 minutes then defrosted. The samples were centrifuged at 13,000 revolution per minute (r.p.m) for 30 min at 4 °C. Then an equal amount of ice-cold isopropanol was subsequently added to the newly formed clear layer (RNA). The samples were centrifuged at 13,000 (r.p.m) for 15 min at 4 °C. The supernant was discarded and the white pellet was washed in 400 µl of ice-cold 70% Ethanol. The samples were centrifuged at 13,000 (r.p.m) for 15 min at 4 °C then the pellet was left to air dry prior to being dissolved in 15µl distilled water.

3.2.2.2 Micro RNA (miRNA) Isolation

MiRNA was extracted from Ishikawa cultures based on Quick-Start protocol-Qiagen by using miRNeasy Mini Kit (Qiagen, Düsseldorf, Germany): 700 µl QIAzol lysis Reagent was added to each sample. Then the homogenate was incubated at room temperature for 5 minutes. Then 200µl of chloroform was added and the tubes were securely closed. The tubes were vigorously shaken for 15 seconds then incubated at room temperature for 2-3 minutes. The samples were centrifuged at 12,000 (r.p.m) at 4 °C for 15 seconds. The upper aqueous phase was transferred to a new collection sterile tube. Next 1.5 volume of 100% ethanol was added and mixed by pipetting up and down. Pipetted up to 700 µl sample into a RNeasy$^{@}$ Mini column in a 2ml collection

tube. Then centrifuged at 12,000 (r.p.m) at 4 °C for 15 seconds. After that 700 µl Buffer RWT was added to RNeasy Mini column and centrifuged at 12,000 (r.p.m) at 4 °C for 15 seconds. 500 µl Buffer RPE as added to the RNeasy Mini column and centrifuged at 12,000 (r.p.m) at 4 °C for 15 seconds. 500 µl of Buffer RPE was added to the RNeasy Mini column then centrifuged at 12,000 (r.p.m) at 4 °C for 2 minutes. The RNeasy Mini column was placed into a new 2 ml collection tube. Then the samples were centrifuged at 12,000 (r.p.m) at room temperature for 3 minutes to further dry the membrane. Transfer the RNeasy Mini column to a new 1.5 ml collection tube. 30 µl RNase-free water was added directly to the RNeasy Mini column membrane and finally centrifuged at 12,000 x g at room temperature for 1 min to elute.

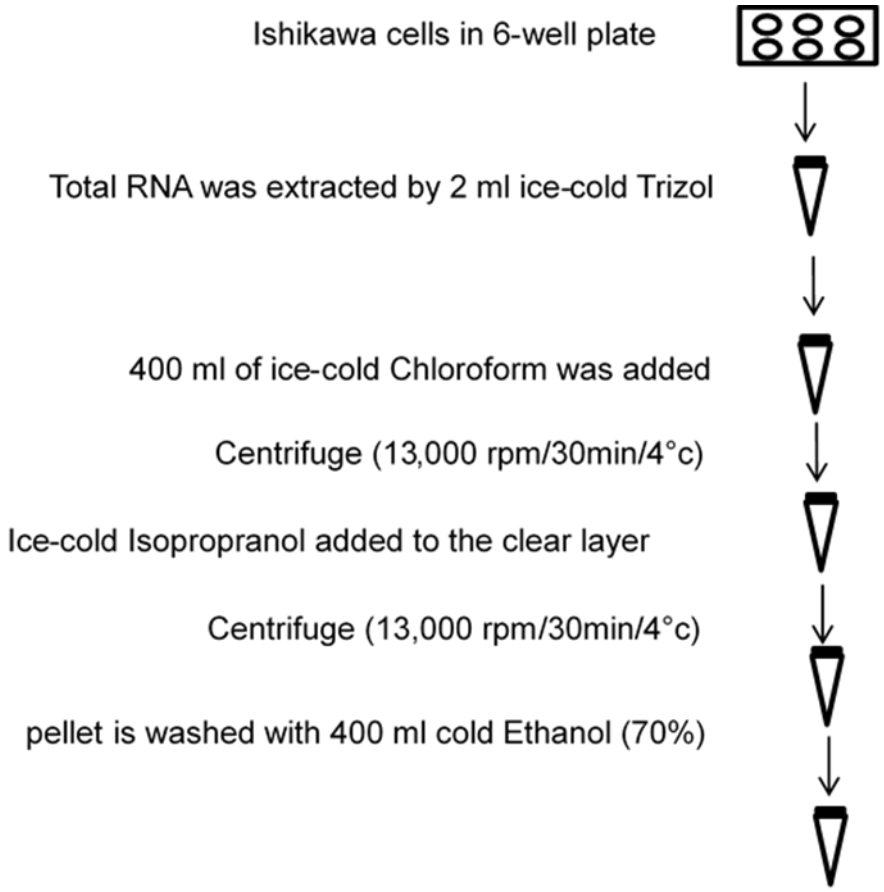

Figure 3.2-1 **Messenger RNA isolation protocol.**

3.2.3 cDNA synthesis

3.2.3.1 From mRNA

RNA was extracted from treated and unstimulated Ishikawa Cells was quantified using a nano-drop BioPhotometer Plus (Eppendorf, Hamburg, Germany) after RNA dilution 1:69 in distilled water. The volume of RNA needed for 2µg of cDNA was calculated. To generate complimentary deoxyribonucleic acid cDNA from total RNA, the Superscript III First-Strand synthesis system for RT-PCR (Invitrogen) was used:

Total RNA was mixed with 1µl oligo (dt) and 1µl of dNTP and the volume made up to 11µl with distilled water. Then incubated at 65°C for 5min and placed on ice for at least 1 min. 9.4 µl of the master mix was added to each RNA/primer mix. And incubated at 50°C for 50min and terminal at 85°Cfor 5 min. cDNA samples were stored at -20°C.

Reagent	**Volume**
10 x RT buffer	2µl
25 mM MgCL2	4µl
0.1 M DTT	2µl
RNase Out (inhibitor)	1µl
Super Script	0.4µl

Table 3.2-1 **The reaction mixture for 2µg of RNA.**

3.2.3.2 miRNA implication

RNA was extracted from treated and unstimulated Ishikawa Cells was quantified using a nano-drop spectrophotometer and the volume of RNA needed for 1 µg of cDNA was calculated. The miRCURY LNA Universal RT

microRNA PCR protocol was used. Thaw gently the 5x RT Buffer and nuclease-free water and immediately placed on ice. Directly before using, the Enzyme mix was removed from freezer and mix by flicking the tube and placed on ice too. The required amount of the reagents was prepared as it is shown in table 3.2-2:

Reagent	**Volume**
5x RT buffer	1 µl
Enzyme mix	0.5 µl
mRNA (100ng)	1 µl
Nuclease-free water	2.5 µl

***Table3.2-2* The reaction mixture for 1µg of mRNA**

The samples were incubated at 42 °C for 60 minutes then at 95 °C for 5 minutes to terminate the reaction.

3.2.4 Amplification of Target cDNA

Detection RNA and miRNA was performed with Kappa Fast-SYBR Green (Peqlab). All reactions were set up in 96-well plate as shown in table 3.2-3 and table 3.2-4:

Reagent	Volume
cDNA	1 µl
SYBR Green Mastermix	5 µl
Forward primer	0.5 µl
Reserve primer	0.5 µl
Distilled water	3 µl

***Table 3.2-3* Total RNA mixture detection for one sample.**

Reagent	Volume
Diluted cDNA	4 µl
SYBR Green Mastermix	5 µl
PCR primer mix	1 µl

Table 3.2-4 **Total miRNA mixture detection for one sample.**

All reactions were carried out by BioRad iCycler iQTM Real-Time PCR Detection System (Bio-Rad Laboratories, München, Germany) for 40 cycles, 10-minute incubations at 95°C (95°C for 15 seconds, 60°C for 1 minute). Data analysis was carried out using the $\Delta\Delta C_T$ method. L19 is a house-keeping gene which served as an internal control and was used to normalize for variances in input cDNA. All measurements were performed in triplicate. The following gene specific primer pairs for housekeeping L19, MKi67, RAC1, mi R-200a were designed using the Primer Blast software.

Gene	**Primer Pairs**
L19	**forward (5'-3'): GCAGCCGGCGCAAA** **reverse (5'-3'): GCGGAAGGGTACAGCCAAT**
MKi67	**forward (5'-3'): CACACTCCACCTGTCCTGAA** **reverse (5'-3'): GACTAGGAGCTGGAGGGCTT**
Rac1	**forward (5'3'):TGCAGACACTTGCTCTCCTATGTAG** **reverse (5'-3'):GAGTTCAATGGCAACGCTTCA**
E-Cadherin	**forward (5'-3'): ACAACGACCCAACCCAAGAA** **reverse (5'-3'): TCACACACGCTGACCTCTAA**

Table3.2-5 **The primers pair sequence.**

3.2.5 Western blotting

3.2.5.1 Protein Analysis

3.2.5.1.1 Globular actin (G-actin) and filamentous actin (F-actin):

Cells were cultured and grown in 6-well plate and treated as described. Ishikawa Cells briefly were washed once with Ice-cold PBS then removed. 100 µl of ice-cold lysis Triton-X 100 extraction buffer was added and incubated on ice for 15 minutes. Soluble G protein was removed by aspiration and collected in 1.5ml tubes. Tubes were centrifuged at 13000 rpm for 15 min at 4 °C then stored in -20 °C. The Triton X-insoluble pellet (F-actin) was extracted by using 100µl ice-cold RIPA buffer and collected in 1.5ml tubes. Tubes were centrifuged at 13000 rpm for 15 min at 4 °C ° and stored in -20 °C.

To measure the amount of protein included in each sample, 800 ml of distilled water was added to 200 ml of Brad Ford (1:5 dilution) and 2µl of protein in the cuvette. The mixture was mixed by pipetting up and down and incubated in room temperature for 20 minutes then measured following the Bradford assay (Bio-Rad Laboratories) by the photometer. Equal amount of proteins (25µg) was diluted 1:1 loading buffer and denatured at 95 °C for 5 minutes.

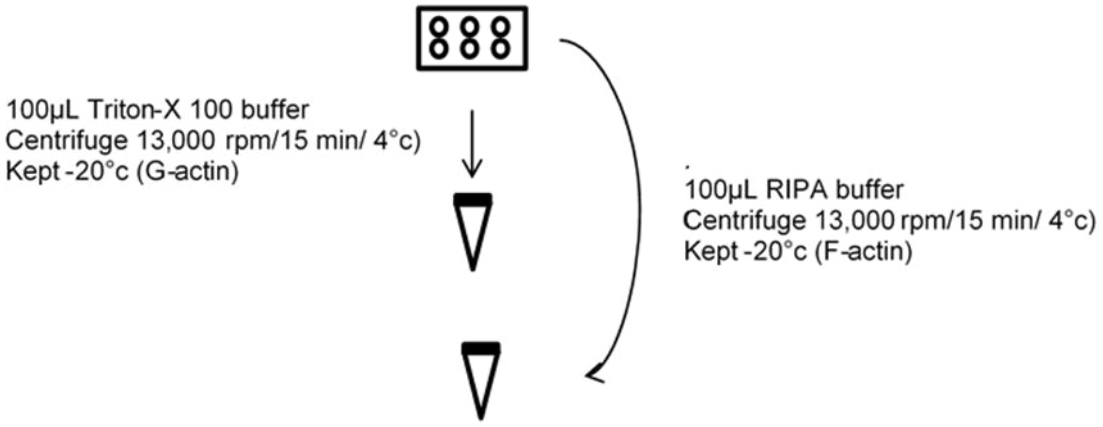

Figure 3.2-2 **G-actin and F-actin extraction from Ishikawa cells.**

3.2.5.1.2 Laemmli Buffer:

Cells were treated as described. Cells were washed once with ice-cold PBS then protein was extracted using 400µl of hot Laemmli buffer (95 °C). Cells were scraped and transferred to 1.5ml tubes and heated to 95 °C for 15 minutes. Samples were kept at -20 °C.

3.2.5.2 SDS polyacrylamide gel electrophoresis (SDS-PAGE)

Proteins were separated on SDS-polyacrylamide gels and transferred using the Invitrogen XCell II II™ Blot Module. Gels were prepared from the solutions as shown in Table 3.2-6 to form the separating and the stacking gels.

Solutions	**Stacking gel**	**12% Separating gel**	**12% Separating gel**
10% Acrylamide	1.67 ml	10.00 ml	8.33 ml
0.5M Tris-HCL pH 6.8	2.1 ml	4.7 ml	4.7 ml
20% SDS	63 µl	175 µl	175 µl
H_2O	7.6 ml	9.5 ml	11.2 ml
TEMED	5µl	6.3 µl	6.3 µl
5% APS	1ml	625 µl	625 µl

Table 3.2-6 **Solutions volumes to prepare stacking and separating gels.**

The separating gel was poured into the empty gel cassettes until it reached approximately 2cm from the top and overlaid with saturated butan-2-ol. After 30

minutes the butan-2-ol was removed and the stacking gel was added on the separating gel and an appropriated comb inserted. Once the stacking gel had set the gel plate fitted into the electrophoresis tank and the running buffer x1 was poured then the comb was removed. A Bradford assay (Bio-Rad Laboratories) determined the proteins concentration required to ensure equal protein loading (25µg). Spectra™ Multicolor Broad Range protein ladder marker was used and the samples were loaded in the wells. Gel was run in 1X Western SDS running buffer at 85 Voltage (V) for 30 minutes then the gel was resolved at 125 V for 60-90 minutes until the dye had migrated to the bottom of the gel. The gels were removed from the cassettes for transfer.

3.2.5.3 Wet transfer

The Invitrogen XCell II II™ Blot Module was used to transfer resolved proteins from a gel onto a polyvinylidene fluoride (PVDF). The PVDF transfer membrane was activated in 100% methanol. The gels, filtered papers and the PVDF were pre-soaked and equilibrated in Ice-cold Western transfer buffer. A sandwich was created as shown in (figure 3.2-3). A voltage gradient of 25 V and 230 mA was created perpendicular to the gel for 90 minutes. After transfer the PVDF membrane was air dried and the reactivated in 100% methanol.

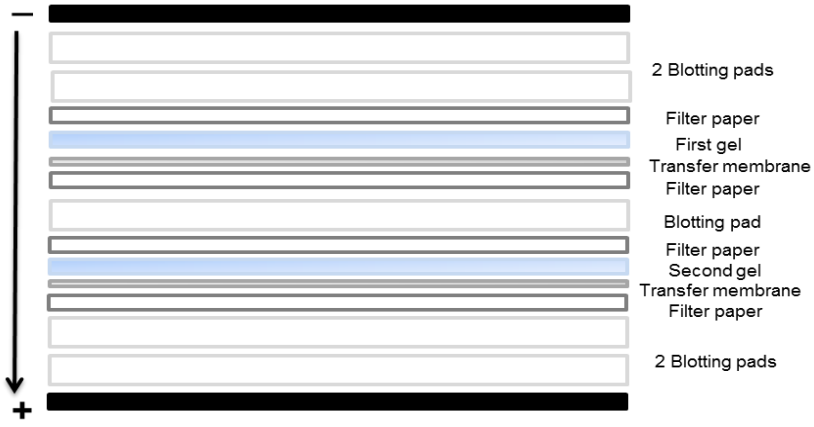

Figure 3.2-3 **The sequence of Western blot sandwich components.**

3.2.5.4 Blocking

The membrane was blocked in 5% non-fat Milk for 1 hour at room temperature on a roller. After that the membrane was washed for 5 min in 1xTBS-T. The primary antibody (see table 2.2) was added to the membrane and kept on the roller at room temperature for 20 min then at 4 °C overnight. The next day the membrane was washed for 5 min in 1XTBS-T then 3 times, each 15 min in TBS-T. The secondary antibody was added for 1 hour at room temperature. Then washed once for 5 min in 1XTBS-T then 3 times, each 15 min in TBS-T. The membrane was subsequently exposed using Amersham Hybond-N+ autoradiology films. Protein complexes were visualized with ECL^{+} chemiluminescent detection kit (Amersham, Little Chalfont, UK).

3.2.6 Flow Cytometry FACS

3.2.6.1 Actin staining

Ishikawa cells (≈1.0×10^{6} cells) were treated as shown and washed three times with PBS then transferred into sterile 15 ml tubes. Then centrifuged at relative centrifugal force (rcf) 600 g for 5 minutes then the pellet was transferred into sterile 96 well-plates. Cells were fixed with 100 µl of 4% paraformaldehyde (PFA) and kept at room temperature in the dark for 15 minutes then permeabilised with 100µl of 1x Permeabilization buffer (eBioscience), and then centrifuged at rcf 600 g for 5 minutes. Cells were stained with 1 µl of fluorescent DNAse1-Alexaflour-488 (50mg/ml) for detection of G-Actin and fluorescent Phalloidin-eFluor® 660 (1000x) (eBioscience, Frankfurt, Germany) for detection of F-Actin and kept in dark at room temperature for 15 min then washed three times with ice-cold PBS. The abundance of the respective labels was measured using green (FL-1) and red channel (FL-4) on a FACSCalibur™ (BD Biosciences, Heidelberg, Germany) and 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.

3.2.6.2 BrdU (5-bromo-2`-deoxyuridine) staining

The BrdU staining for flow cytometry was used for identifying and examining proliferating cells by flow cytometric analysis. BrdU (5-bromo-2`-deoxyuridine) is a synthetic analog of thymidine which incorporates into newly synthesized genomic DNA during the S-phase of mitosis. Then by following DNA denaturation, the cells were stained for BrdU incorporation along with any other cell surface and/or intracellular targets of interest.

The BrdU staining was done based on BrdU staining for flow cytometry FITC (eBioscience). The cells were treated in 6-well plate as described. To harvest the cells 250 µl of trypsin was added, and cells were transferred to 15ml tubes and add PBS up to 10ml. Then centrifuged at 300xg for 5 min at room temperature. The PBS was removed from the tubes and the cells were transferred to 96-well plate. Then centrifuged at 300xg at room temperature for 5 min. The plate was flicked and 200µl of Fixation Buffer was added and incubated at room temperature for 15 minutes, protected from light. After incubation, the plate was centrifuged at 300xg at room temperature for 5 min then washed twice with staining buffer. 25 µl DNase I was added to 75 µl of staining buffer and incubated at 37 °C for 1 hour, protected from light. The cells were washed twice with the staining buffer. For intracellular staining, 2 µl BrdU antibody was added and mixed gently and incubated at room temperature for 30 minutes in the dark. Then centrifuged at 300xg at room temperature for 5 min and then 200 µl of PBS was added to each sample and transferred to FACS tubes for analysis.

3.2.6.3 Annexin V staining

The Annexin staining was done based on Annexin V staining for flow cytometry (eBioscience). Ishikawa cells were treated in 6-well plate as described. Cells

were washed once with 200 µl of PBS, and once with 1 X binding buffer. Then cells were suspended in 150 µl of 1 X binding buffer and 2µl of fluorochrome-conjugated Annexin V was added. The plate was incubated for 15 minutes at room temperature, protected from the light. Wash cells with 1 X binding buffer and Resuspend in 200 µl 1 X binding buffer then the cells were transferred to FACS tubes, and 2 µl of Propidium Iodide (PI) Staining Solution was added. The samples were analyzed with flow cytometry within 4 hours.

3.2.7 Immunofluorescence for Confocal for Actin and E-CAD staining:

Immunofluorescence is the method which is used to visualize a specific fluorescently labelled protein. Cells were treated and grown in sterile 4 –well Tissue culture chambers (Sarstedt) then were washed with ice-cold PBS once then fixed in 4% paraformaldehyde (PFA) for 15 minutes at room temperature. Cells were washed with ice-cold PBS x3 then permeabilised 10X Permeabilization buffer (eBioscience) for 15 minutes at room temperature.

Actin staining: Actin was stained with eflour660-phalloidin (1:200, Invitrogen) for 1 hour at room temperature and with SYTOX® Green dye (1:5000, Invitrogen) for nuclei staining for 30 min in the dark then the slides were mounted with ProLong Gold antifade reagent (Invitrogen).

E-Cadherin staining: E-CAD Primary antibody (diluted in Permeabilization buffer (1:100) was placed and kept at 4°C overnight. Cells were washed with ice-cold PBS (x4) then incubated with the secondary antibody (1:200) for 1hour at room temperature. Then wash three times with ice-cold PBS. Then DRAQ5^{TM} (eBioscience), an anthraquinone nuclei dye with high affinity for double-stranded DNA, was added. Finally, to mount ishikawa cells CC/Mount (Sigma^{TM}) was added and the cover slip was placed.

Confocal microscopy was performed with a confocal laser-scanning microscope (LSM 5 Exciter, Carl Zeiss, Germany) with a C-Apochromat 63/1.3 NA DIC

water immersion objective. The mean fluorescence from six related cells of each picture was quantified by ZEN software (Carl Zeiss, Germany)

3.2.8 Migration assay:

The Ishikawa Cells were counted and grown in sterile 6 well plates. Each well was scratched with sterile 10µl pipette tip then washed one time with PBS. Fresh 2% medium was placed and cells were treated as shown. The locations at which wounds were to be measured were marked with a marker on the undersurface of the wells to make sure that the measurements were taken from the same place. The wound width was measured by phase-contrast microscopy (Nikon Diaphot 300, Amsterdam, The Netherlands) immediately 0h and after 48 h and photos were taken by Bresser Mikrocam (Bresser GmbH, Rhede, Germany) camera using MikroCamLab software. Data was analysed with ImageJ software. With ImageJ software the total area refers to the white area (the wound). Results were taken as 1-total area to refer to the black area (the cells), in other words to the migration. Wound closure was calculated and expressed as a percentage of initial wound width.

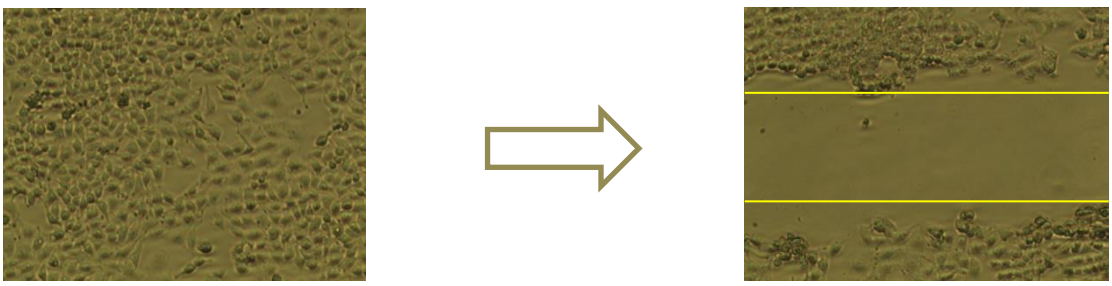

Figure 3.2-4 **Ishikawa Cells before (Left) and after (right) the artificial wound.**

3.2.9 Statistics

Results are provided as means ± SEM; *n* represents the quantity of independent experiments. Results were tested for significance depending on Student's unpaired two-tailed *t*-test. The results with $P < 0.05$ were referred as statistically significant.

4. Results

The next two chapters of results were published in the following 2 articles.

LeftyA decreases Actin Polymerization and Stiffness in Human Endometrial Cancer Cells. Salker MS, Schierbaum N, Alowayed N, Singh Y, Mack AF, Stournaras C, Schäffer TE, Lang F. Sci Rep. 2016 Jul 11;6:29370. doi: 10.1038/srep29370.

LEFTY2 Controls Migration of Human Endometrial Cancer Cells via Focal Adhesion Kinase Activity (FAK) and miRNA-200a. Alowayed N, Salker MS, Zeng N, Singh Y, Lang F. Cell Physiol Biochem. 2016;39(3):815-26. doi: 10.1159/000447792. Epub 2016 Aug 9.

4.1 Results I

4.1.1 The impact of LEFTY2 in Ishikawa cells stiffness by its effect on actin polymerization

To test whether the role of LEFTY2 on cell shape, volume and stiffness was paralleled by the changing of actin polymerization dynamics, Ishikawa cells were incubated with LEFTY2 (25ng/ml) for 2 h then the ratio G-actin over F-actin (G/F) was measured by Western blotting (A, B Fig.4.1-.1) and flow cytometry (C, D Fig.4.1-1). Significantly the amount of G-actin over F-actin increased, reflecting a depolymerization of actin in LEFTY2 treated ishikawa cells. Fluorescent images of F-actin organization and its concomitant changes under LEFTY2 show a profound reorganization of the actin cytoskeleton (E, F Fig.4.1-1). (as shown in Salker. et.al., 2016).

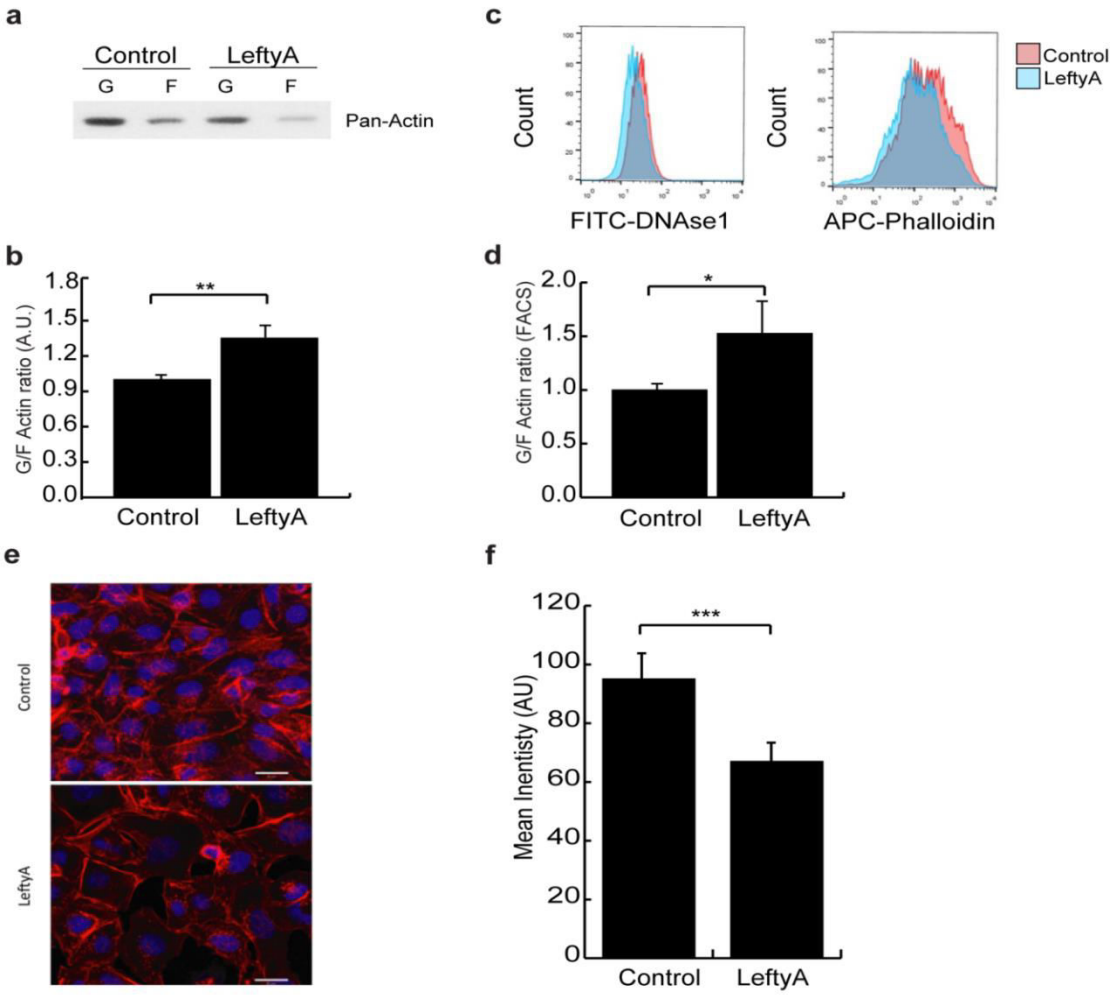

Figure 4.1-1 **The effect of LEFTY2 on actin polymerization in Ishikawa cells**

(A) Representative Western blotting of soluble G-Actin over filamentous F-Actin in human endometrial cancer Ishikawa cells after a 2 hour treatment without (-LEFTY2) and with (+LEFTY2) Lefty A (25 ng/ml) **(B)** means ± SEM (n=6) of G-Actin over F-Actin ratio in Ishikawa cells after a 2 hour treatment without and with LEFTY2 (25 ng/ml). **(C)** Representative original histogram of DNase1 (G-Actin; Left) and Phalloidin (F-Actin; Right) binding in Ishikawa cells after a 2 hour treatment without (-LEFTY2) and with (+LEFTY2) LEFTY2 (25 ng/ml) **(D)** means ± SEM (n=6) of G-Actin over F-Actin ratio in Ishikawa cells after a 2 hours treatment without/with (white bar/black bar) LEFTY2 (25 ng/ml).*, P<0.05; **, P<0.01; ***, P<0.001 by Student's t-test. **(E)** Confocal figuers of eflour660- Phalloidin bind to F-Actin and SytoxGreen for nuclei (red and blue) in Ishikawa cells treated with/without LeftyA (white bar 20 μ m) **(F)** means ± SEM (n=6) of actin fluorescence in Ishikawa cells with and without LeftyA treatment (as shown in Salker. et.al., 2016).

4.1.2 LEFTY2 has a negative effect on Small G protein or GTPase RAC1 levels and activity

Small G protein or GTPase Rac1 is a member of Rho family. Rac1 is known as a regulator for many different signaling pathways which are involved in cytoskeleton reorganization. It has the main role in lamellipodium formation during the cell motility. Rac1 overexpression was shown in many different classes of carcinomas and metastasis, which suggests Rac1 role in the pathogenesis of these cancers and the distant metastasis pointing to its role in cell migration. To test the role of LEFTY2 on Rac1 expression in Ishikawa cells we treated the cells with LEFTY2 (25ng/ml) for 2 hours. The treatment was followed significantly by downregulation of Rac1 transcript levels normalized to L19 transcript levels (Fig.4.1-2 A). The expression of Rac1 protein was also measured by Western blotting and showed a significant downregulation of total Rac1 and phospho Rac1 in LEFTY2 treated Ishikawa cells comparing to nontreated Ishikawa cells (Fig.4.1-2 B, C). (As shown in Salker. et.al., 2016).

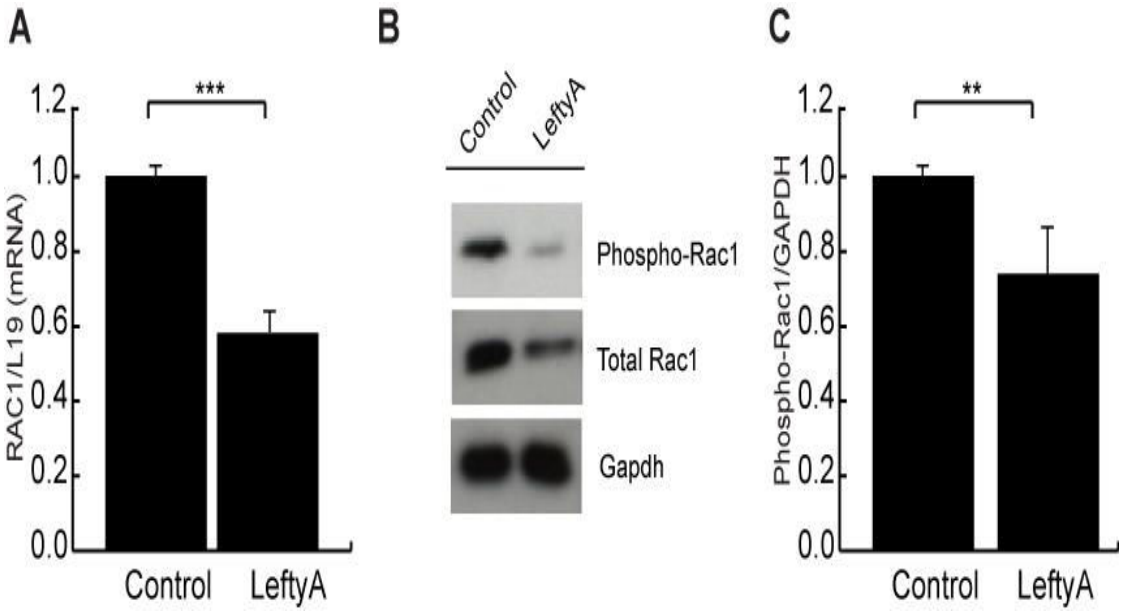

Figure 4.1-2 **The effect of LEFTY2 on Small G protein or GTPase Rac1 (Rac1) transcript level and activity in Ishikawa cells.** **(A)** means ±SEM (n=4) of Rac1 normalized to L19 transcript levels in human endometrial cancer Ishikawa cells following treatment with LEFTY2 (25ng/ml) for 2 hours. Data are shown as fold induction relative to transcript levels of untreated samples. *(P<0.05); **(P<0.01) indicate statistically significant difference, Students t-test. **(B)** Western- blots showing activated Rac1 & total Rac1 protein abundance in Ishikawa cells after 2 h incubation without and with LEFTY2 (25 ng/ml). **(C)** means ±SEM (n=4) of phospho-Rac1 protein ratio normalized to GAPDH. *, P<0.05; **, P<0.01; ***, P<0.001 by Student's *t-test* (as shown in Salker. et.al., 2016).

4.1.3 The negative effect of LEFTY2 on RAC1 indeed contributed to the polymerization of actin in Ishikawa cells

After observing that LEFTY2 affected the cytoskeleton polymerization by downregulation the G/F ratio and LEFTY2 lead to a downregulation of Rac1 in Ishikawa cells. Rac1 is well acknowledged as a key regulator of various pathways that regulate the (re)organization of the cytoskeleton, we wanted to investigate whether this negative effect of LEFTY2 on Rac1 indeed contributed to the polymerization of actin in Ishikawa cells. To test that, Ishikawa cells were treated with LEFTY2 (25ng/ml) and with pharmacologic Rac1 inhibitor (NSC23766) trihydrochloride (100 µM) and with both LEFTY2 and Rac1 inhibitor for 2 hours. The ratio G-Actin over F-Actin was measured by western Blotting (Fig.4.1-3; A,B) and flow cytometry (Fig.4.1-3; C,D). The treatment showed a significant rise of G/F actin in human endometrial cancer, an effect thus mimicking the effect of LEFTY2 treatment. In the presence of the Rac1 inhibitor the additional administration of LEFTY2 (25 ng/ml) did not lead to a significant further rise of G-Actin over F-Actin Ratio in Ishikawa cells. (Fig.4.1-3) (as shown in Salker. et.al., 2016).

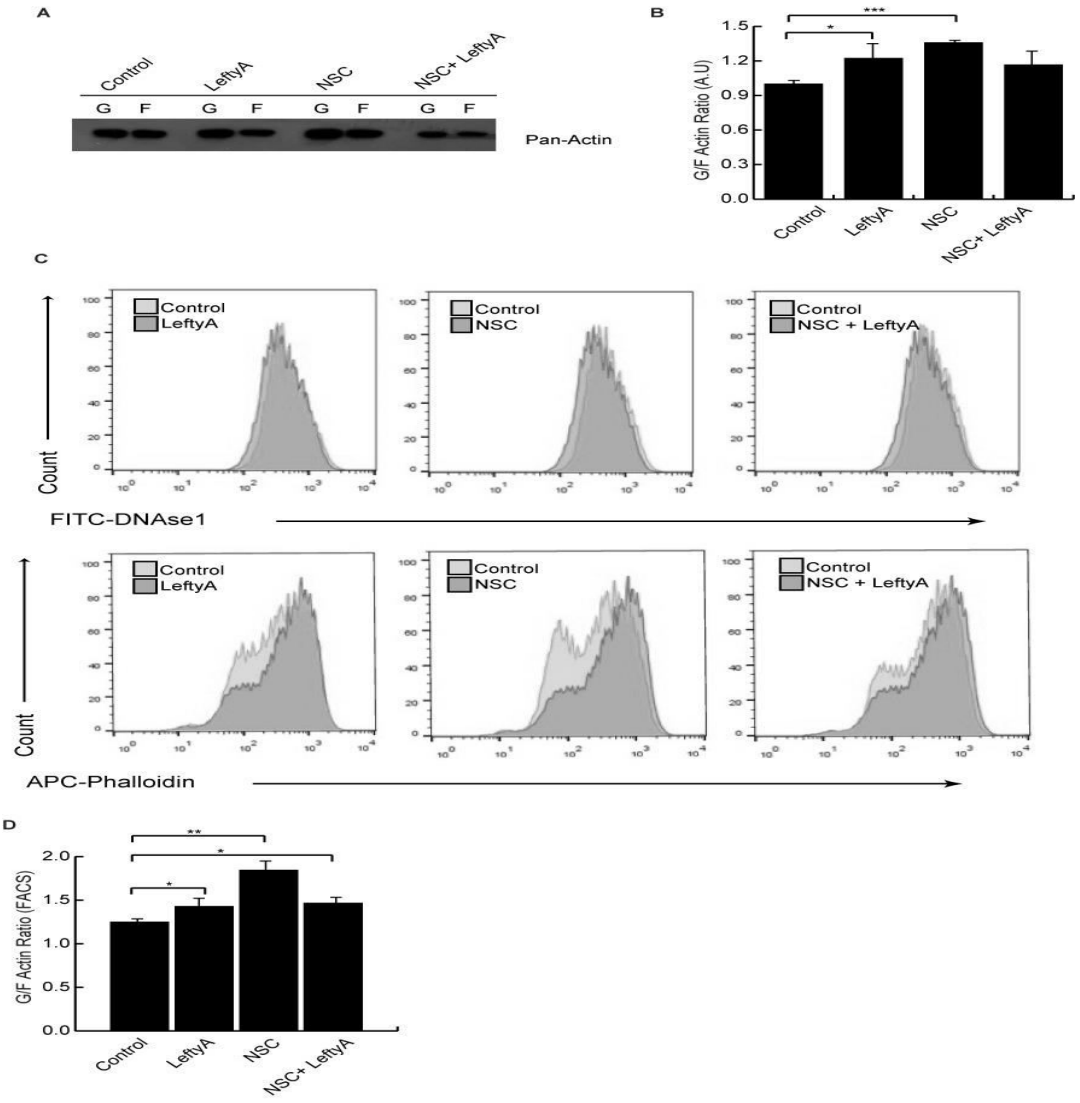

Figure 4.1-3 **The effect of LEFTY2 on actin polymerization in Ishikawa in absence or presence of Rac1 inhibitor (A)** Representative Western-blotting of G-Actin over F-Actin in human endometrial cancer Ishikawa cells after a 2h treatment without and with LEFTY2 (25 ng/ml) in without/with Rac1 inhibitor NSC (100 µM). **(B)** means ± SEM (n=3) of G-Actin over F-Actin ratio in Ishikawa cells after a 2h treatment without/with LEFTY2 (25 ng/ml) without/with Rac1 inhibitor NSC23766 trihydrochloride (100µM). **(C)** Representative histogram of DNase1 (G actin; Upper) and Phalloidin (F actin; Lower) binding in Ishikawa cells after a 2h treatment without and with LEFTY2 (25 ng/ml) without/with Rac1 inhibitor NSC23766 trihydrochloride (100µM). **(D)** means ±SEM (n=5) of G-Actin/F-Actin ratio in Ishikawa cells after a 2h treatment without and with LEFTY2 (25 ng/ml) without/with Rac1 inhibitor NSC23766 trihydrochloride (100µM) *, P<0.05; **, P<0.01; ***, P<0.001 by Student's t-test. (as shown in Salker. et.al., 2016).

4.1.4 The effect of LEFTY2 on p21 protein-activated kinase 1 (PAK1) expression Ishikawa cells

Membrane- bound RAC1-GTP auto-phosphorylate PAK1. PAK1 expression has been studied in the endometrial cancer. It has been shown that PAK1 was overexpressed in this cancer comparing to the normal tissues pointing to its role in the pathogenesis of the endometrial cancer. PAK1 takes part in different cellular processes, e.g. the cytoskeleton polymerization and cells proliferation. To investigate whether LEFTY2 affects PAK1 expression in Ishikawa cells, treatment of LEFTY2 (25ng/ml) for 2 hours was applied. It shows that the expression of phospho PAK1 was downregulated in treating cells comparing to non-treating cells. This downregulation of PAK1 phosphorylation points to downregulation in the activity as well. (Fig.4.1-4). (as shown in Salker. et.al., 2016).

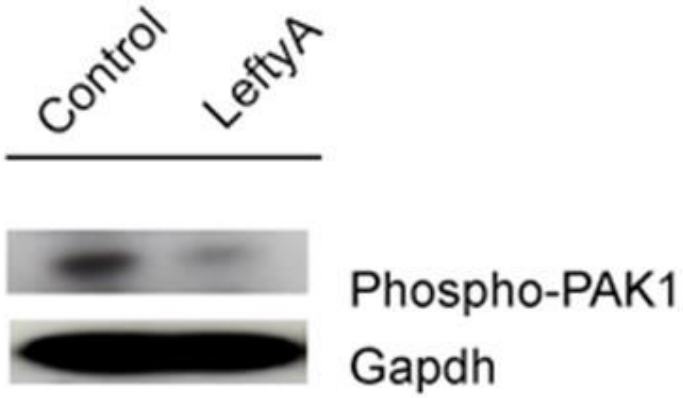

Figure4.1-4 **The effect of LEFTY2 on phospho p21 protein-activated kinase 1 (pPAK1) expression Ishikawa cells.** Representative Western-blotting showing phospho PAK1 protein abundance in human endometrial cancer after 2h incubation without/with LEFTY2 (25 ng/ml) (as shown in Salker. et.al., 2016).

4.1.5 The negative effect of LEFTY2 on pPAK1 indeed contributed to the polymerization of actin in Ishikawa cells

Next series of experiments explored whether this decrease of PAK1 phosphorylation indeed contributes the actin polymerization in Ishikawa cells. IPA-3 (50 μM), the pharmacological inhibition of PAK1, was followed by a significantly increase of G-actin over F-actin in human endometrial cancer Ishikawa cells, and the same effect has been shown with the treatment of LEFTY2 (25 ng/ml) for two hours (Fig.4.1-5) The doubled treatment of IPA-3 and LEFTY2 was not followed by a significant increase of G/F-actin (as shown in Salker. et.al., 2016).

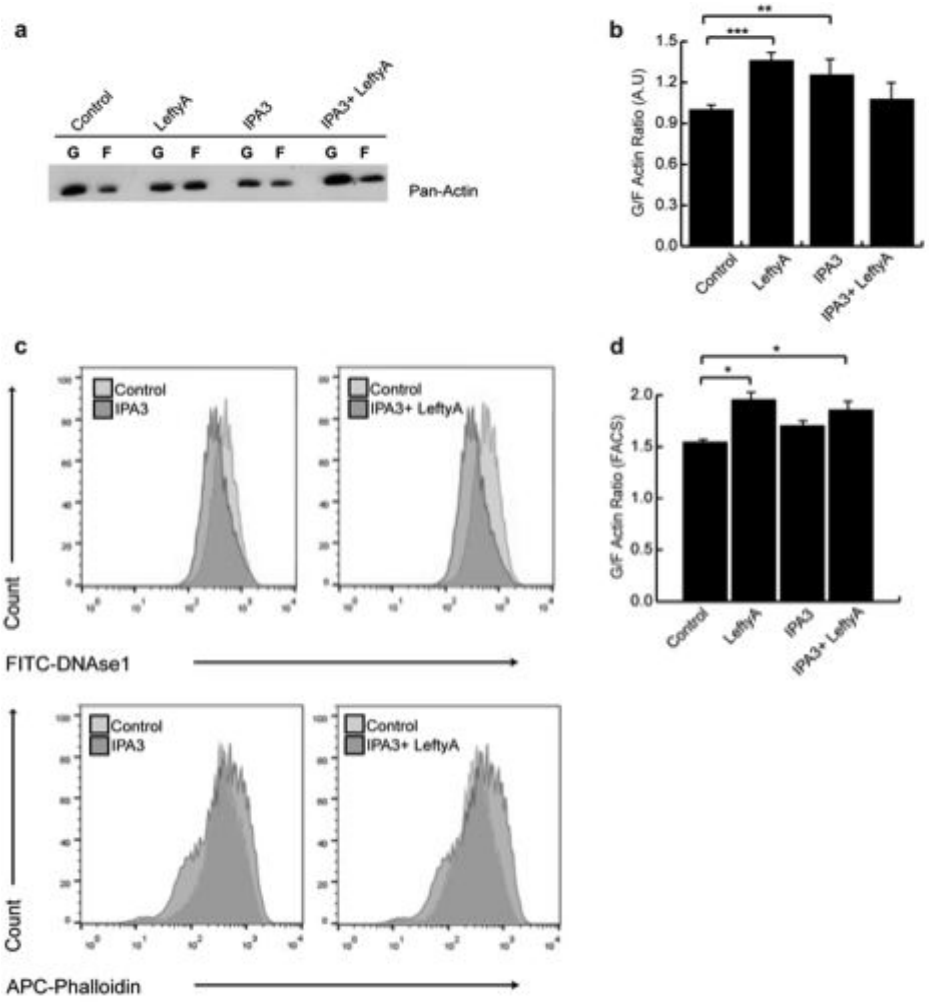

Figure 4.1-5 **The effect of LEFTY2 on actin polymerization in Ishikawa in absence or presence of PAK1 inhibitor.** **(A)** Representative Western blotting of G/actin over F/actin in human endometrial cancer Ishikawa cells after a 2-hour treatment without and with LEFTY2 (25ng/ml) in without/with PAK1 inhibitor IPA-3 (50 μM). **(B)** Arithmetic means±SEM (n = 6) of G/actin over F/actin ratio in Ishikawa cells after a 2hour treatment without and with Lefty A (25ng/ml) 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 2-hour treatment without and with LEFTY2 (25 ng/ml) aswell without and with PAK1 inhibitor IPA-3 (50μM). **(D)** Arithmetic means ± SEM (n = 5; arbitrary units) of G/actin over F/actin ratio in Ishikawa cells after a 2hour treatment without and with LEFTY2 (25ng/ml) without and with PAK1 inhibitor IPA-3 (50μM). *, P< 0.05; **, P<0.01; ***, P<0.001 using Students t-test (as shown in Salker. et.al., 2016).

4.1.6 The effect of LEFTY2 compared to Cytochalasin D

The effect of LEFTY2 on Ishikawa cells was compared with the effect of Cytochalasin D, which induces the rapid actin depolymerisation. Ishikawa cells were treated with LEFTY2 (25ng/ml), and Cytochalasin D (10 µM, Sigma, Germany), LEFTY2 and Cytochalasin D for two hours. Treated-Ishikawa cells showed increase in the amount of G-actin over F-actin ratio reflecting depolymerization of the actin filaments compared to non-treated cells. (Fig 4.1-6). (as shown in Salker. et.al., 2016).

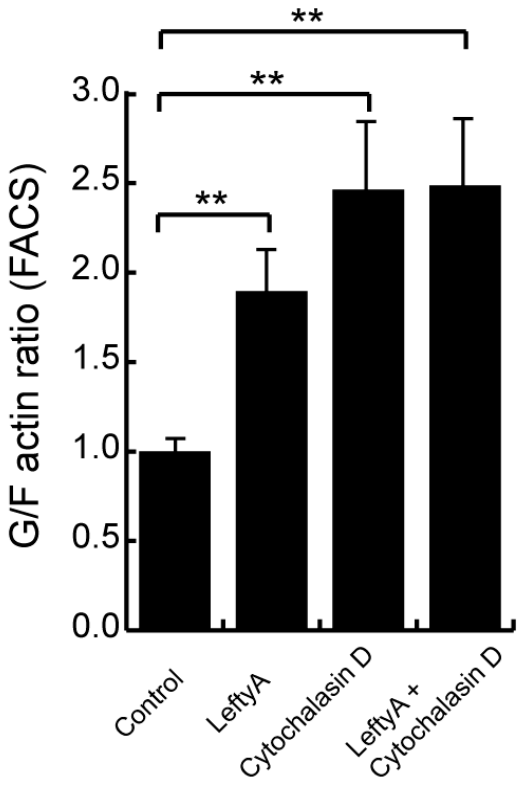

Figure 4.1-6 **The effect of LEFTY2 on the actin polymerization compared to Cytochalasin D.** Arithmetic means±SEM (n =4) of G actin over F actin ratio in Ishikawa cells after a 2hour treatment without (control) and with LEFTY2 (25 ng/ml) without and with Cytochalasin D (10 µM) *, P< 0.05;**, P<0.01;***,P<0.001 by Student's *t-test.* (as shown in Salker. et.al., 2016).

4.1.7 The effect of LEFTY2 on actin filaments is temporary

Treatment with LEFTY2 (25ng/ml) for two hours lead to increase in the amount of G actin over F actin in Ishikawa cells pointing to depolymerisation of actin filaments. To investigate whether this effect is permanent or long lasted, we treated Ishikawa cells with LEFTY2 (25ng/ml) for two hours and then G/F actin ratio was measured with FACS machine, once without washing and once with washing with normal medium for two hours. Interestingly, this effect of LEFTY2 on the actin filaments was reversible in washed cells, pointing to temporary effect of LEFTY2. (Fig 4.1-7) (as shown in Salker. et.al., 2016).

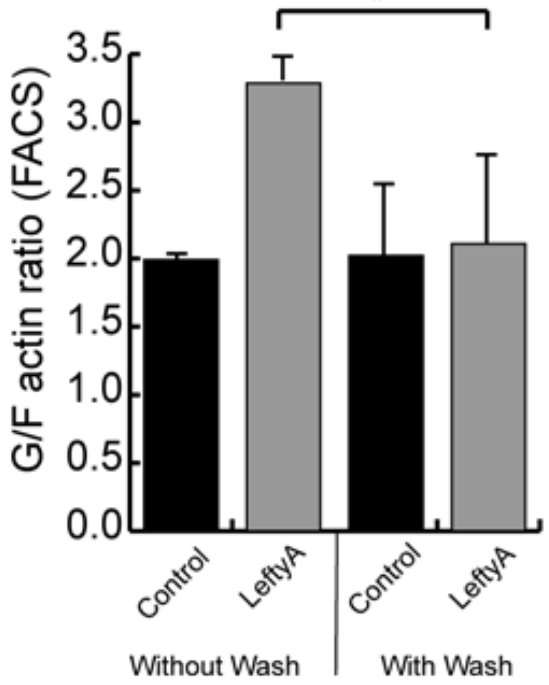

Figure 4.1-7 **The effect of LEFTY2 on actin filaments in Ishikawa cells was temporary.** Arithmetic means ± SEM (n =4) of G actin over F actin ratio in Ishikawa cells after a 2 hour treatment without (control) and with LEFTY2 (25 ng/ml) without wash (on the Left) and with wash (on the Right) *, P<0.05;**,P<0.01; ***, P<0.001 using Student's *t-test.* (as shown in Salker. et.al., 2016).

4.2 Results II:

4.2.1 LEFTY2 significantly decreased the transcript levels of the proliferation marker MKi67

The next experiments explored effects of LEFTY2 on proliferation of human En Ca (Ishikawa) cells. To this end, Ishikawa cells were incubated with 25ng/ml of recombinant LEFTY2 for 48 hours. As illustrated in (Fig 4.2-1), LEFTY2 significantly decreased the transcript levels of the proliferation marker MKi67. To investigate the effect of LEFTY2 during cell proliferation on Ishikawa cells BrdU assay was implemented. As shown in (Fig 4.2-1B), the proliferative ability of cells was decreased (34%) after a 48h treatment with LEFTY2. (as skhown in Alowayed. et.al., 2016).

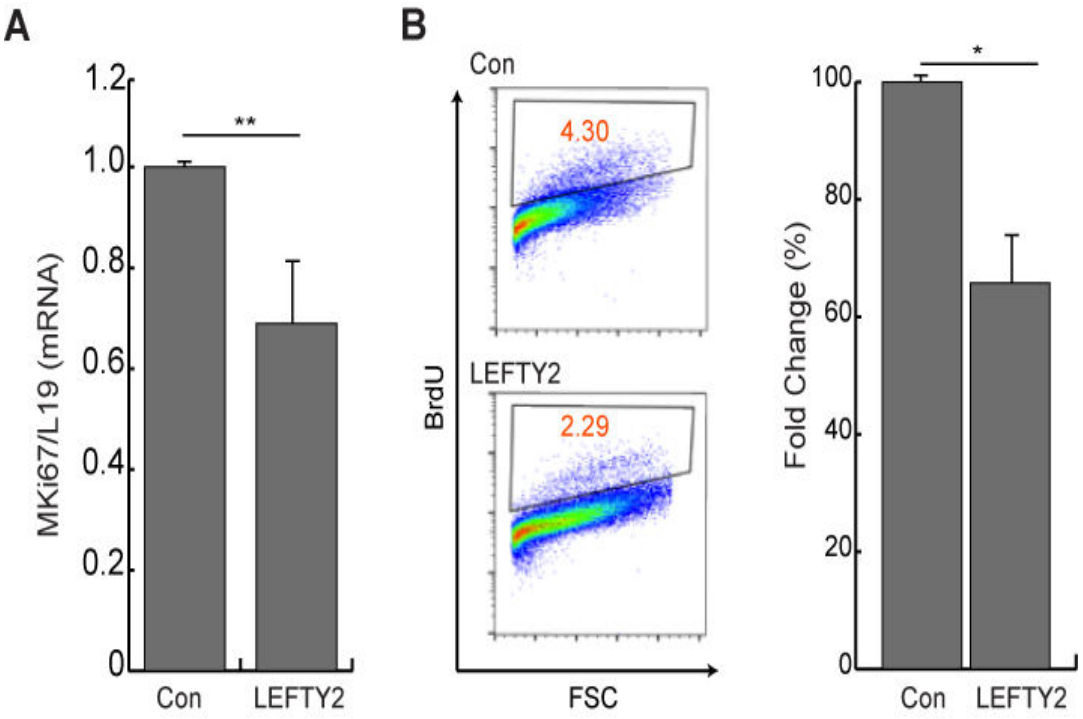

Figure 4.2-1 **LEFTY2 decreased the transcript levels of the proliferation marker MKi67. (A)** means±SEM (n = 4) of MKi67 transcript levels. **(B)** Original FACS plot of BrdU staining (% of proliferative cells). C. Fold Change (%±SEM) of cell proliferation (n = 4). *(*P*<0.05) indicates significant difference, Student's *t-test.* (as shown in Alowayed. et.al., 2016).

4.2.2 LEFTY2 significantly decreases migration in Ishikawa cells

In order to test whether LEFTY2 affects migration, a wound-healing assay was performed. To this end, confluent Ishikawa cells were treated by mechanical wounding and subsequently treated with or without LEFTY2 (25ng/ml) for 48 hours. Wound widths have been measured at 0 h and at 48 h, and wound closure was calculated (Fig. 4.2-2 A, B). Wound closure in the LEFTY2 treated cells was significantly lower when compared with the control-samples. (as shown in Alowayed. et.al., 2016).

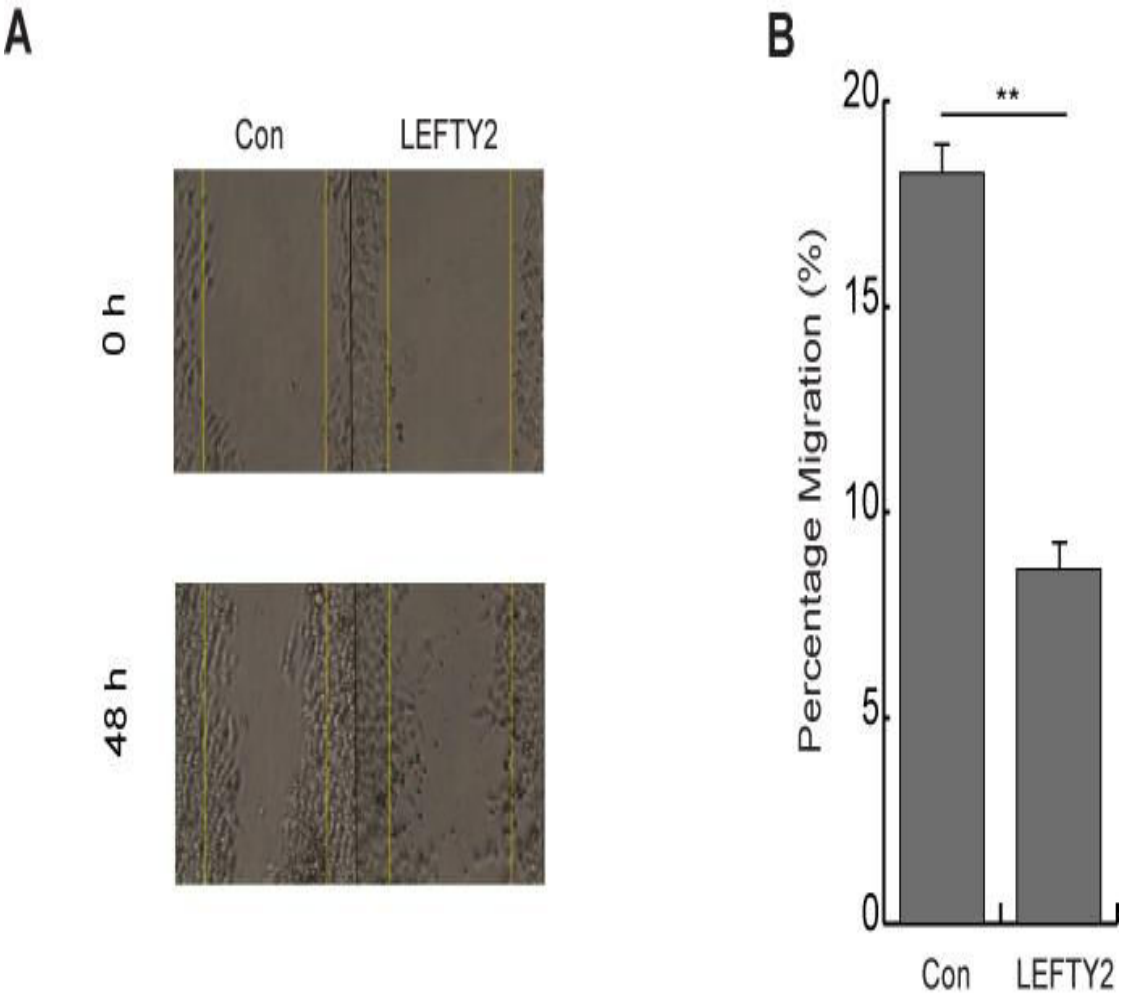

Figure 4.2-2 **LEFTY2 decreases migration in Ishikawa cells.** **(A)** Original photographs illustrating migration of human endometrial cancer Ishikawa cells without (left) and with (right) of LEFTY2 (25ng/ml) in a wound-healing assay **(B)** means±SEM (n=10) of the % of wound closure determined by image analysis without and with LEFTY2 (25ng/ml). ***(*P*<0.001) indicates significant difference, Student's *t-test.* (as shown in Alowayed. et.al., 2016).

4.2.3 The effect of LEFTY2 on migration was paralleled by an influence on the activity of focal adhesion kinase (FAK)

Next, the effect of LEFTY2 on cell migration was by of Focal Adhesion Kinase (FAK) was tested. To this end, confluent cultures were incubated as above and total protein lysates harvested for Western blot analysis. FAK activity was quantified from FAK-phosphorylation (Tyr397), utilizing a specific phospho antibody. Total FAK levels remained unchanged. As illustrated in (Fig 4.2-3 A, B), the incubated Ishikawa cells with LEFTY2 were followed by a significant reduction of FAK phosphorylation, in other words decreasing of FAK activity. (as shown in Alowayed. et.al., 2016).

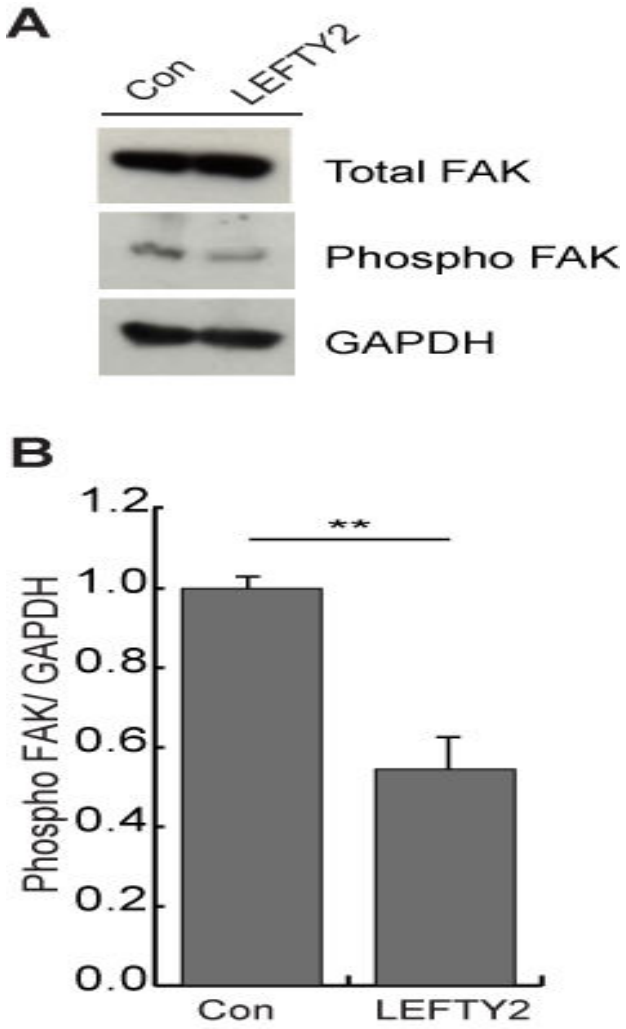

Figure.4.2-3 **The effect of LEFTY2 on migration was paralleled by an influence on the activity of focal adhesion kinase (FAK).** ***(A)*** Representative original Western blots showing phosphorylated-FAK (Tyr397), total FAK and GAPDH protein abundance in human endometrial cancer cells after 48 h culture without and with LEFTY2 (25ng/ml). ***(B)*** means±SEM (n=4) of phosphorylated-FAK/ total FAK ratio normalized to GAPDH in the cells after 48 h culture without and with LEFTY2 (25ng/ml). **($p<0.01$) indicates significant difference from absence of LEFTY2; Student's *t-test.* (as shown in Alowayed. et.al., 2016).

4.2.4 The effect of LEFTY2 on FAK activity with presence and absence of FAK inhibitor PF-573228 in Ishikawa cells

In additional experiments Ishikawa cells were incubated for 48 h with LEFTY2 (25 ng /ml) either with or without FAK inhibitor (PF 573228) (50μM) or left untreated (control). Western blotting was done to assess levels of total FAK and phosphorylated FAK (Tyr397). As shown in Figure 4.2-4, there was significantly reduction in phospho-FAK in cells treated with LEFTY2 with or without inhibitor PF 573228, compared to the control. No change was observed in total FAK levels (Fig4.2-4). (as shown in Alowayed. et.al., 2016).

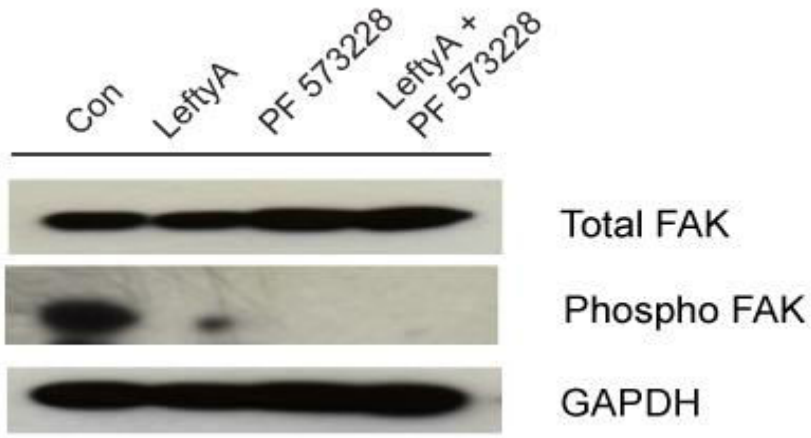

Figure 4.2-4 **The effect of LEFTY2 on FAK activity with presence and absence of FAK inhibitor PF-573228 in Ishikawa cells.** Representative original Western blots showing phosphorylated-FAK (Tyr397), total FAK and GAPDH protein abundance in human endometrial cancer cells after 48 h treatment without and with LEFTY2 (25ng/ml) ± FAK inhibitor PF-573228 (50 μM). (as shown in Alowayed. et.al., 2016).

4.2.5 The inhibition of FAK phosphorylation was paralleled by inhibition of migration

Next, we investigated whether inhibition of FAK phosphorylation was paralleled by inhibition of migration. To this end, a wound-healing assay was performed on Ishikawa cells treated with LEFTY2 with or without PF-573228 or control cells. As shown in Figure 4.2-5, the percentage of wound closure was significantly decreased 8% by a 48 hours treatment with FAK inhibitor PF 573228, which thus fully mimicked the effect of LEFTY2 on migration. The additional presence of LEFTY2 (25 ng/ml) did not lead to a further significant decrease of migratory activity, indicating that LEFTY2 was effective in large part by inhibiting FAK (Fig 4.2-5 A, B). (as shown in Alowayed. et.al., 2016).

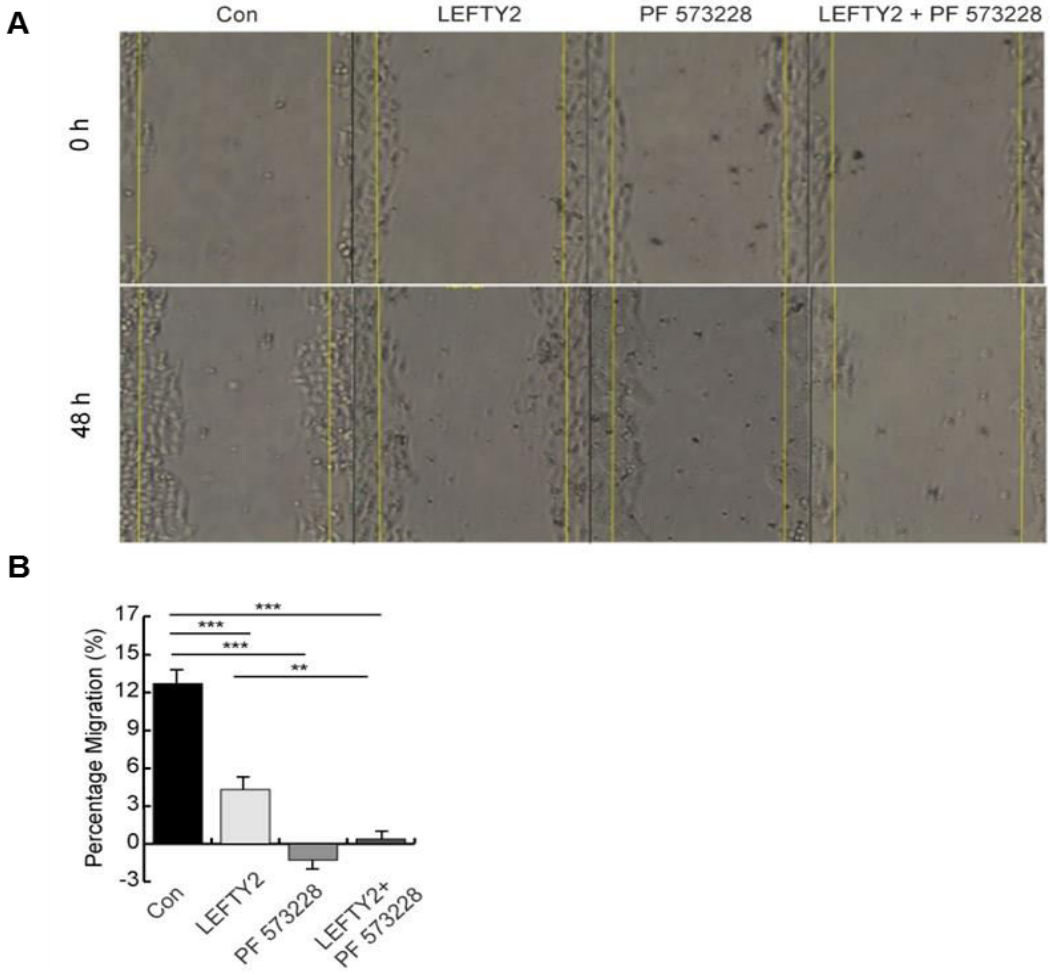

Figure 4.2-5 **The inhibition of FAK phosphorylation was paralleled by inhibition of migration (A)** Original photographs illustrating migration of human endometrial cancer Ishikawa cells without and with LEFTY2 (25ng/ml), FAK inh. PF-573228 alone (50 μM) and LEFTY2 (25ng/ml) together with FAK inhibitor PF 573228 (50 μM) at 0 h and 48 h . (**B)** means± SEM (n = 4) of the % of wound closure in the absence and presence of LEFTY2 (25ng/ml), FAK inhibitor PF 573228 (50 μM) and LEFTY2 (25ng/ml) together with FAK inhibitor PF 573228 (50 μM). **(*P*<0.01),***(*P*<0.001) indicates statistically significant difference, Student's *t-test*. (as shown in Alowayed. et.al., 2016).

4.2.6 The effect of LEFTY2 on cell proliferation and apoptosis with and without the presence of FAK inhibitor.

To investigate if the effect of migration was dependent upon cell proliferation, we performed qPCR for MKi67. As illustrated in (Fig. 4.2-6 A), incubation with LEFTY2 again decreased MKi67 transcript levels. However, treatment with the FAK inhibitor slightly decreased MKi67 levels, though not reaching statistical significance.

Flourescently labeled Annexin V provides a method for studying the cellular-apoptosis. FAK inhibitor (PF 573228) treated cells showed a slight increase in apoptotic cells (Fig 4.2-6 B) (as shown in Alowayed. et.al., 2016).

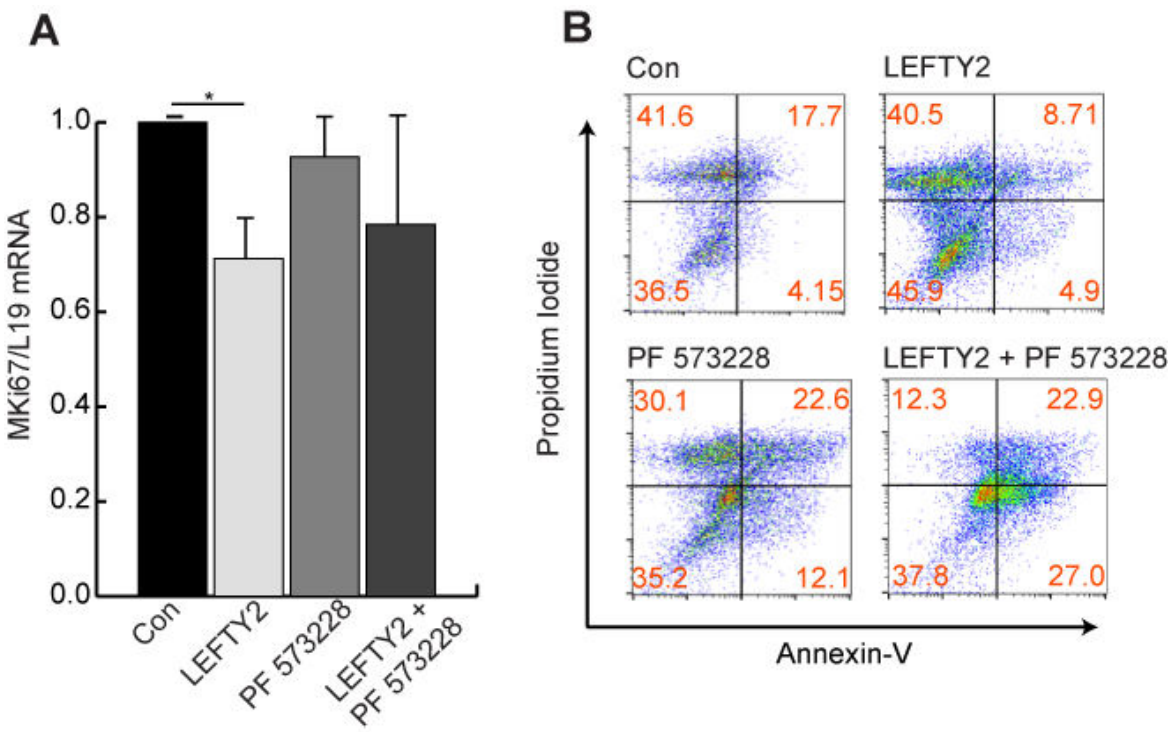

Figure 4.2-6 **The effect of LEFTY2 on cell proliferation and apoptosis with and without the presence of FAK inhibitor.** **(A)** Human endometrial cancer Ishikawa cells without and with LEFTY2 (25ng/ml), FAK inhibitor PF-573228 alone (50 μM) and LEFTY2 (25ng/ml) together with FAK inhibitor PF-573228 (50 μM) for 48 hours. Expression levels of MKi67 were measured using qRT-PCR. (**B)** In parallel experiments cells were subjected to Propidium Iodide (PI) and Annexin-V staining. Original FACS plots are shown. **P*<0.05 indicates statistically significant difference, Student's *t-test*. (as shown in Alowayed. et.al., 2016).

4.2.7 LEFTY2 influences the expression of E-cad and miRNA-200a

Invasion and metastasis (migration) in carcinoma cells is associated with overexpression of FAK activity. Previously, it has been reported that migration is inhibited by adhesion molecule E-Cadherin which is upregulated by miRNA-200. The next experiments determined whether LEFTY2 could affect the expression of E-cad and miRNA200-a in Ishikawa cells. To this end, Ishikawa cells either remained untreated or were treated with LEFTY2 (25ng/ml) with or without FAK inhibitor PF-573228 for a total of 48 hours and mRNA were collected. As a result, miR-200a was significantly upregulated in LEFTY2, PF-573228 and LEFTY2+ PF 573228 treated cells compared to the control (Fig4.2-7 A). In parallel cultures, confocal microscopy revealed that LEFTY2 with or without PF-573228 treatment further increased E-Cadherin protein abundance (Fig4.2-7 B) (as shown in Alowayed. et.al., 2016).

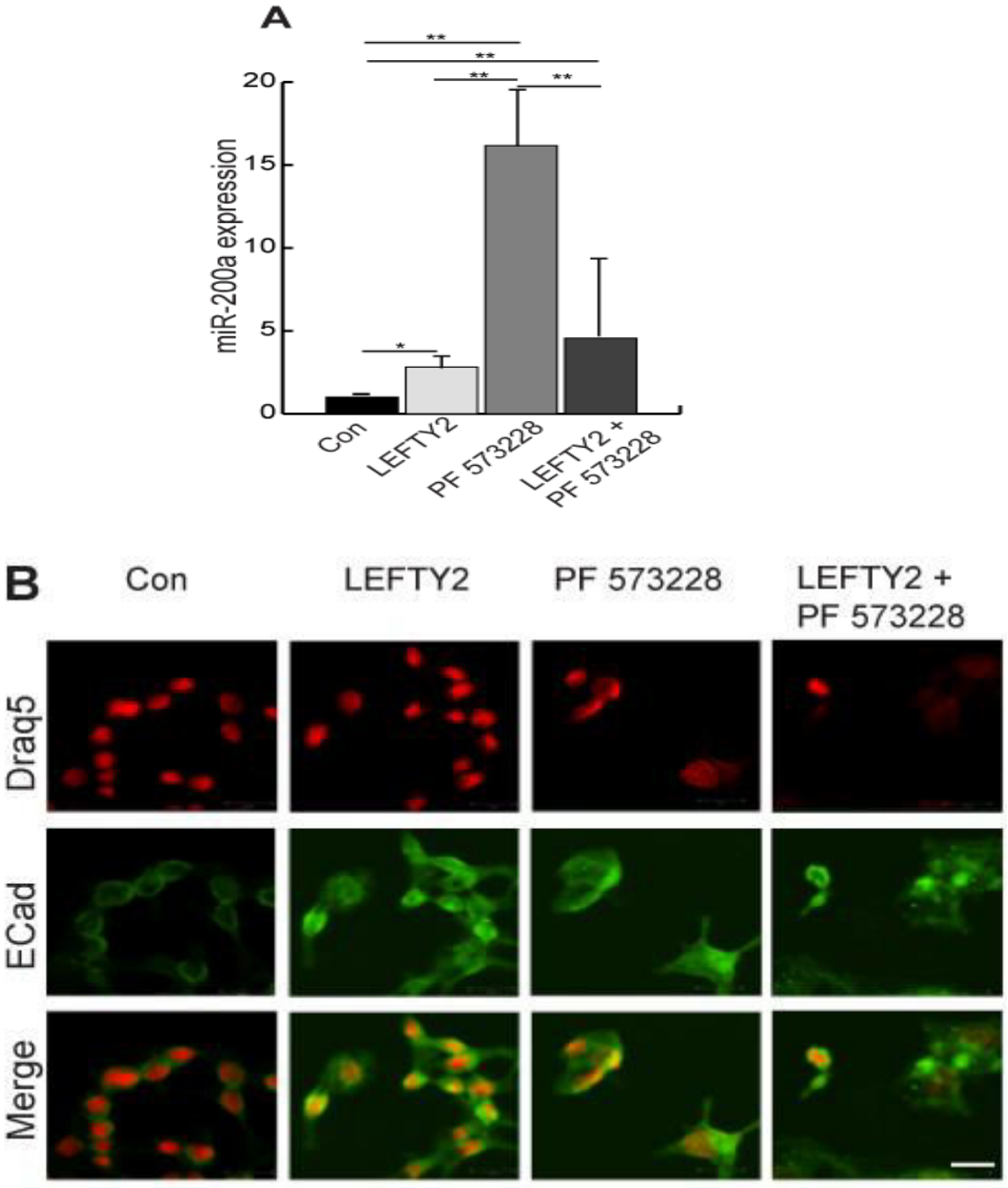

Figure 4.2-7* LEFTY2 influences the expression of E-cadherin and miRNA-200a.** **(A)** Human endometrial cancer cells without and with LEFTY2 (25ng/ml), FAK inhibitor PF-573228 alone (50 μM) and LEFTY2 (25ng/ml) together with FAK inhibitor PF 573228 (50 μM) for 48 hours. Expression levels of miR-200a/5S rRNA were measured using qRT-PCR. **(B)** Confocal Images of E-Cadherin (E-Cad) with and without LEFTY2 treatment with and without FAK inhibitor PF573228 (50 μM). Green (E-Cad), Red (Draq5; nucleus). **P*<0.05,P*<0.01 indicate statistically significant difference, Student's *t-test* (as shown in Alowayed. et.al., 2016).

5. Discussion

The discussion is based on my two publications:

LEFTY2 Controls Migration of Human Endometrial Cancer Cells via Focal Adhesion Kinase Activity (FAK) and miRNA-200a. Alowayed N, Salker MS, Zeng N, Singh Y, Lang F. Cell Physiol Biochem. 2016;39(3):815-26. doi: 10.1159/000447792. Epub 2016 Aug 9.

LeftyA decreases Actin Polymerization and Stiffness in Human Endometrial Cancer Cells. Salker MS, Schierbaum N, Alowayed N, Singh Y, Mack AF, Stournaras C, Schäffer TE, Lang F. Sci Rep. 2016 Jul 11;6:29370. doi: 10.1038/srep29370.

*5.*1 Discussion I

LEFTY2 is a member of the TGF beta superfamily (Cornet et al., 2002). LEFTY2 active forms are able to inhibit TGF-β signaling (Cornet et al., 2002; Ulloa et al., 2001). It was shown that LEFTY2 can reprogram cancer cells (Cavallari et al., 2013), inhibiting cell proliferation, stimulating the apoptosis and thereby, suppression the tumor growth (Cavallari et al., 2013; Sun et al., 2014). These lines of data show LEFTY2 as a strong suppressor of tumor cell activity (Papageorgiou et al., 2009; Saito et al., 2013).

The actin cytoskeleton provides the structural scaffold of a cell and mainly determines mechanical cellular properties (Bao and Suresh, 2003; Fletcher and Mullins, 2010) alteration of actin polymerization is in turn anticipated to modify cell stiffness. In this study, I observed that LEFTY2 significantly downregulated the cellular stiffness (Alesutan et al., 2013), volume and area compared to controls. I also showed that LEFTY2 has a negative effect of LEFYT2 on the actin filaments polymerization. Previously, researchers showed that the depolarization is related with a decrease in the stiffness of endothelial cells (Callies et al., 2011). In keeping with previous studies, I observed that LEFTY2 significantly increased the G/F actin ratio pointing the powerful effect of LEFTY2 in F actin filaments depolymerization compared to the controls.

LEFTY2 can inhibit Na^+/H^+ exchanger 1 (NHE1) activity (Salker et al., 2015). Regulators of NHE1 activity include Rac1 (Paradiso et al., 2004). Rac1 is a major controller of actin cytoskeleton and promotes the formation of lamellipodia (Nobes and Hall, 1995), the essential step for cell motility(Small et al., 2002). Rac1 can stimulate actin polymerization, either itself or by leading to auto-phosphorylation of PAK1 (Papakonstanti and Stournaras, 2002; Ridley, 2006). I tested the role of LEFTY2 on Rac1 and PAK1. LEFTY2 downregulated Rac1 and PAK1 expression and activity compared to the controls. In my study however, I showed that LEFYT2 can decrease of Rac1 (and PAK1) phosphorylation, indicating deactivation of these signaling effectors. Inhibition of these two key molecules is further expected to decrease of lamellipodia formation, which could explain the decrease of the cell area and volume.

This effect of LEFTY2 was compared to cytochalasin D drug, the cytoskeletal drug which induces rapid actin depolymerization. Indeed, in keeping with previous findings, I observed that LEFTY2 showed an increase in the G/F actin ratio reflecting depolymerization of the actin filaments compared to controls. Interestingly, washing one group of the cells with normal medium showed that the effect of LEFTY2 was reversible pointing to the temporary and not permanent effect of LEFTY2. These rapid effects, were reversible upon washout, could reflect an early response of actin cytoskeleton dynamics in receiving and mediating extracellular signals as this was previously reported as well for cytokines, growth factors and steroid hormones.

5.2 Discussion II

The results of the second part of this study uncover the negative role of LEFTY2 on activation of FAK. FAK integrin-associated phosphorylation is a regulator of actin polymerization (Koukouritaki et al., 1999) through activating downstream molecules such as PAK1 (Manser et al., 1997). It regulates many cellular processes like (cell) growth, migration in normal & cancer cells, and cell shape and stiffness (Cohen and Guan, 2005; Guan et al., 1991; Mitra and Schlaepfer, 2006; Schlaepfer et al., 2004). A previous study suggested, that decreased G/F actin in HUVECs is paralleled by a significant decrease in pFAK (Alesutan et al., 2013). In my thesis, I observed the role of LEFTY2 on FAK expression and activity. In Ishikawa cells, LEFTY2 did not show any effect on total FAK levels. On the other hand, LEFTY2 significantly downregulated phospho FAK compared to controls, pointing to an inhibitory effect of LEFTY2 on FAK activity. FAK is located in the focal adhesions and makes a link between the ECM to the cytoskeleton (Mitra et al., 2005). Therefore, by regulating the activity of FAK, LEFTY2 could contribute to regulation of other physiological processes beyond regulating actin polymerization, cell stiffness and adhesion.

Upregulation of FAK has been reported in endometrial hyperplasia and carcinoma pointing to its role in epithelial mesenchymal transition (EMT) and migration (van Nimwegen and van de Water, 2007; Zhao and Guan, 2011). EMT is the process by which the epithelial cells gain the properties of the mesenchymal cells by undergoing morphological changes (Kong et al., 2011; Thiery, 2002) and becomes more invasive. Deletion or inhibition of FAK impairs migration of fibroblasts (Mitra et al., 2005). In keeping with previous study, treatment with LEFTY2 or FAK inhibitor (PF-573228) decreases migration of Ishikawa cells. FAK inhibitor (PF 573228) treated cells showed a slight increase in apoptotic cells

LEFTY2 is a suppressor of proliferation and a stimulator of cell death (Branford and Yost, 2004). In parallel with previous reports, this thesis observed the suppressor effect of LEFTY2 on the proliferation in Ishikawa cells. qPCR was

employed to measure the MKi67, the proliferation marker. LEFTY2 showed a significant decrease of MKi67 compared to controls. BrdU staining assay was performed to study the effect of LEFTY2 during Ishikawa cells proliferation. I observed that LEFTY2 significantly downregulated the proliferation compared to controls. The proliferative ability of the cells was decreased by 34% in LEFTY2 treated cells compared to controls.

It has been shown that overexpression of LEFTY2 antagonizes TGF-β signaling and blocks EMT-induced changes, including decrease E-cadherin in human proximal tubule epithelial cells (HK-2) (Li et al., 2010). MiRNA-200 family suppresses EMT by the upregulation of E-Cad and inhibits migration in BrCa cells and HeLa cells (Korpal et al., 2008). I explored further whether LEFTY2 effects the expression of E-cadherin and miRNA-200, and I observed that treatment with LEFTY2 with or without FAK inhibitor was followed by a significant upregulation of miR-200a compared to the controls. LEFTY2 with or without FAK inhibitor treatment further increased E-Cadherin protein abundance compared to controls. That could explain another mechanism by which LEFTY2 can inhibit cell migration.

5.3 Discussion III

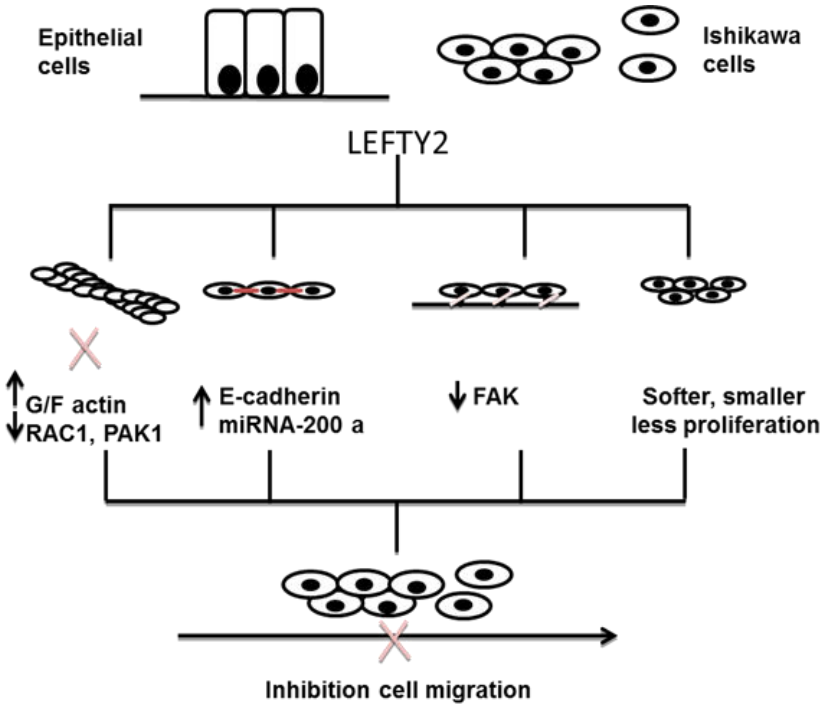

Figure 5.1 The suggested mechanisms of LEFTY2 on Ishikawa cells by which LEFTY2 may inhibit the migration of these cells. This figure is drawn by me as a summary of the results.

It has been shown in this study; LEFTY2 has an inhibitory effect on Ishikawa cell migration. I suggest that LEFTY2 may influence the migration by different mechanisms. By affecting mechanically, the cytoskeleton and by making it smaller in volume and softer which could lead to weakness in the cells ability to migrate.

Another mechanism could be, that LEFTY2 effects on Rac1 and PAK1 levels and activity, which are the trigger to cells motility process, by their critical role in the formation of the Lamellipodium which is considered as the first step in cell migration.

Reorganization of the cytoskeleton requires regulation of the actin proteins. LEFTY2 treated Ishikawa cells showed increase in G/F actin which points to the inhibitory effect on the polymerization of filamentous actin. This end could explain how LEFTY2 inhibits the formation of Lamellipodium which requires the elongation of F actin to apply the pressure on the cell membrane.

FAK is a major intermediary of signalling cascades between cells and the ECM. Current findings show that FAK could have an important role in EMT and cell migration/invasion, in this study I further suggested that LEFTY2 could inhibit the signaling between cells and the ECM by inhibiting the FAK activity, which in turn could inhibit cell migration and the actin polymerization. I observed that the migration of cells was inhibited by LEFTY2 and/or by FAK inhibitor (PF 573228), pointing to the same pathway.

LEFTY2 may inhibit the migration of Ishikawa cells by its inhibitory effect on the cell proliferation as it has been shown in the second part of this study.

It has been revealed that TGFβ induces EMT. LEFTY2 can antagonise TGF- β signaling. I showed that LEFTY2 shows increases E-Cad and miRNA-200a in Ishikawa cells. This end suggests that LEFTY2 could antagonise TGF-β signaling in EMT.

The ability of cancer cells to invade the surrounding tissue and to metastasize at a secondary site requires a highly dynamic reorganization of the actin cytoskeleton. By whatever mechanism, LEFTY2 inhibits the migration of Ishikawa cells, which could make LEFTY2 a novel therapeutic target in the future to inhibit the metastasis of primary endometrial carcinoma to the lymph nodes or inhibit invading the surrounding. The effect of LEFTY2 is temporary and not permanent which could make LEFTY2 a therapeutic target to inhibit cancer cells from metastasis by performing cyclic therapy.

Clearly, further investigations are required to fully uncover role of LEFTY2 in EMT and the diverse process MET.

5.4 Future work:

The exact mechanism of the negative effect of LEFTY2 on the ishikawa cell migration is still not totally understood. Further reserch is required to uncover the exact role of LEFTY2 in cancer progression and suppression.

- This study has been done on Ishikawa cells *in vitro*. I suggest in the future studying the effect of LEFTY2 directly in the mouse models of cancer P53 with endometrial cancer to check the effect of LEFTY2 *in vivo*.
- Another suggestion could be to obtain biopsies from the human endometrial cancer and check the levels of LEFTY2.
- Previous study showed that the overexpression of LEFTY2 was absent in the adenocarcinomas of colon metastatic to the liver, that has not been studied in the endometrial cancer. I suggest checking the expression of LEFTY2 in the invaded lymph notes and in the distant metastasis of endometrial cancer.
- Further research is required to check the effect of MiRNA 200-a treatment in Ishikawa cell on migration, the proliferation and the apoptosis.

5.5 Limitation of the study:

During this study, I acquired a number of laboratory skills ranging from cell culture to qRT-PCR analysis, Western blot analysis, Immuno-staining and wound healing migration assay. Neverless, I faced some small complications. For example, during migration study, the difficulty was in taking photos by the microscopy camera from the same wound region. This problem was easily resolved by taking three photos for each sample each time and analyse the data by taking the average.

Furthermore, there was no access to clinical samples or to apply the treatment on mouse models.

6. Summary

- This summary is based on the summary of the two publications of this study (Salker.et.al., 2016) and (Alowayed. et.al., 2016) as mentioned in the declaration.

Cancer is unregulated cell growth with the ability to invade the neighboring tissues and could metastasize to distant organs. Endometrial carcinoma is an epithelial cancer which arises from the endometrium with a potential to invade the myometrium of the uterus or/ and the cervical stroma as well as. Each year, 320 000 women worldwide are diagnosed with endometrial cancer and 76 000 will die. An urgent medical innovation is required.

In healthy endometrium, LEFTY2 is at low concentrations, but its expression levels are increased prior and during menstrual bleeding. LEFTY2 overexpression is associated not only with infertility but also with dysfunctional uterine bleeding (DUB).

LEFTY2 regulates stemness and embryonic differentiation. It is also able to down-regulate cellular growth and migration/invasion, which is dependant on actin re-organization. This process is controlled by Rac1 and PAK1.

LEFTY2 can inhibit Na^+/H^+ exchanger1 (NHE1) activity. Regulators of NHE1 activity include Rac1. NHE1 contributes further the stabilization and localization of actin. F-actin, plays a major role in maintenance cellular shape, therefore change of pH affect F-actin.

This thesis tested whether LEFTY2 can modulate the cytoskeletal elements (G/F actin) in EnCa (Ishikawa cells). Changes in G over F Actin was tested by Western blotting and flow cytometry (FACS). We showed that LEFTY2 has an inhibitory role in the actin polymerization. We used quantativeRT-PCR method to measure the mRNA levels of Rac1 and PAK1, phospho Rac1 and PAK1 were measured by immunoblotting. We show that a 2h treatment with LEFTY2 significantly reduced the transcript levels and function of RAC1 and PAK1, leading to depolymerization of the actin filaments.

Further, the function of LEFTY2 on polymerization was mirrored inhibition of both Rac1 (NSC 23766) and PAK1 (IPA-3). LEFTY2 together with either of Rac1 / PAK1 inhibitors did not show further significant depolymerization of actin.

We next tested whether LEFTY2 influences MKi67 (a celluler marker of proliferation), FAK activity, E-cad , and wound healing in our endometrial cancer cell line.

We next tested miR-200a as a possible regulator of E-Cad. We show that LEFTY2 treatment reduces MKi67, FAK and wound healing. Further, this 2h treatment increased miR-200a and E-Cad levels. Finally, we show that inhibition with PF 573228 (FAK inhibitor) mirrored the effects of LEFTY2.

In closing, this study describes the novel function of LEFTY2 in downregulating the activity of Rac1 & Pak1, which is follwed by rapid depolymerization. Furthermore, LEFTY2 reduced the expression of MKi67 and the function of FAK. On the other hand, LEFTY2 increased miR-200a and E-cad levels in keeping with its role as a negative controller of cellular proliferation and migration in EnCa.

Zusammenfassung

Ungeregeltes Zellwachstum führt zur Krebserkrankung, die die Fähigkeit in Nachbargeweben einzudringen entwickelt und auf andere Organe metastasieren könnte. Das Endometriumkarzinom ist ein Epithelialkrebs, der aus dem Endometrium ausgeht, zum Myometrium der Gebärmutter oder / und dem zervikalen Stroma eindringen könnte. Jährlich wird bei 320000 Frauen weltweit das Endometriumkarzinom diagnostiziert und 76000 Frauen sterben daran. Eine therapeutische Intervention wird deshalb dringend benötigt. LEFTY2, auch Endometriumblutungen-assoziierter Faktor (EBAF) genannt, ist ein Mitglied der Transforming Growth Factor (TGF) -β Familie. LEFTY2 ist im gesunden Endometrium in geringer Konzentration vorhanden, aber das Expressionsniveau wird vor und während der Regelblutung erhöht. LEFTY2 Überexpression steht nicht nur im Zusammenhang mit Unfruchtbarkeit, sondern auch mit einer dysfunktionalen uterinen Blutung (DUB). LEFTY2 reguliert den Stammzellenphänotyp und die embryonale Differenzierung und kann Zellproliferation und Migration hemmen. Zellproliferation und Motilität benötigen Aktinreorganisation, die unter der Kontrolle des ras-verwandten C3 Botulinumtoxin-Substrats 1 (Rac1) und p21-Protein-activated kinase 1 (PAK1) stehen.

LEFTY2 hemmt die Aktivität des Na + / H + Austauscher NHE1 und führt in Ishikawa-Zellen zu einer Verringerung des cytosolischen pH. NHE 1 Aktivität wird von Rac1 kontroliert. NHE1 trägt außerdem zur Stabilisierung und Lokalisierung von filamentösem Aktin (F-aktin) bei, das ein Zytoskelett-Struktur-Protein ist und eine entscheidende Rolle beim Aufrechterhalten der zellulären Form spielt.

Die vorliegende Arbeit untersucht, ob LEFTY2 das Aktin-Zytoskelett, die Form und die Steifheit von Ishikawa-Zellen (eine gut differenzierte Endometriumkarzinom-Zelllinie) modulieren kann. Die Wirkung von LEFTY2 auf das globuläre/filamentäre Aktin-Verhältnis wurde unter der Verwendung von

Western-Blot und Durchflusszytometrie bestimmt. Wir zeigten, dass LEFTY2 einen hemmenden Effekt auf die Aktin-Polymerisation hat. Rac1 und PAK1 Transkriptlevel wurden mittels qRT-PCR und phospho Rac1 und PAK1 wurden mittels Immunoblotting gemessen. Nach 2 Stunden Behandlung mit LEFTY2 (25 ng / ml) folgte eine signifikante Verringerung des Rac1 und PAK1 Transkriptniveaus und Aktivität, und eine Depolymerisierung des Aktin. Ferner wurde der Effekt von LEFTY2 auf die Aktin-Polymerisation durch pharmakologische Hemmung von Rac1 und PAK1 nachgeahmt. LEFTY2 zusammen mit Rac1 oder PAK1 Inhibitor führten zu keiner signifikanten weiteren Aktin-Depolymerisation.

Maligne Transformation kann durch den Verlust von Zell-Zell-Adhäsion sowie durch Erwerb der Migrationseigenschaften zur Migration führen. Die Signaltransduktion inklusive von focal adhesion kinase (FAK) und Adhäsionsmoleküle E-Cadherin waren in der Migration, Proliferation und Verbreitung von Krebszellen involviert.

Wir untersuchten als nächstes, ob LEFTY2 die Proliferationsmarker MKi67, FAK-Aktivität, E-Cadherin Menge und die Migration von Ishikawa-Zellen beeinflusst.
Darüber hinaus untersucht die Arbeit die Beteiligung von microRNA-200a (miR-200a), die durch gezielte Beeinflußung von E-Cadherin, die Zelladhäsion reguliert. Zu diesem Zweck wurde FAK-Aktivität mittels FAK-Phosphorylierung durch Western blotting, Migration durch Verwendung eines Wundheilungs Assay, miR-200a und MKi67 Expressionsniveaus unter Verwendung von qRT-PCR, Zellproliferation und Apoptose unter Verwendung von BrdU und Annexin V-Färbung bzw. und E-Cadherin (E-CAD) Menge, mit der konfokalen Mikroskopie untersucht. Als Ergebnis führte LEFTY2 (25 ng / ml, 48 Stunden) Behandlung zur Abnahme der MKi67 Expression, FAK-Aktivität sowie Migration. LEFTY2 steigert miRNA-200a und E-Cadherin levels in Ishikawa-Zellen hoch. Die Wirkung von LEFTY2 auf Migration wurde mittels FAK-Inhibitors (PF 573228) (50 µ M) bestätigt. Die Zugabe von LEFTY2 in

Gegenwart von PF-573228 führte nicht zu einem weiteren deutlichen Rückgang der Migration.

Abschließend beschreibt diese Arbeit die neue Funktion von LEFTY2 bei der Herunterregulierung der Aktivität von Rac1 und Pak1, mit daraus resultierender Aktin Depolymerisation, Zell Steifigkeit und Zellschrumpfung. Darüberhinaus vermindert LEFTY2 die MKi67 Expression und die FAK-Aktivität, erhöht miR-200a und E-Cadherin und zusammengefasst ist somit ein negativer Regulator der endometrialen Proliferation und Migration.

7. Reference list

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8. Publications:

LEFTY2 Controls Migration of Human Endometrial Cancer Cells via Focal Adhesion Kinase Activity (FAK) and miRNA-200a. Alowayed N, Salker MS, Zeng N, Singh Y, Lang F. Cell Physiol Biochem. 2016;39(3):815-26. doi: 10.1159/000447792. Epub 2016 Aug 9.

LeftyA decreases Actin Polymerization and Stiffness in Human Endometrial Cancer Cells. Salker MS, Schierbaum N, Alowayed N, Singh Y, Mack AF, Stournaras C, Schäffer TE, Lang F. Sci Rep. 2016 Jul 11;6:29370. doi: 10.1038/srep29370.

9. Declaration

I Nour Alowayed certify that:

1. The thesis being submitted for examination is my own account of my own research and it has been written by me in its entirety.

2. My research has been conducted ethically.

3. The data and results presented are the genuine data and results actually obtained by me during the conduct of the research.

4. Where I have drawn on the work, ideas and results of others this has been appropriately acknowledged and referenced in the thesis.

5. Results I is taken from “LeftyA decreases Actin Polymerization and Stiffness in Human Endometrial Cancer Cells”. Sci Rep. 2016 Jul 11;6:29370. doi: 10.1038/srep29370.

6. Results II is taken from “LEFTY2 Controls Migration of Human Endometrial Cancer Cells via Focal Adhesion Kinase Activity (FAK) and miRNA-200a”. Cell Physiol Biochem. 2016 Aug 9;39(3):815-826. doi:10.1159/000447792

7. The experimental work which incorporated in this thesis has been carried out in the Institute of Physiology I, University of Tübingen, Germany. (Figure 4.1-2E) confocal image has been measured by Dr Madhuri Salker, and Figures with flow cytometry method have been measured by Dr. Yogesh Singh.

8. The thesis submitted is within the required word limit as specified by Medical Faculty, Eberhard Karls University, Tübingen and This thesis has not been submitted for the award of any degree or diploma in any other tertiary institute.

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Curriculum vitae

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