Protective Role of Klotho against Endometrial Cell Disease

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Protective Role of Klotho against Endometrial Cell Disease

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ABBREVIATIONS

$[Ca^{2+}]_i$	Intracellular calcium
αSMA	α-smooth muscle actin
2-APB	2-aminoethoxydiphenyl borate
ADAM10	ADAM metalloproteinase domain 10
ADAM 17	ADAM metalloproteinase domain 17
AKI	Acute kidney injury
Akt	V-akt murine thymoma viral oncogene homolog
ANG II	Angiotensin II
ATP	Adenosine triphosphate
AT1R	Angiotensin type 1 receptor
Alb	Albumin
ALT	Alanine aminotransferase
AMPK	5' AMP-activated protein kinase
AngII	Angiotensin II
ARBs	Angiotensin receptor blockers
ASICs	Acid-sensing ion channels
AST	Aspartate transaminase
ATP	Adenosine triphosphate
BFA	Brefeldin A
bFGF	Basic fibroblast growth factor
BoNT	Botulinum neurotoxin
BDL	Bile duct ligation
CCh	Carbachol
ccRCC	Clear cell renal cell carcinoma
CKD	Chronic kidney disease
CPA	Cyclopiazonic acid
Cr	Creatinine

CRAC	Calcium release activated calcium channel
CsA	Cyclosporin A
CYP	Cytochrome P450 superfamily
CCC	Cation chloride cotransporter
CCh	Carbachol
CFTR	Cystic fibrosis transmembrane conductance regulator
ColIα	Collagen type I α
CREB	cAMP response element binding protein
DAG	Diacylglycerol
DAPI	4′,6-diamidino-2-phenylindole
DHE	Dihydroethidium
DMEM	Dulbecco’s modified Eagle’s medium
DN	Diabetic nephropathy
DTT	Dithiothreitol
EDA	Anhidrotic ectodermal dysplasia
EGF	Epidermal growth factor
EGR 1	Early growth response 1
ER	Endoplasmic reticulum
Erk1/2	Extracellular signal-regulated kinases1/2
ECL	Enhanced chemiluminescence
ECM	Extracellular matrix
EDTA	Ethylene diamine tetra acetic acid
EGTA	Ethylene glycol tetra acetic acid
EMT	Epithelial-mesenchymal transition
ENaC	Epithelial Na^+ channel
ET-1	Endothelin 1
FBS	Fetal bovine serum
FFPE	Formalin fixed paraffin embedded
FGF	Fibroblast growth factor
FGFR	Fibroblast growth factor receptor

FPE	Foot process effacement
FSGS	Focal segmental glomerulosclerosis
Fhht	Familial hyperkalemic hypertension
FSGS	Focal segmental glomerulosclerosis
FXR	Farenoid X receptor
GBM	Glomerular basement membrane
GEnC	Glomerular endothelial cells
GIIS	Glucose-induced insulin secretion
GPCR	G-protein coupled receptor
GRB2	Growth factor receptor-bound protein 2
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GPCR	G protein-coupled receptors
HRP	Horseradish peroxidase
HSCs	Hepatic stellate cells
H_2O_2	Hydrogen peroxide
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HCC	Hepatocellular carcinoma
HEK293	Human embryonic kidney 293
HRP	Horseradish peroxidase
IB	Immunoblotting
IGF-1R	Insulin-like growth factor-1 receptor
IHC	Immunohistochemistry
Ins	Insulin
IP	Immunoprecipitation
IGF-1	Insulin like growth factor _1
IHC	Immunohistochemistry
IL-1β	Interleukin 1- β
IR	Insulin receptor
KCC	K^+-Cl^- cotransporters
KS-WNK1	Kidney specific-WNK1

LY294002	2-(4-morpholinyl)-8-phenylchromone
Lctl	Lactase-like
LGCC	Ligand gated calcium channel
MAPK	Mitogen-activated protein kinases
MMC	Mitomycin C
MRTF-A	Myocardin-related transcription factor A
MTC	Masson's trichrome stain
M3R	Muscarinic acetylcholine receptor type 3
MAPK	Mitogen-activated protein kinase
MKK3	Mitogen-activated protein kinase kinase 3
MKK4	Mitogen-activated protein kinase kinase 4
mTOR	Mammalian target of rapamycin
NaF	Sodium fluoride
NAFLD	Nonalcoholic fatty liver disease
NASH	Nonalcoholic steatohepatitis
NCC	Na^+-Cl^- cotransporter
NCX1	Na^+-Ca^{2+} exchanger 1
NFAT	Nuclear factor of activated T-cells
NGF	Nerve growth factor
NKCC1	Na^+-K^+-$2Cl^-$ cotransporter 1
NOX2	NADPH oxidase 2
NOX4	NADPH oxidase 4
NPSS	Normal physiological salt solution
NaPi-IIa	Type IIa sodium phosphate cotransporter
NSCLC	Non-small cell lung carcinoma
OAG	1-Oleoyl-2-acetyl-sn-glycerol
OSR1	Oxidative stress responsive-1
OAG	1-oleoyl-2-acetyl-*sn*-glycerol
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction

PDGK	Platelet-derived growth factor
Pi	Inorganic phosphate
PI(3,4,5)P3, PIP3	Phosphatidylinositol-(3,4,5)-triphosphate
PI(4,5)P2, PIP2	Phosphatidylinositol-4,5-bisphosphate
PI3K	Phosphatidylinositol-4,5-bisphosphate 3-kinase
PKB	Protein kinase B
PKCα	Protein kinase Cα
PLC	Phospholipase C
PPAR	Peroxisome proliferator-activated receptor
PTH	Parathyroid hormone
PVDF	Polyvinylidene difluoride membrane
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PDGF	Platelet-derived growth factor
PIP_2	Phosphatidylinositol-4,5-bisphosphate
PLC	Phospholipase
PMSF	Phenylmethylsulfonyl fluoride
Q-PCR	Quantitative polymerase chain reaction
ROCE	Receptor-operated calcium entry
ROS	Reactive oxygen species
RTK	Receptor tyrosine kinases
RAS	Renin-angiotensin system
RhoA	Ras homolog gene family, member A
RAAS	Renin-angiotensin-aldosterone system
RCC	Renal cell carcinoma
RIG-1	Retinoic-acid-inducible gene-1
ROCC	Receptor-operated Ca^{2+} channel
ROMK	Renal outer medullary potassium channel
RTK	Receptor tyrosine kinase
RT-PCR	Reverse transcription polymerase chain reaction

SGK1	Serum glucocorticoid-induced kinase 1
SKF	SKF96365
SOCC	Store-operated calcium channel
SOCE	Store-operated calcium entry
SRF	Serum response factor
STIM	Stromal interaction molecule
SCID	Severe combined immunodeficiency
Ser473	Serine473
SERCA	Sarcoplasmic/endoplasmic reticulum Ca^{2+} ATPase
SGK-1	Serum and glucocorticoid-regulated kinase-1
SNAP	Synaptosome-associated protein
SNARE	Soluble *N*-ethylmaleimide-sensitive-factor attachment protein receptors
SOCC	Store-operated Ca^{2+} channel
SOCE	Store-operated Ca^{2+} entry
SOS	Son of sevenless
STIM1	Stromal interaction molecule 1
T2DM	Type 2 diabetes mellitus
TEM	Transmission electron microscopic
TeNT	Tetanus toxin A
TGF-β	Transforming growth factor β
Thr308	Threonine 308
TRPA	Transient receptor potential channel (Ankyrin)
TRPC	Transient receptor potential channel (Canonical)
TRPM	Transient receptor potential channel (Mucolipin)
TRPML	Transient receptor potential channel (Melastatin)
TRPP	Transient receptor potential channel (Polycystin)
TRPV	Transient receptor potential channel (Vanilloid)
TAA	Thioacetamide

TAZ	Transcriptional coactivator with PDZ-binding motif
TGFβ	Transforming growth factor beta
TRP	Transient receptor potential
TRPC	Transient receptor potential channel (Canonical)
VEGF	Vascular endothelial growth factor
VGCC	Voltage-gated calcium channel
VEGF	Vascular endothelial growth factor
VDR	Vitamin D receptor
VSMCs	Vascular smooth muscle cells
Yap1	Yes-associated protein 1
WB	Western blot
WNK	With No lysine (K)
WB	Western blot
WMN	Wortmannin
WT-1	Wilms' tumor-1

ABSTRACT

Protective Role of Klotho against Endometrial Cell Disease

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Endometriosis is a condition in which endometrial tissue, which is normally located in the inner layer of the uterine lumen of the female reproductive tract, is present outside the uterine lumen. It is a progressive disease that induces various symptoms such as pelvic pain and further devastates the patient. It occurs in 10 to 15% of women of reproductive age. The cause, pathophysiology, diagnosis, treatment, and prevention are still not precisely known. Because the cause of endometriosis is reported by various hypotheses, a clear treatment has not been suggested.

Several literature reviews have reported that SGK1, IGF1 and Orai 1 are pathogenic mediators that induce endometriosis, adenomyosis, and endometrial cancer. We are trying to elucidate pathogenic pathway of endometriosis with these mediators. Inflammation signaling has been reported to induce endometriosis by increasing Ca^{2+} entry (SOCE) through NFB in several reports. IGF1 was observed to activate Orai1 by increasing

Ca^{2+} entry (SOCE) through the PI3K/Akt pathway. Inhibition was observed to inhibit the pathway to endometriosis. SGK1 was observed to be highly expressed in uterus adenomyosis and endometrial cancer and to activate Orai1.

The αKlotho gene showed various lifespan-related phenotypes including growth retardation, vascular calcification, osteoporosis, and premature death in mice of the hypomorphic klotho allele. Conversely, mice with overexpression of the klotho gene show a lifespan extension that supports the evidence that they are anti-aging genes. αKlotho is a type 1 transmembrane anti-aging protein. αKlotho-deficient mice have premature aging phenotypes and an imbalance of ion homeostasis including Ca^{2+} and phosphate. Soluble αKlotho is known to regulate multiple ion channels and growth factor-mediated phosphoinositide-3-kinase (PI3K) signaling. Store-operated Ca^{2+} entry (SOCE) mediated by pore-forming subunit Orai1 and ER Ca^{2+} sensor STIM1 is a ubiquitous Ca^{2+} influx mechanism and has been implicated in multiple diseases. However, it is currently unknown whether soluble αKlotho regulates Orai1-mediated SOCE via PI3Kdependent signaling.

Among the Klotho family, αKlotho downregulates SOCE while βKlotho or γKlotho does not affect SOCE. Soluble αKlotho suppresses serum-stimulated SOCE and Ca^{2+} release-activated Ca^{2+} (CRAC) channel currents. Serum increases the cell-surface abundance of Orai1 via stimulating vesicular exocytosis of the channel. The serum stimulated SOCE and cell-surface abundance of Orai1 are inhibited by the preincubation of αKlotho protein or PI3K inhibitors. Moreover, the inhibition of SOCE and cell-surface abundance of Orai1 by pretreatment of brefeldin A or tetanus toxin or PI3K inhibitors prevents further inhibition by αKlotho. Functionally, we further show that soluble αKlotho ameliorates serum-stimulated SOCE and cell migration in breast and lung cancer cells.

These results demonstrate that soluble αKlotho downregulates SOCE by inhibiting PI3K-driven vesicular exocytosis of the Orai1 channel and contributes to the suppression of SOCE-mediated tumor cell migration. In the following experiments, Klotho expression was negatively correlated with tumorigenesis and serum- or IGF-1-mediated endometriosis progression indicating that Klotho may have a protective role in tubular cell as well as in endometrial cell. Altogether, these results provide novel perspectives on the mechanisms linking Klotho and growth factors and hormone receptor-mediated Ca^{2+} channel activation and offer new therapeutic strategies for the treatment of diseases caused by dysregulation of Ca^{2+} signaling.

Keywords: Endometriosis, Klotho, Orai1, IGF1, SGK1, Ca^{2+} signaling, Ishikawa cell

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I. INTRODUCTON

1.1. Endometriosis

1.1.1. Endometrial Cell induced Problems

In clinical practice, the dysfunctional problem that originates from endometrial cells is endometriosis, which occurs when endometrial cells grow in pelvic organs other than the uterus, and endometrial cells proliferate in the muscular layer of the uterus, leading to hypertrophy of the uterine muscle and bleeding. Adenomyosis and endometrial cancer that cause pain and discomfort. Obstetrically, it causes unexplained infertility due to endometrial dysfunction, recurrent miscarriage, and premature birth with premature labor and premature rupture of the membranes. It can be seen that the tissue of the endometrium

causes various problems, from benign and malignant disease as well as gynecological obstetric problems to organs other than the uterus as well as the uterus, and the diagnosis encompassing all these concepts is endometriosis. Endometriosis is a disease with evidence of progression from adenomyosis of the uterine wall to endometrial cancer and from endometrioma of the ovary to ovarian cancer. In addition, the endometrial tissue contains blood vessels and nerves, so it is accompanied by pain and excessive bleeding, so it is a diagnosis that most covers various problems caused by endometrial cells.

1.1.2. What is Endometriosis?

Endometriosis is a condition in which endometrial tissue, which is normally located in the inner layer of the uterine lumen of the female reproductive tract, is present outside the uterine lumen. It is a progressive disease that induces various symptoms such as pelvic pain and further devastates the patient. It affects 5–10% of women of reproductive age, up to 80% of women with pelvic pain, and 20–50% of women with infertility [1,2]. It is a progressive disease that induces various symptoms such as pelvic pain and further devastates the patients. Endometriosis is relatively common in women of reproductive age and potentially during the before menstrual period. It is a debilitating condition that affects women.

Endometriosis is an estrogen-dependent, benign inflammatory disease caused by ectopic endometrial glands and stroma, and therefore accompanies fibrosis. These glands and stroma are typically pelvic organ and cavity, but are also found in below the diaphragm organ. There are three subtypes of endometriosis: superficial peritoneal lesions, deep infiltrating lesions, and cysts (endometriomas) containing blood and endometrium-

like tissue [3].

Symptom of pains typically include painful intercourse (deep dyspareunia), dysmenorrhea, bladder and bowel pain, and chronic pelvic pain. In addition, endometriosis is often associated with infertility, and even in asymptomatic women, endometriosis is commonly found during infertility tests [4]. Symptoms of endometriosis continue to cause long term adverse effects on interpersonal relationships, quality of life, and work productivity for individuals until menopause. Symptoms caused by endometriosis increase women's loss of work productivity and medical costs, and among them, pain, infertility, and persistent disease are the factors that lower women's quality of life the most.

1.1.3. What are the cause of Endometriosis?

The cause, pathophysiology, diagnosis, treatment, and prevention are still not precisely known. However, among several hypotheses proposed for the development of endometriosis, representative theories include menstrual regression, immune changes, metaplasia of the embryonic epithelium, and metastatic spread [5]. Recent studies suggest stem cells and genetic origins as well. Among the several hypotheses proposed to explain the pathogenesis of endometriosis, the hypothesis that endometrial tissue flows back into the pelvic cavity through the fallopian tubes during menstruation has received the most attention [7]. However, while this hypothesis could explain how endometrial cells could reach this ectopic region, the factors involved in their growth were unclear. Also, the explanation of whether some women develop endometriosis among menstruating women and protect others is not sufficient. As a hypothesis for this phenomenon, abnormalities in the immune system of patients with endometriosis have been proposed, and the quality of

stimulation of endometrial protein antigen in women prone to endometriosis was more immunogenic than in women without endometriosis. It can be thought that an immune system abnormality may be the cause. Indeed, numerous cell-mediated and humoral immune system abnormalities have been observed in patients with endometriosis [5,6]

Functional defects of natural killer cells in patients with endometriosis [8,9,10], macrophage count and Increased activity [11], lack of T lymphocyte-mediated cytotoxic action on endometrial cells [12]. have been reported, and nonspecific antibodies such as antinuclear antibody and antilupus factor in the blood of patients was detected [13]. In addition, the presence of auto-antibodies against endometrial tissue, anti-endometrium antibodies, is confirmed by a method of immune diffusion [14,15], immunofluorescence and immunochemistry [16,17,18], passive hemagglutination [19,20], western blot [21,22,23], enzyme immunization method [24,25]. Kim et al. [25] reported that anti-endometrial antibodies in the blood were observed in 48.1% and 55.1% of endometriosis patients using double-label immunohistochemistry and western blot. It has been suggested that endometrial protein may be a specific antigen. Like other organs in the pelvis, endometrial implants are in constant and continuous contact with the intraperitoneal fluid, and it has been reported that the intraperitoneal fluid of patients with endometriosis causes more proliferation of normal human endometrial stromal cells than that of patients without endometriosis. [26] suggested the possibility that certain components in the abdominal fluid may play an important role in the growth and maintenance of endometrial hyperplasia.

In endometriosis patients, a difference in the concentration of sex steroid hormones, which has long been known as an important growth factor for endometrial implants, was not observed in the abdominal fluid of endometriosis patients and control patients [27,28,29]. Recently, it has been revealed that local growth factors are involved in

cell proliferation and differentiation in many tissues, including those dependent on sex steroid hormones, but epidermal growth factor (EGF) and fibroblastic growth factor (FGF) have It was reported that there was no difference with their concentrations in the intraperitoneal fluid of patients without endometriosis [30].

Meanwhile, insulin-like growth factor (hereinafter abbreviated as IGF) has two types, IGF1 and IGF2, which act through cell receptors [31]. The role of IGF as a mediator of various growth factors such as estrogen, transforming growth factor (TGF), EGF, and FGF has been revealed depending on the tissue [32] In addition, IGF is involved in the differentiation and proliferation of endometrial stromal cells in vitro [33] and human endometrial tissue [34,35], alveolar megakaryocytes [36], IGF can be produced in the intraperitoneal megakaryocytes of mice [37], but the activity of these megakaryocytes has been reported in patients with endometriosis. Furthermore, recently Giudice et al. [34] suggested the presence of IGF in the abdominal fluid of 7 infertile women, and Kim et al. [38] reported that the intraperitoneal fluid concentration of endometriosis patients was higher than that of control patients.

1.1.4. Clinical diagnosis and treatment strategy of Endometriosis

a. Natural course of symptomatic endometriosis

It is difficult to assess the natural course of symptomatic endometriosis. First of all, invasive testing is essential to exclude the diagnosis of endometriosis in adolescent girls who complain of severe menstrual pain. In addition, it is not clear whether

endometriosis is a progressive disease and if so, which factors regulate progression, and whether initial treatment reduces disease progression in endometriosis is also unknown.

b. Other conditions commonly associated with endometriosis

In the survey data of the group diagnosed with endometriosis, an increase in autoimmune disease, chronic fatigue, allergies, asthma, and fibromyalgia were self-reported. Although endometriosis is a benign disease, histologically, the association with endometriosis and clear cell or endometrioid ovarian cancer is small but consistently reported [39].

c. Clinical diagnosis of endometriosis

Although the clinical manifestations of endometriosis vary widely, the most common clinical features that should raise suspicion are pelvic pain and infertility that begin immediately after menarche. Although endometriosis cannot be definitively diagnosed by clinical examination, pain on vaginal examination, tender nodules in the posterior fornix, adnexal masses, and immobility of the Findings such as uterus, particularly fixed retroversion, etc. are important diagnostic pointers.

A comparative study between clinical examination, transvaginal ultrasonography, and magnetic resonance imaging (MRI) in bimanual examination lacked sensitivity and specificity in the diagnosis of endometriosis, with less than 50%. Transvaginal ultrasonography was superior to MRI in terms of sensitivity (95% v 76%), specificity (98% v 68%), and accuracy (97% v 71%) [40].

d. Treatment strategy of endometriosis

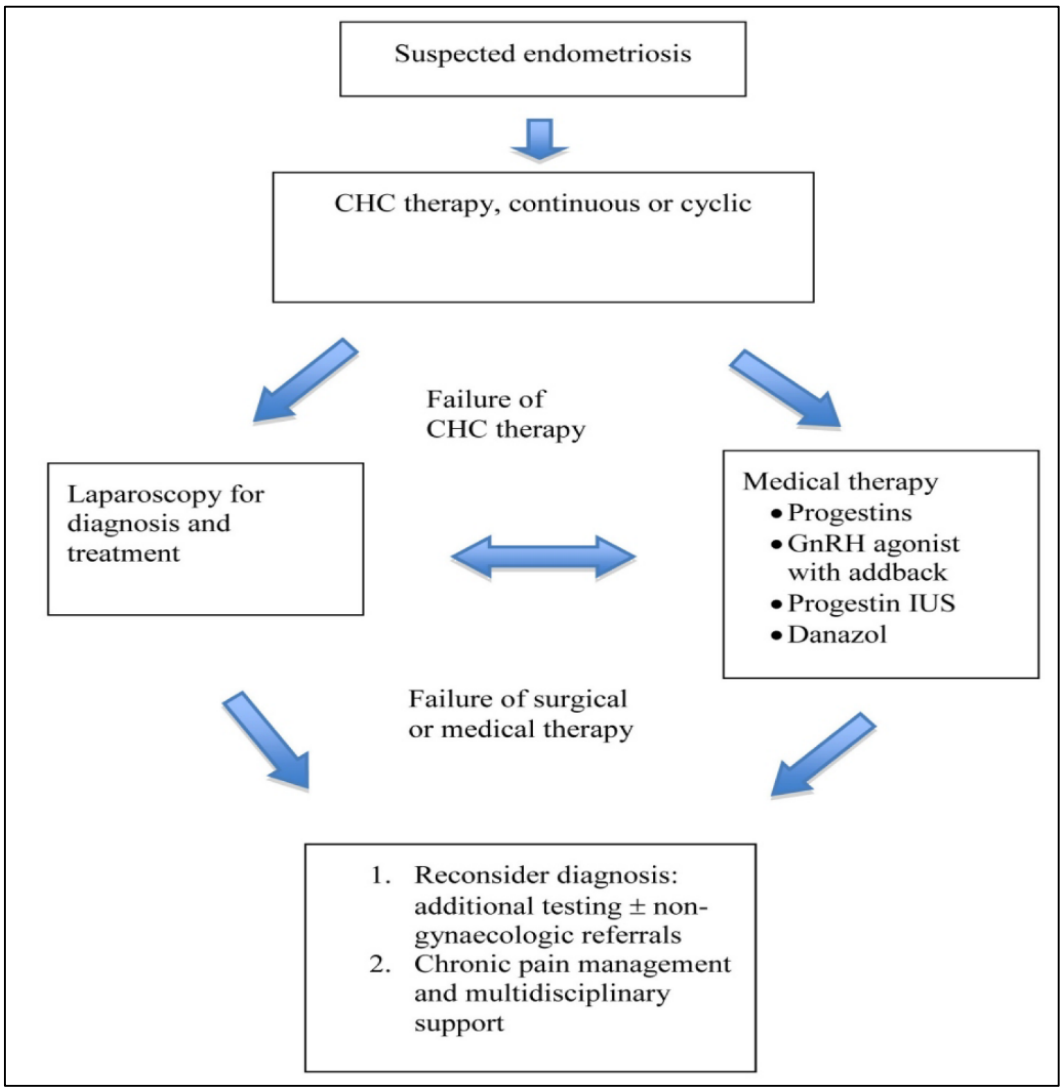
The purpose of endometriosis treatment is to relieve pain and improve fertility, which can achieve good treatment results by removing all possible endometriosis lesions. Since endometriosis is a chronic disease and has a high recurrence rate despite surgery and drug treatment, it is important to establish a long-term treatment strategy aimed at improving the patient's quality of life. In particular, pain can persist even after appropriate medical and surgical treatment of the disease.

Despite many studies over a long period of time, the molecular and cellular pathophysiology of endometriosis and the ultimate treatment have not yet been clearly elucidated. It is a disease with character. For endometriosis, it is necessary to confirm the lesion through a questionnaire, pelvic examination, and laparoscopy for confirmation.
Surgical treatment of endometriosis requires careful consideration of indications for surgery, preoperative evaluation, surgical method, and operator's experience. When endometriosis is discovered, if the lesion is surgically removed, pain reduction within 1 year of surgery is observed in 50-95% of cases, so it is a principle to remove it [41]. Indications for surgical treatment include pelvic pain, suspected or accompanying ovarian endometrioma, suspected anatomical damage due to endometriosis in infertile patients, or discovered endometriosis during diagnostic laparoscopy or other operations. Drug treatment for endometriosis is effective when it is accompanied by pain, and if it is accompanied by infertility, it may be helpful to use a gonadotropin agonist for a certain period before assisted reproductive surgery.

Indications for drug treatment can be divided into the case of pain and the case of infertility. If medication is administered after surgical removal, the recurrence of pain can be delayed [42,43]. When endometriosis is confirmed by surgery, if the lesion remains or

pain persists, when it recurs, or even if there is no lesion or pain, drug treatment may be considered to reduce the recurrence. For drug treatment, oral contraceptives, progestogens such as dienogest medroxyprogesterone acetate, gonadotropin agonists, danazole, and gestrinone can be considered as first-line drugs. In the treatment of pain caused by deep endometriosis lesions and adenomyosis, levonorgestrel-releasing intrauterine devices may be effective.

In patients with endometriosis accompanied by infertility, surgical treatment can increase the likelihood of spontaneous pregnancy after surgery. After surgery, try natural pregnancy for at least 12 months and, if unsuccessful, try assisted reproductive technology. If hydrocephalus, endometrioma of a medium or larger ovary, or pelvic pain are accompanied, surgical treatment is performed immediately. let it be implemented



(July JOGC JUILLET 2010)

Fig.1. Management of pain Associated with suspected endometrioisis

1.2. Klotho

1.2.1. History

The klotho gene was originally identified as being mutated in a mouse strain in which inherited phenotypes closely resemble human aging [44]. Mice homozygous for a hypomorphic klotho allele (kl/kl) displayed multiple aging-like phenotypes including growth retardation, vascular calcification and osteoporosis and died prematurely at around two to three months of age [44]. Conversely, overexpression of the klotho gene extends the life span in mice, which supports the notion that klotho is an aging-suppressor gene [45]. Since the discovery of Klotho, two related paralogs, βKlotho and γKlotho (or Lctl or KLPH), have been identified as Klotho family members [46,47]. Klotho is also called αKlotho in order to distinguish it from the other two members [48]. In this review, Klotho is simply used to refer to αKlotho. This review introduces the structure and function of Klotho and summarizes the current knowledge of Klotho as it relates to human aging and disease.

1.2.2. Structure and expression of Klotho

The *Klotho* gene is composed of 5 exons [49,50] and encodes a type-1 single-pass transmembrane glycoprotein (1014 and 1012 amino acids in mouse and human, respectively) that is located at the plasma membrane [51,50] and Golgi apparatus [52] (Fig.2). The intracellular domain is very short (~10 amino acids) without functional domains. The extracellular domain has two internal repeats, KL1 and KL2, which have amino-acid sequence homology to family 1 glycosidases that hydrolyze-glycosidic linkage in saccharides, glycoproteins and glycolipids [51, 53, 54]. Klotho is expressed in multiple tissues and cell types, particularly high in the kidney (Fig.3). Klotho is abundantly expressed in distal convoluted tubule in the kidney and choroid plexus in the brain [51]. It is also expressed in renal proximal tubule [55], parathyroid gland [50, 56, 57] and several sex organs including ovary, testis and placenta[51].

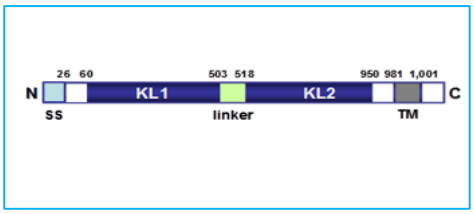


Fig.2. structure of Klotho: klotho is type1 TM glycoprotein, extracellular domain, KL1

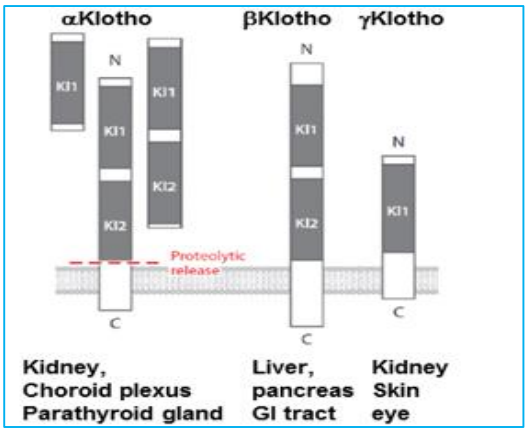


Fig.3. Klotho family : αklotho(=klotho),
βklotho (bile acid synthesis)
γ klotho (lactase-like (Lctl) gene

1.2.3. Function of Klotho

1. Membrane bound Klotho

Klotho forms complexes with diverse FGFRs (FGFR1c, FGFR3c and FGFR4) and increases their affinity selectively to FGF23, a bone-derived phosphouretic hormone. FGF23 acting on the Klotho-FGFRs complex plays an important role in Ca^{2+} and phosphate homeostasis [58,59]. Membrane-bound Klotho is involved in FGF23 action, thereby promoting Pi excretion followed by low serum Pi. In addition, FGF23 acting on the Klotho-FGFRs complex at the basolateral side stimulates renal Ca^{2+}reabsorption via the TRPV5 channel, which is expressed in the apical membrane of the distal convoluted tubule.

βKlotho contributes to the regulation of energy metabolism as an obligatory co-receptor for FGF15 (the mouse ortholog of human FGF19 and FGF21 [59,61]. Expression of FGF15/19 in the intestine is regulated by bile acid [62]. This intestine-liver endocrine axis mediated by FGF19 and βKlotho is indispensable for maintaining bile acid homeostasis, as evidence by the fact that mice lacking FGF15, βKlotho or FGFR4 exhibit increased Cyp7α1 expression and bile acid synthesis in the liver [62]. By contrast, FGF21 is secreted from the liver upon fasting and acts on adipose tissue to promote lipolysis [63]. Thus, βKlotho is required to regulate energy metabolism in the fasting state. ɣKlotho is highly and selectively expressed in brown adipose tissue and the eye and can function as an additional co-receptor for FGF19 in cultured cells [60].

2. Intracellular Klotho

Although Klotho is present on the cell surface, large amounts of Klotho immune reactivity are detectable in the cytoplasm in mouse kidneys and human parathyroid glands [64]. In these tissues, Klotho binds Na+-K+-ATPase and stimulates its surface abundance and activity. Klotho interacts physically with Na+-K+-ATPase in intracellular organelles, not at the plasma membrane. The intracellular negativity and low [Na+]i created by Na+-K+-ATPase activation provide driving force for transepithelial Ca^{2+} transport in the choroid plexus and the kidney [64].

It is well established that senescence is associated with increased expression of pro-inflammatory cytokines such as IL-6 and IL-8, which is mediated by retinoic-acid-inducible gene-I (RIG-I). Klotho suppresses RIG-I-mediated senescence-associated inflammation, suggesting that Klotho functions as an intracellular anti-inflammatory and anti-aging factor [65].

3. Secreted Klotho

The secreted (or soluble) form of Klotho functions as a humoral factor that targets multiple tissues and organs independent of FGFRs. Although Klotho functions as a co-receptor for FGF23, secreted Klotho may not function as a
soluble receptor for FGF23 [66]. The Klotho-FGFR complex has high affinity for FGF23, but not secreted Klotho or FGFR alone, indicating that secreted Klotho exerts its biological effect independent of FGF23 [66].
Secreted Klotho exerts anti-aging and organ protection effects role with
pleiotropic actions. First, secreted Klotho downregulates the signaling of growth factors

and cytokines such as insulin, IGF-1, TGF-β and IFNɣ [67,68,69]. Second, secreted Klotho maintains ion homeostasis by regulating ion channels and/or transporters. Secreted Klotho modifies the N-glycan of channels and transporters via its β-glucuronidase and/or sialidase activity [70,71,72].

1.2.4. Klotho in Human Aging and Disease

Klotho is an anti-aging protein with pleiotropic actions that exerts organ protection [73,74]. Several lines of evidence support the notion that Klotho functions as a human aging-suppression molecule. Polymorphisms of KLOTHO are correlated with life span [75], coronary artery disease [76], atherosclerosis [76] and osteoporosis [77] in humans. Klotho is also associated with severe calcinosis and stroke [78,79]. Klotho deficiency is involved in acute and chronic kidney diseases [74], cancers [80] and salt-sensitive hypertension [81]. Actually, the serum level of Klotho decreases with aging in humans [82]. However, the biological function of Klotho and the way in which Klotho deficiency contributes to age-related diseases remain elusive.

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II. SPECIFIC AIMS AND RESEARCH DESIGNS

2.1. Rationale and hypothesis

In the pathogenesis of endometriosis and endometrial cancer, inflammation may be a therapeutic strategy for endometriosis development, including cell adhesion, invasion, angiogenesis, inflammation and apotosis, as reported in the above journal.

First, In the pathogenesis of endometriosis and endometrial cancer, inflammation may be a therapeutic strategy for endometriosis development, including cell adhesion, invasion, angiogenesis, inflammation, and apotosis, as reported in the above journal. NFkB transcriptional activity modulates key cell processes contributing to the initiation and progression of endometriosis. Because endometriosis is a multifactorial disease, inhibiting NFkB appears to be a promising strategy for future therapies targeting different cell functions involved in endometriosis development such as cell adhesion, invasion, angiogenesis, inflammation, proliferation, and apoptosis. Upcoming research will elucidate these hypotheses [83]. In vitro and in vivo studies show that NFkB–mediated gene transcription promotes inflammation, invasion, angiogenesis, and cell proliferation and inhibits apoptosis of endometriotic cells. Constitutive activation of NFkB has been demonstrated in endometriotic lesions and peritoneal macrophages of endometriosis patients. Agents blocking NFkB are effective inhibitors of endometriosis development and some drugs with known NFkB inhibitory properties have proved efficient at reducing endometriosis -associated symptoms in women. Iron overload activates NFkB in macrophages. NFkB activation in macrophages and ectopic endometrial cells stimulates synthesis of proinflammatory cytokines, generating a positive feedback loop in the NFkB

pathway and promoting endometriotic lesion establishment, maintenance and development. [83]

The second, the association of IGF 1 with endometriosis has been reported, and it is especially important in young women, and there has been a mention of severe pain in endometriosis. Case-control studies that evaluated associations between serum or peritoneal fluid IGF1 or IGFBP-3 levels and endometriosis risk reported inconsistent findings [84, 85-88, 89]. Gurgan et. al. [84] investigated the association between serum and peritoneal fluid IGF1 and IGFBP-3 levels among 44women and found that mean serum and peritoneal IGF1 levels of controls and early-stage endometriosis cases were significantly lower than those of late-stage cases, while there were no significant differences in mean IGFBP-3 levels between the groups. Kim et al. [85] studied peritoneal IGF1 and IGFBP-3 among 43 women and found that mean IGF1 levels were significantly higher in cases than in controls, whereas mean IGFBP-3 levels were significantly lower in cases than in controls.

Third, it has been reported that SGK1 promotes endometrial cell proliferation and migration and is highly expressed in endometrial cancer. In nature medicine, SGK1 plays a critical role in fertility in the endometrium, but SGK1 is upregulated in unexplained infertility and habitual abortion. It was reported that the recurrent miscarriage was down-regulated. The serum-and-glucocorticoid-inducible-kinase-1 (SGK1) is ubiquitously expressed and under genomic control by cell stress, hormones and further mediators. A most powerful stimulator of SGK1 expression is transforming growth factor TGFβ1. SGK1 is activated by IGF1 via phosphatidylinositol-3-kinase and the 3-phosphoinositide-dependent kinase PDK1. As shown recently, SGK1 increases the store-operated Ca^{2+}entry (SOCE), which is accomplished by the pore-forming ion channel unit Orai1. Most recent

observations further revealed that SGK1 plays a critical role in the regulation of fertility. SGK1 is up-regulated in the luminal epithelium of women with unexplained infertility but down-regulated in decidualizing stromal cells of patients with recurrent pregnancy loss. The present study explored whether Orai1 is expressed in endometrium and sensitive to regulation by SGK1 and/or TGFβ1. To this end, Orai1 protein abundance was determined by western blotting and SOCE by fura-2 fluorescence. As a result, Orai1 was expressed in human endometrium and in human endometrial Ishikawa cells. Orai1 is expressed in the human endometrium and is up-regulated by SGK1 and TGFβ1. The present observations thus uncover a novel element in SGK1-sensitive regulation of endometrial cells [90].

Therefore, assuming the pathologic mediator signaling pathway of IGF1 and SGK1, it can be hypothesized that acute and chronic stimulation will lead to inflammation, fibrosis, and tumor progression through Orai1 activation and ca entry by IGF1 and SGK1 (Fig. 4).

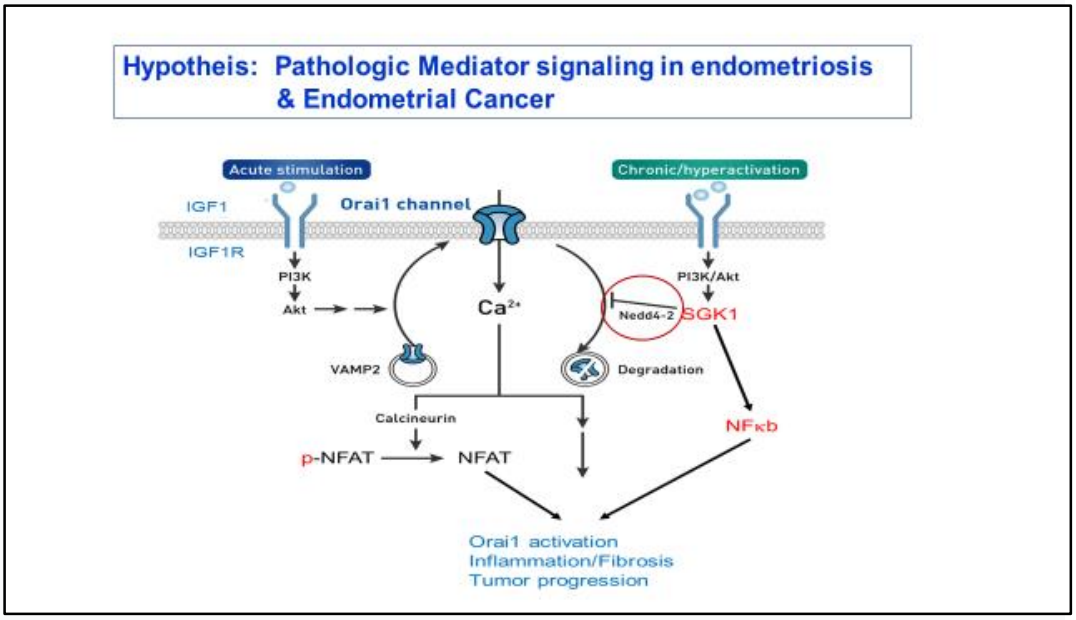

Fig. 4. Summarize of the hypothesis.

2.2. Specific aims

Specific Aim 1: To examine the TNFα upregulates Orai1 expression and SOCE Ishikawa cell. To determine whether TNFα by infammatory stimulation upregulates Orai1 expression and ca entry. The purpose of this study was to test that Orai1 is overexpressed by TNFα and Ca^{2+} signaling and SOCE are increased in Ishikawa cells, an endometrial cancer cell.

Specific Aim 2: To examine Klotho suppressed TNFα-induced Orai1 overexpression and NFκb signaling in endometrial cells.

Specific Aim 3: To examine expression of SGK1 and IGF1 receptor and Orai1 in Human endometriosis and endometrial cancer tissue, and role of Klotho. According to the references that SGK1 and IGF1 receptor and Orai1 are overexpressed in human endometrial cancer and endometriosis tissue, ovarian endometrioma and endometrial To check the expression in cancer tissue, and to observe the expression level of Klotho in endometriosis and endometrial cancer.

2.3. Research design

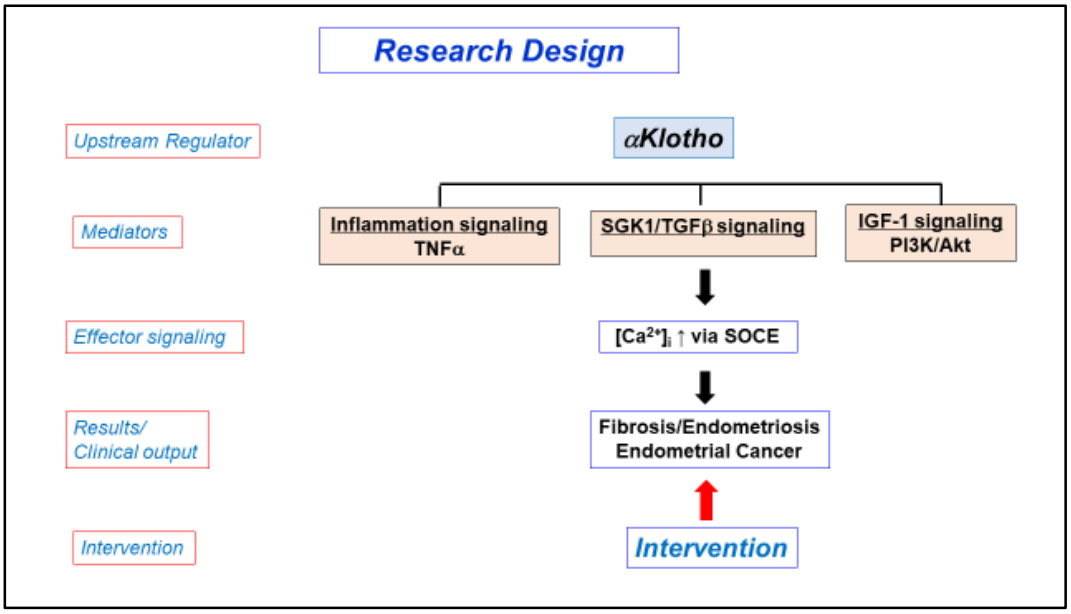

Fig. 5. Summarize of the Research Design.

In this paper, the process of Ca^{2+} entrance and Orai1 activation through SOCE by pathologic mediators such as Inflammation signaling such as TNFα and IGF1 signaling through SGK1 and PI3K/Akt in the process of inducing endometriosis and endometrial cancer is researched. It was done by design.

III. MATERIALS AND METHODS

3.1. Ethnics of human experiments

According to the Consolidated Standards of Reporting Trials (CONSORT), we conducted a randomized controlled trial of patients who underwent laparoscopic ovarian cystectomy between January 2019 and September 2019 in the Department of Obstetrics and Gynecology at the Yonsei Wonju Severance Christian Hospital. The study was approved by the Institutional Review Board (IRB) of the Yonsei Wonju Severance Christian Hospital (CR118035). All procedures were conducted in adherence to the Declaration of Helsinki and CONSORT.

3.2. Ethnics of animal experiments

The animal experimental protocol used in the present study was approved by the Institutional Animal Care and Use Committee, Yonsei University Wonju College of Medicine. The approval number for TRPC6 knockout mouse is YWC-151228-1 and for fibrosis animal models are YWC-140902-1 and YWC-150908-2.

3.3. Patients and tissue samples

The tissue samples were collected from patients diagnosed with diabetic nephropathy or ccRCC who had undergone surgery at the Yonsei University Wonju Severance Christian Hospital. Formalin-fixed paraffin embedded (FFPE) were collected for

immunohistochemistry (IHC). Pathologic reports and clinical records were reviewed. Fresh tissues were also collected in liquid nitrogen for immunoblotting (IB) assay.

3.4. Animal models

Thirty female mice (C57B6/J) aged 12 weeks and around 21–23 g were randomly divided into three groups according to hemostasis techniques: the sham (no artificial incision injury and treatment to the ovary) group, the bipolar coagulation group, and the Epi-pledget compression group (n=10 per group). After an operation was performed to create an artificial incision injury to the ovary, bipolar coagulation, and Epi-pledget compression techniques were applied to the injured ovary. Five mice in each group were sacrificed at postoperative day 1. The remaining five mice were sacrificed at postoperative day 4. Except for the sham group, the ovary wounds in all other groups were treated with hemostasis. The hair on the backs of the mice was shaved 2 days before surgery. Mice were anesthetized via inhalation followed by a transverse incision at the lower back of the mouse (Fig. 4A) and entry into the retroperitoneal space to approach the ovary. A partial incision of the ovaries resulted in wound production and bleeding of the ovary. To stop the bleeding, bipolar coagulation and diluted epinephrine (0.5 mg/mL of epinephrine in 50 mL of saline solution)-soaked cotton pledget compression were introduced. When the stoppage of bleeding was confirmed in the damaged lesion, the skin was closed by suture and the mice were checked for vital signs and complications at 1 and 4 days. Histological evaluations of ovaries and determination of ovarian reserve. Ovaries were harvested and included normal and wounded areas. All tissues were fixed in 4% paraformaldehyde for

48 h at room temperature. After fixation, ovaries underwent dehydration in graded ethanol and xylene and then were embedded in paraffin. Sections were cut with a thickness of 5 μm and attached onto slides. The sections were deparaffinized in xylene and rehydrated in graded alcohol. After hydrating with deionized water, the sections were stained with hematoxylin and eosin (H&E), Masson's trichrome (MTC) for detecting fibrosis, or Ki-67 for detecting proliferation. Stained sections were examined in a bright field with an Olympus microscope and photographed with a DP73 camera; the images were exported to CellSens Software. Fibrotic areas were measured and analyzed using Image J software (http://imagej.nih.gov/ij/imdex/html)

3.5. Cell culture and Transfection

A HEK293 cell line with an inducible mCherry-STIM1-T2A-Orai1-eGFP (provided from Dr. Chan Young Park (UNIST, Korea)) [91] and HEK293FT cells were cultured under high glucose DMEM medium (cat no. SH30243, Hyclone, Logan, UT, USA) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin. The human breast cancer cell line MDAMB231 and the human lung cancer cell line H1693 cells were cultured under RPMI1640 (cat no. SH30027, Hyclone, Logan, UT, USA) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin. All DNA plasmids were transfected by using X-tremeGENE HP DNA transfection reagent® (Roche, Mannheim, Germany) following the manufacturer's instructions. Experiments were conducted 48 h after transfection. For knockdown by siRNA, oligonucleotides were transfected into MDA-MB231 and H1693 cells with DharmaFect (cat no. T-2001-03, Horizon Discovery Ltd., Cambridge, UK) following the

manufacturer's instructions. Cells were trypsinized, and re-seeded on the poly-lysine coated cover glasses after 48 h for live-cell Ca^{2+} imaging or on the 6-well plate for in vitro wound-healing assay. Experiments were conducted after 24 h re-seeding the cells.

3.6. Materials and DNA constructs

2-(4-morpholinyl)-8-phenylchromone (LY294002) (cat no. 19-142) was purchased from Calbiochem (San Diego, CA, USA) and wortmannin (WMN) (cat no. W1628), brefeldin A (BFA) (cat no. B7651), and tetanus toxin A (TeNT) (cat no. T3194) were purchased from Sigma-Aldrich (St Louis, MO, USA). Recombinant αKlotho (human) protein was provided from R&D Systems (cat no. 5334-KL-025, Minneapolis, MN, USA). Non-targeting control oligonucleotides (cat. n. SN-1003) and small interfering RNA (siRNA) against human Orai1 (cat. n. M-014998-01-0005) were obtained from Bioneer (Daejeon, Korea) and Horizon Discovery Ltd. (Cambridge, UK), respectively. Expression vectors for the transmembrane full-length mouse αKlotho (KLFL), an extracellular domain of mouse αKlotho (KL△TM), βKlotho, and γKlotho was a kind gift from Prof. Makoto Kuro-o (Jichi Medical University, Japan) [92, 93, 94]. Orai1 (mCherry-3xFlag-Orai1) and STIM1 (YFP-STIM1) plasmids were kindly provided from Drs. Joseph Yuan (University of North Texas, USA)

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3.7. Real-time quantitative PCR analysis

Purified total RNA was extracted from the trypsinized pellets of HEK293FT cells through Hybrid-RTM total RNA purification kit (cat. n. 305-101, GeneAll, Seoul, South

Korea) according to the manufacturer's instructions. Complementary DNA (cDNA) was synthesized from 1 μg of total RNA by using a ReverTraAce® qPCR RT Master Mix with gDNA Remover (cat. n. FSQ-301, Toyobo, Osaka, Japan). The mRNA abundance was analyzed by real-time quantitative PCR with SYBR Green (cat. n. 204143, Qiagen, Germantown, MD, USA) using the following sequence specific human primers: ORAI1, forward (F) 5′-TTGA GCCGCGCCAAGCTTAAA-3′, reverse (R) 5′-CATT GCCACCATGGCGAAGC-3′; ORAI2, F-5′-AAGT GCTTGGATGCGGTGCTG-3 ′ R-5 ′ -GGAG CCAGGCAGGTCATTTATACG-3′; ORAI3, F-5′-TCAG CCGGGCCAAGCTCAAA-3 ′, R-5 ′ -CATG GCCACCATGGCGAAGC-3′; STIM1, F-5′-GTAC ACGCCCCAACCCTGCT-3′, R-5′-AGGCTAGGGGACTG CATGGACA-3′; STIM2, F-5′-TGGACCTCTAACAC GCCCACCT-3′, R-5′-CTGCGTATAAGCAAACCAGC AGCC-3′. For the analysis of each gene expression, the experiments were performed in triplicate in a real-time PCR system (7900HT, Thermo Fisher Scientific). Data were analyzed following the 2-ΔΔCt method with 18S as the reference gene.

3.8. Quantitative real-time polymerase chain reaction (Q-PCR)

Quantitative polymerase chain reaction (Q-PCR) or real-time PCR is a technique to amplify and simultaneously quantify the gene of interest. The procedure follows the general principle of polymerase chain reaction with detection of amplified product in "real time" using a specific DNA-intercalating fluorescence dye such as SYBR green. Real-time PCR was performed to measure the mRNA levels of candidate genes using sequence-specific primers (Table 1). β-actin and 18s were used as the reference

control. For the analysis of each gene expression, experiments were conducted in triplicate in a real time PCR system (7900HT, Applied Biosystems, Foster City, CA, USA) using SYBR green PCR master mix (catalog no. 204143, Qiagen, Hilden, Germany). The PCR reaction was performed with 10 µl total reaction volume containing 5 µl of 2X SYBR green PCR master mix (final conc. = 1 x), 1 µl of 10 µM forward primer (final conc. = 1 µM), 1 µl of 10 µM reverse primer (final conc. = 1 µM), 1 µl of template cDNA (50 ng/µl) (final conc. = 5 ng/µl) and 2 µl of DNase-RNase free H_2O. PCR was started with two holding temperatures at 50 °C for 2 min and 95 °C for 15 min as manufacture's instruction. In each PCR experiment, 40 cycles were considered including denaturation at 95 °C for 15 s, annealing at 58 °C for 30 s and extension at 72 °C for 30 s. Data was analyzed following $2^{-\Delta\Delta CT}$ method.

3.9. Intracellular Ca^{2+} ($[Ca^{2+}]_i$) measurement

Intracellular Ca^{2+}concentration ($[Ca^{2+}]$ I) measurement was previously described [5]. A normal physiological salt solution was used for bath solution that contained (in mM) 135 NaCl, 5 KCl, 1 MgCl2, 2 CaCl2, 10 HEPES, and 10 glucose (pH 7.4). Fura-2 signals were obtained by alternating excitation at 340 or 380 nm, and detecting emission at 510 nm. Data acquisition and analysis were performed using the MetaFluor (Sutter Instruments, Novato, CA, USA) software. All $[Ca^{2+}]$i measurements were performed at ~ 37 °C.

3.10. Electrophysiological recordings

For recording Ca^{2+} release-activated Ca^{2+}] (CRAC) currents, HEK293FT cells were co-transfected with cDNAs for mCherry-3xFlag-tagged Orai1 and YFP-STIM1 (0.5 μg each per 35 mm dish). The bath and pipette solution for Orai1 currents contained (in mM) 130 NaCl, 5 KCl, 10 CaCl2, 2 MgCl2, 10 HEPES and 10 glucose (pH 7.4), and 140 Cs-Asp, 10 BAPTA, 6 EGTA, 6 MgCl2 and 10 HEPES (pH 7.2), respectively. Currents were recorded using the whole cell-dialyzed configuration of the patch-clamp technique as described previously [5]. Whole-cell currents were recorded under voltage-clamp using an EPC-9 patch-clamp amplifier (Heka Electronik, Lambrecht, Germany). The patch electrodes were coated with silicone elastomer (Sylgard 184; Dow Corning, Midland, MI, USA), fire-polished, and had resistances of 2–3 MΩ when filled with the pipette solution. The cell membrane capacitance and series resistance were compensated (>80%) electronically using the EPC9 amplifier. Data acquisition was performed using the PatchMaster software (Heka Electronik). All electrophysiological recordings were performed at room temperature (~20–24°C).

3.11. Western blot and surface biotinylation assay

Western blotting and cell-surface biotinylation assay as described previously [95]. Briefly, HEK293FT cells were mechanically homogenized in RIPA lysis buffer with protease and phosphatase inhibitors. Primary antibodies were used following as: Orai1 (HPA016583, ATLAS antibodies, Stockholm, Sweden), STIM1 (11565-1-AP, ProteinTech Group Inc., Chicago, IL, USA), GFP (ab137687, Abcam, Cambridge, UK), αKlotho (clone KM2076,

KAL-KO603, Cosmo Bio Co., Ltd., Tokyo, Japan), βKlotho (GTX45558, Gene Tex, Inc., Irvine, CA), γKlotho (AF5984-SP, R&D Systems, Minneapolis, MN, USA), Flag-HRP (A8592, Sigma-Aldrich, St. Louis, MO, USA), β-actin (ab6276, abcam, Cambridge, UK), pAktSer407 (#9271), p-AktThr308 (#2965), and Akt (#9272) were provided from Cell Signaling Technology (Beverly, MA, USA). Bands in the immunoblotting were detected and quantified using ChemiDoc XRS+ Imaging System and the Image Lab software (version 5.2.1, Bio-Rad Laboratories, Hercules, CA, USA) and the Image J software (NIH, USA), respectively. Pretreatment of αKlotho protein and all reagents was processed 1 h before adding serum. Total cellular and biotinylated cell-surface proteins were analyzed by SDS-PAGE followed by western blot. These experiments were performed three times with similar results

3.12. Confocal microscopy

For immunofluorescence staining, HEK293 cells with an inducible mCherry-STIM1-T2A-Orai1-eGFP were grown on poly-L-lysine-coated coverslips. eGFP-Orai1 and mCherry-STIM1 protein were induced after 12~24 h tetracycline (5 μM) treatment [34] and were fixed with 4% paraformaldehyde in PBS for 15 min at room temperature. GFP and mCherry fluorescent images were obtained using a laser scanning confocal microscope (Zeiss, LSM 800, Jena, Germany) with Airyscan. Super-resolution image of the Orai1 expression on the plasma membrane and Airyscan image processing was acquired using the ZEN 2.3 software

3.13. Biotinylation assay

For biotinylation assay, HEK293 cells were prepared in a 6 well-plate. For endogenous Orai1 and TRPC channels, podocytes were plated at 1 x 10^3 per well in a type I collagen-coated 100 mm culture dish and grown to confluence for 10 days at 37 °C. 48 h after siRNA transfection (control oligo and siRNA TRPC6, VAMP2), serum starving for 18 h and then treated reagents on the cells as described below. Klotho (1 nM, 1 h), serum (10%, 1 h), insulin (100 nM, 1 h) and/or other reagents (WMN, LY294002, BFA and TeNT) were treated after serum deprivation (SD). Cells were rinsed twice with ice-cold Ca^{2+}-Mg^{2+}-PBS on the ice. 750 μl of biotinylation solution with 1.5 mg/ml biotin (Sulfo-NHS-SS-Biotin, cat no. 21331, Pierce, Rockford, IL, USA) were added on the cells and then the dish was incubated for 1 h at 4 °C. 1 ml of quenching buffer was added on the cells, and which incubated on shaker for 20 min at 4 °C. After 20 min, the cells were washed two times more and harvested by scraping into 250 μl ice-cold lysis buffer with protease inhibitor. 100 μl supernatant of the cell lysates was mixed with 100 μl bead (streptavidin agarose resin, cat no. 20353, Thermo Scientific, Rockford, IL, USA) and incubated in a rotary shaker for 1 h in the cold chamber. The mixture was centrifuged (6000 rpm, 4 °C, 10 min) and washed 3 times with ice cold Triton X 100-TBS (140 mM NaCl, 10 mM Tris-HCl-pH 7.4, 5 mM KCl, Triton X-100 1%). Captured proteins were recovered by boiling the beads in 40 μl of 2.5X sample buffer including 500 mM DTT for 5 min at 95 °C. Total and biotinylated proteins were analyzed by SDS-PAGE followed by western blot. These experiments were performed three times with similar results.

3.14. *In vitro* wound-healing assay

A wound-healing assay was conducted as described previously [96, 97]. Briefly, MDA-MB231 and H1693 cells were plated at 1 × 107 per well in a 6-well plate until grown to confluence. The cells were incubated with recombinant αKlotho protein (1 nM) in only 1% penicillin contained RPMI1640 media for 30 min and then exchanged with complete media with or without αKlotho protein. To distinguish cell migration from proliferation, all wound-healing assays were performed in the presence of anti-tumor drug mitomycin C (M4287, Sigma-Aldrich, a final concentration of 0.1 μg/ml) to prevent proliferation. The image was captured by a microscope after 24 h of drug treatment (time 0, initial time point). The migrated cells were counted using an ImageJ 1.48 (NIH, USA).

3.15. Immunohistochemistry (IHC)

Tissue microarray (TMA) preparation

A total of 126 FFPE samples were used for TMA. A representative tumor site without necrosis, hemorrhage, or artifact was marked in the paraffin blocks. The selected tumor area was harvested using a 5 mm Quick-ray tip-punch (Unitma, Seoul, Korea), placed on a 20 pore TMA mold (Unitma), and re-embedded with paraffin. 4 μm sections of TMA blocks were cut and attached onto coated slides. Staining was performed using a Ventana Benchmark XT (Roche Diagnostics, Basel, Switzerland) automatic immunostaining machine. The sections were deparaffinized in xylene, rehydrated in graded alcohols, and subjected to pretreatment with CC1 (Roche Diagnostics). The sections were washed with reaction buffer followed by incubation with primary IR-β antibody (Abcam, Cambridge, MA, USA) at a 1:100 dilution for 60 min at 42°C. Bound antibody was detected with the Ultra

View Universal DAB kit (Roche Diagnostics) and sections were counterstained with hematoxylin (Roche Diagnostics) according to the manufacturer's instructions. Positive and negative control stains were also performed.

3.16. Immunofluorescence

Experiments were performed with differentiating podocytes on 12 mm coverslips (cat no. 01 115 20, Marienfeld GmbH & Co. KG, Lauda-Kőnlgshofen, Germany) with type I collagen coat for 14 days at 37 °C without interferon-γ. Podocytes plated on the coverslips were washed twice with phosphate-buffered saline (PBS) and fixed with 4% paraformaldehyde in PBS for 15 min at 37 °C. The coverslips were incubated with Alexa-549-phalloidin (1:40 dilution) (A12381, Molecular Probes-Invitrogen, Carlsbad, CA, USA) for 20 min at room temperature without light. Next, the cells were permeabilized with 0.25% Triton X-100 in PBS for 7 min and blocked with 1% BSA in PBS for 1 h. Specific antibodies for rabbit-Orai1 (1:100 dilution), goat-synaptopodin (1:40 dilution) or rabbit-paxillin (1:250 dilution) were incubated overnight at 4 °C followed by incubation with secondary antibody, Alexa fluor 594 goat anti-rabbit IgG (1:200 dilution), Alexa flour 488 rabbit anti-goat IgG (1:200 dilution) or Alexa flour 488 goat anti-rabbit IgG (1:200 dilution) for 1 hr at RT in a dark room. The cells were washed and counterstained with 1 μg/ml DAPI for 5 min and mounted on a glass slide (cat no. 10 006 12, Marienfeld GmbH & Co). Fluorescence images were obtained using a confocal laser-scanning microscope (TCS SPE, Leica Microsystems GmbH, Wetzlar, Germany).

3.17. Colony formation and cell viability assays

In order to carry out the colony formation assay, ccRCC cells were first transfected with Orai1, STIM1 and control siRNA. One day after transfection, cells were trypsinized, and re-plated into new 6-well plates (100 cells/well) and allowed to grow for ten days until they formed colonies. Cells were also cultured in the same maneuver in the presence of IGF-1, Klotho, SKF96365 and 2-APB. Medium was changed every three days and colonies were visualized with 1% methylene blue.

For cell viability assay, cells were transfected with Orai1 and STIM1 siRNA alongside control siRNA. Two days after transfection, cells were trypsinized and re-plated into new 6-well plates (400 cells/well). After 9 days, cells were counted using a hemocytometer.

3.18. Data analysis and statistics

Results are presented as mean ± SEM. Statistical analysis was performed using a two-tailed unpaired Student's t test and one-way ANOVA followed by Tukey's multiple comparison tests by the GraphPad Prism Software (version 5.0, GraphPad Software, San Diego, CA, USA). p values less than 0.05 and 0.01 were considered significant for single and multiple comparisons, respectively. All experiments were repeated independently 3–4 times with similar results

Table 1. List of human primer sequences for PCR

Name	Gene Bank Accession No.		Sequence (5'-3')	Size (bp)
18s	NR_003278.1	F	AACCCGTTGAACCCCATT	149
		R	CCATCCAATCGGTAGTAGCG	
αSMA	NM_001613.3	F	CACTGCCTTGGTGTGTGACAAT	146
		R	CGTAGCTGTCTTTTTGTCCCATTC	
Col1α	NM_000088.3	F	CACACGTCTCGGTCATGGTA	91
		R	AAGAGGAAGGCCAAGTCGAG	
MRTF-A	NM_001318139.1	F	TTGGAAGAGACCTCGGCTGA	127
		R	TCTTCTCCACCAGCTCCATG	
NCX1	NM_001112802.1	F	ATGATTGGCCTACTGACAGC	130
		R	TGGCAAATGTGTCTGGCACT	
NKCC1	NM_001256461.1	F	ACAAGAGAAATCTCCTGGCAC	122
		R	CCTCTTCCTCAACTTTGTGTGT	
NKCC2	NM_000338.2	F	TTGGATGGGTGAAAGGTGTG	109
		R	ACTCCAAGACCAATTCCAGC	
OSR1	NM_005109.2	F	CTGGTGGTGATATTACCCGA	90
		R	CCACGGACCTGTTCCATAAC	
SPAK	NM_013233.2	F	TGGAGTTCTGGAAGAGGCAA	93
		R	TCCCTGTGAATCTGACCGTT	
SRF	NM_003131.3	F	GACCAAGGACACACTGAAGC	105
		R	CTGCTGACTTGCATGGTGGT	
TRPC1	NM_001251845.1	F	GAGGTGATGGCGCTGAAGG	78
		R	GCACGCCAGCAAGAAAAGC	
TRPC3	NM_001130698.1	F	CTGGGTCTGCTTGTGTTCAATG	76
		R	AGTCAGTAACTGTGATATTGGGCAGC	
TRPC4	NM_001135958.2	F	TCAGCACATCGACAGGTCAGAC	76
		R	CCACGGTAATATCATCCACTCGAC	
TRPC5	NM_012471.2	F	CCTCTCATCAGAACCATGCCAA	72
		R	GCGTTTGCTTGATGACTCAGC	

Continued

Name	Gene Bank Accession No.		Sequence (5'-3')	Size (bp)
TRPC6	NM_004621.5	F	CCTTGCTGTTGCCATTGGA	74
		R	TCTTCCCCATCTTGCTGCAT	
TRPC7	NM_001167577.1	F	TGGGCATGCTGAATTCCAAA	73
		R	GCTGTCAGATTTTCAGAATTCCTCA	
WNK1	NM_018979.3	F	CCTTTTCGTTCACGAATCCGAG	94
		R	AACCGGGAGTGCTGCTCTGCTT	
WNK2	NM_006648.3	F	TTCAGCGCCCTATAAAGACCAGC	71
		R	CTCAGAGCTGCTGACTGGCTAGA	
WNK3	NM_020922.4	F	GGACCATTCCAGCCAGGGATGAA	86
		R	CATTTAGGACCAGGAGGGATTGTGG	
WNK4	NM_032387.4	F	AGGGAAAGCCGCAGCTTGTTG	91
		R	AGAGTGGGGGATGTTGGCTGCAA	

F, forward primer
R, reverse primer
bp, base pairs
Annealing Tm: 60 °C

Table 2. List of siRNA sequences

Name	Cat. No.	siRNA sequence (5' - 3')	Manufacturer
Control Oligo		UGGUUUACAUGUCGACUAAUU	Bioneer
Human TRPC6	M-004192-03-0005	GCACAAAACUCCUUCCUAA GGACACGGUUCUCCCAUGA GCAGAUAUCACUUGGAAGA	Dharmcon
Mouse TRPC6	sc-42673	UGAACGGCCUCAUGAUUAU GUCAUUCCCUCAAUGUUAAtt UUAACAUUGAGGGAAUGACtt CAAGGAGCUCAGAAGAUUAtt UAAUCUUCUGAGCUCCUUGtt CUAGCAGAGCUCAUUAGAAtt UUCUAAUGAGCUCUGCUAGtt	Santa Cruz Biotech
Human MRTF-A	M-015434-01-0005	GGUGAACUAUCCCAAAGUA GGACAGAGGACUAUCUCAA AAACUGAGCUGAUUGAGCG	Dharmcon
Human WNK1		CCAUGGACACCUCGGAAUU UGUCUAACGAUGGCCGCUUtt TGTCTAACGATGGCCGCTTtt	Bioneer

Table 3. List of primary and secondary antibodies for Western Blot

Antibody Name	Dilutions	Cat. No.	Manufacturer	Host
αSMA	1:200	ab7817	Abcam	Mouse
αSMA	1:1000	ab5694	Abcam	Rabbit
AMPKα	1:1000	2532S	Cell signaling	Rabbit
GAPDH	1:3000	sc-25778	Santa Cruz Biotechnology	Rabbit
MRTF-A	1:1000	sc-398675	Santa Cruz Biotechnology	Mouse
NFATc1	1:200	sc-13033	Santa Cruz Biotechnology	Rabbit
phospho-AMPKα (T172)	1:3000	2535S	Cell signaling	Rabbit
phospho-NFATc1 (S259)	1:200	sc-32979	Santa Cruz Biotechnology	Rabbit
TGFβ	1:250	ab64715	Abcam	Mouse
TRPC6	1:1000	ACC-017	Alomone	Rabbit
TRPC6	1:1000	ACC-120	Alomone	Rabbit
TRPC6	1:1000	ab63038	Abcam	Mouse
TRPC6	1:1000	18236-1 AP	ProteinTech	Rabbit
TRPC6	1:1000	ab62461	Abcam	Rabbit
anti-mouse-IgG$_k$ BP-HRP	1:3000	sc-516102	Santa Cruz Biotechnology	Mouse
mouse anti-rabbit IgG-HRP	1:3000	sc-2313	Santa Cruz Biotechnology	Mouse
Flag-HRP	1:3000	A8592	Sigma	Mouse

Table 4. List of primary and secondary antibodies for Immunostaining and IHC.

Antibody Name	Dilutions	Cat. No.	Manufacturer	Host
a-SMA	1:200	ab7817	Abcam	Mouse
a-SMA	1:200	ab5694	Abcam	Rabbit
a-SMA	1:300	NB300-978	Novus	Goat
CD-68 (FA-11)	1:50	MCA1957T	Serotec	Rat
DAPI	1:1000	D9542	Sigma	
Desmin	1:100	5332	Cell signaling	Rabbit
DHE	5 μM	D1168	Thermo scientific	
GFAP	1:25	12389S	Cell signaling	Rabbit
TRPC6	1:50	ACC-120	Alomone	Rabbit
TRPC6 (for IHC)	1:50	ACC-120	Alomone	Rabbit
Alexa Fluor® 488	1:200	A-31627	Thermo scientific	Rabbit
Alexa Fluor® 488	1:200	A-31619	Thermo scientific	Mouse
Alexa Fluor® 488	1:200	A-11006	Thermo scientific	Rat
Alexa Fluor® 488	1:200	A-11055	Molecular Probes	Goat
Alexa Fluor® 594	1:200	A-11012	Thermo scientific	Rabbit
Alexa Fluor 594 phalloidin	1:40	A-12381	Thermo scientific	
Cy3-AffiniPure Goat Anti-Rabbit IgG	1:200	111-165-144	Jackson	Rabbit

Table 5. List of siRNA sequences

Name	Cat. No.	siRNA sequence	Manufacturer
Control Oligo		5' UGGUUUACAUGUCGACUAAUU 3'	Bioneer
siOrai1 (mouse)	M-056431-01	5' GCACCUGUUUGCCCUCAUG 3'	Dharmacon
		5' GGCGCAAGCUCUACUUAAG 3'	
		5' CACCAAGCCUCCCGCUGAA 3'	
		5' ACAUCGAGGCUGUGAGCAA 3'	
siOrai2 (mouse)	M-057985-01	5' GGGCAUGGAUUACCGAGAC 3'	Dharmacon
		5' UGGAACUCGUCACGUCUAA 3'	
		5' GCGCCACAACCGUGAGAUC 3'	
		5' GUGGAAGCGGUGAGCAACA 3'	
siOrai3 (mouse)	M-054417-01	5' GUGACUGUCUCCCUUAGUU 3'	Dharmacon
		5' GCGGCUACCUGGACCUUAU 3'	
		5' UGGAGAACGAUCAUGAAUA 3'	
		5' CUGUGGGACUAGUGUUUAU 3'	
siVAMP2 (human)	M012498-01	5' UAGUUUACUUCAGCUCUUA 3'	Dharmacon
		5' GCGCAAAUACUGGUGGAAA 3'	
		5' CCUCCAAACCUCACCAGUA 3'	
		5' CAGCAUGUUAAUUAGCGUA 3'	
siVAMP2 (mouse)	M-041975-01	5' CCUCCAAACCUUACUAGUA 3'	Dharmacon
		5' CAAGUGCAGCCAAGCUCAA 3'	
		5' AUGAUCAUCUUGGGAGUGA 3'	
		5' CGCAAAUACUGGUGGAAAA 3'	
siTRPC6 (mouse)	sc-42673	5' GUCAUUCCCUCAAUGUUAAtt 3'	Santa Cruz Biotech
		5' UUAACAUUGAGGGAAUGACtt 3'	
		5' CAAGGAGCUCAGAAGAUUAtt 3'	
		5' UAAUCUUCUGAGCUCCUUGtt 3'	
		5' CUAGCAGAGCUCAUUAGAAtt 3'	
		5' UUCUAAUGAGCUCUGCUAGtt 3'	
siSGK-1 (mouse)	10620318	5' GGGCUGUCCUGUAUGAGAUGCUCUA 3'	Life Technology
		5' UAGAGCAUCUCAUACAGGACAGCCC 3 '	

Table 6. List of primary and secondary antibodies

Antibody Name	Dilutions	Cat. No.	Manufacturer	Host
Klotho	1:2000	KM2076	Gift from Kuro-O M.	Rat
Gok-STIM1	1:1000	610954	BD Science	Mouse
Paxillin (N-term)	1:250	1500-1	Epitomics	Rabbit
Flag	1:100	A00170	GenScript	Rabbit
Alexa-594-Phalloidin	1:40	A12381	Molecular Probes-Invitrogen	-
β-actin	1:10000	ab6276	Abcam	Mouse
Klotho	1:1000	ab75023	Abcam	Rabbit
Orai1	1:100 or 1:1000	ab86748	Abcam	Rabbit
TRPC6	1:100 or 1:1000	ab63038	Abcam	Mouse
VAMP2	1:1000	ab181869	Abcam	Rabbit
His-HRP	1:1000	ab1187	Abcam	Rabbit
GAPDH	1:3000	sc25778	Santa Cruz Biotech	Rabbit
p-SGK-1 (S422)	1:500	sc16745	Santa Cruz Biotech	Goat
SGK-1 (total)	1:500	sc15885	Santa Cruz Biotech	Goat
Synaptopodin	1:40	sc21537	Santa Cruz Biotech	Goat
Anti-goat HRP	1:3000	sc2354	Santa Cruz Biotech	Mouse
Anti-rat HRP	1:3000	sc2032	Santa Cruz Biotech	Goat
Anti-mouse HRP	1:3000	sc2314	Santa Cruz Biotech	Donkey
Anti-rabbit HRP	1:3000	sc2313	Santa Cruz Biotech	Donkey
Anti-Rabbit Alexa Flour 594	1:200	A31631	Life Tech	Goat
Anti-Rabbit Alexa Flour 488	1:200	A31627	Life Tech	Goat
Anti-Goat Alexa Flour 488	1:200	A11078	Life Tech	Rabbit

Continued

Antibody Name	Dilutions	Cat. No.	Manufacturer	Host
Flag-HRP	1:1000	A8592	Sigma Aldrich	Mouse
Myc-HRP	1:1000	A5598	Sigma Aldrich	Rabbit
InsulinRβ	1:1000	30025	Cell Signaling	Rabbit
p-Erk1/2 (T202/204)	1:2000	9101	Cell Signaling	Rabbit
Erk1/2 (total)	1:2000	9012	Cell Signaling	Rabbit
p-Akt (S473)	1:2000	9271	Cell Signaling	Rabbit
p-Akt (T308)	1:2000	2965	Cell Signaling	Rabbit
Akt (total)	1:2000	9272	Cell Signaling	Rabbit

IV. RESULTS AND DISSCUSSION

4.1. Inflammation

4.1.1. Background

Endometriosis is an estrogen-dependent inflammatory disease characterized by the presence of endometrial glands and stroma outside the uterine cavity. It affects 5–10% of women of reproductive age, up to 80% of women with pelvic pain, and 20–50% of women with infertility [98,99]. Affected women experience impaired quality of life due to chronic pelvic pain and other clinical symptoms such as dysmenorrhea, menorrhagia, dyspareunia, dysuria, and dyschezia [100]. Ovarian endometriomas could alter ovarian function through space-occupying and local effects [101]. The cystic fluid within an endometrioma is a rich source of proinflammatory cytokines (IL-6, IL-8), iron, reactive oxygen species (ROS), growth factors such as TGF-β, and matrix metalloproteases (MMPs) [101]. These cystic fluid contents could affect adjacent ovarian function through diffusion into surrounding tissue. Structural alterations were identified in the neighboring tissue of an ovarian endometrioma, with lower follicular density, greater fibrosis, and loss of cortex-specific stroma [102]. Both TGF-β1 and ROS promote fibrosis and adhesion formation through myofibroblast differentiation and profibrotic gene expression of plasminogen activator inhibitor-1 [103].

The effect of endometriosis on endometrial receptivity is based on inflammation. Progesterone exhibits anti-inflammatory activity, and in normal circumstances, an inflammatory response occurs after progesterone withdrawal in the late secretory phase of the menstrual cycle. Decreased progesterone leads to decreased prostaglandin

metabolism and increased ROS, which activate an NFκB-mediated inflammatory cascade that induces the processes required for menstruation [104]. Stromal cells derived from the endometrium of a woman with endometriosis have a reduced decidualization capacity [105], possibly because of progesterone resistance and inflammatory cytokines TNF-α and IL-1 Several preclinical studies have shown that the inflammatory microenvironment maintained by endometriosis compromises implantation ability in animal models. [106] Microenvironmental inflammatory cytokines and growth factors may cause significant changes in cellular behavior, promoting stemness, EMT, invasion, and malignant transformation. The interplay between inflammatory niche and stemness has been widely studied in cancer biology. These inflammatory cytokines can be secreted by tumor-associated immune cells, cancer stromal cells, or cancer cells function via NFκB. [107,108]. Both the MAPK and phosphoinositide 3-kinase (PI3K)/Akt pathways are enriched in the eutopic and ectopic endometria [109–111], and their relationships with chronic inflammation have been established [112]. These pathways are involved in inflammation, cell cycle progression, cell proliferation and migration, angiogenesis, and fibrosis [112].

Overactivation of these pathways compromises endometrial cells' ability to decidualize, promote progesterone resistance, and increase proliferation and migration of endometrial cells [113]. Inflammatory cytokines can be secreted by tumor-associated immune cells, cancer stromal cells, or cancer cells function via NFκB- or STAT3-mediated pathways [114,115]. Inflammation may also promote stemness properties by stimulation of growth factor production.

The functions of Klotho are related to its binding to fibroblast growth factor

receptor (FGFR) and its involvement in various intracellular signal transduction pathways. In this regard, the effects of Klotho are associated

with antioxidant activity through increased transcription of FoxO1 (forkhead box) and, conversely, antiinflammatory effects due to the negative regulation of nuclear factor κB (NFκB) [116]. NF-κB is a critical transcription factor regulating inflammatory responses [117]. In response to stimuli via the canonical pathway, the IKK complex is activated, which causes IκBα phosphorylation and subsequent degradation. The dissociation of IκBα from the sequestered cytoplasmic complex results in the translocation of the active NF-κB subunit p65 from the cytoplasm to the nucleus, where it then binds to NF-κB promoter sequences and triggers inflammatory gene expression. Inflammatory processes have been linked to lower Klotho expression, whereas the inhibition of proinflammatory cytokine production reverses this pattern and restores circulating Klotho levels. Thus, inflammation downregulates Klotho, generating oxidative stress [118]

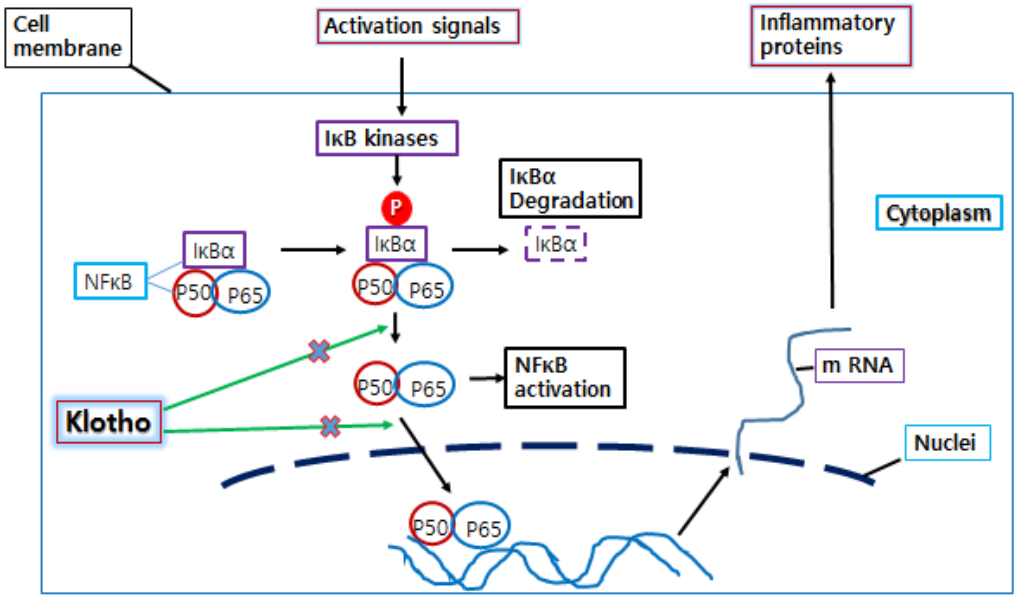

Fig. 7. Response to stimuli via the canonical pathway and anti-inflammatory effects Of klotho

4.1.2. Results

In this study, in ishikawa cell, an endometrial cancer cell, Ca^{2+}entrance was increased by TNFα signaling, an inflammatory stimulation, and Orai1 was overexpressed (Fig. 8.). In order to understand the role of Klotho in this process, Klotho was treated with TNFα and NFκb in ishikawa cells, and it was confirmed that klotho inhibited TNFα-induced overexpression of Orai1 and NFκb signaling during the phosporylation process. (Fig. 9.)

In addition to suppressing inflammatory cells, Klotho suppresses the proliferation and migration of cancer cells, confirming the role of klotho in malignant cells. In endometrial cancer cells, migration and proliferation were significantly reduced in klotho-treated cell

plates. (Fig. 10)

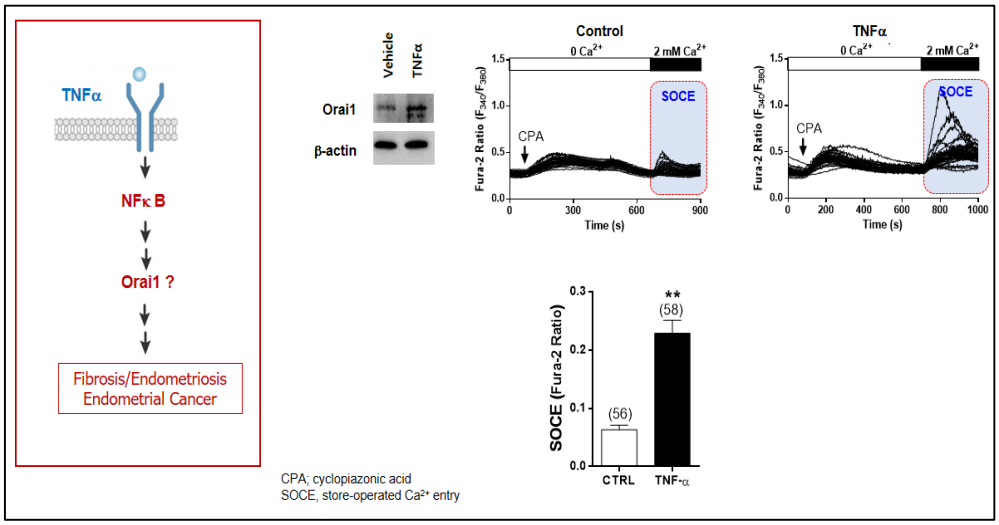

Fig. 6. TNFα upregulates Orai1 expression and SOCE in Ishikawa cell

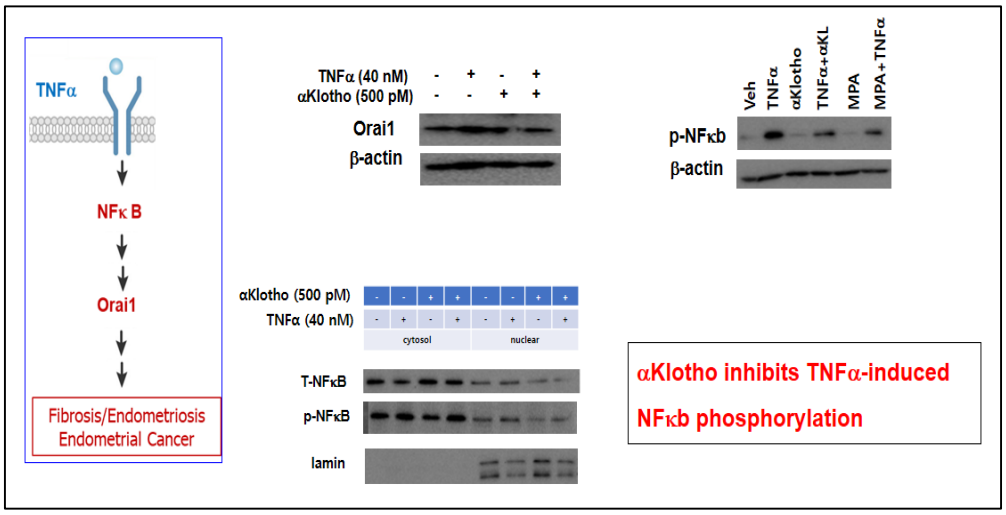

Fig. 7. αKlotho suppresses TNFα-induced Orai1 overexpression and NFκb signaling in Ishikawa cells

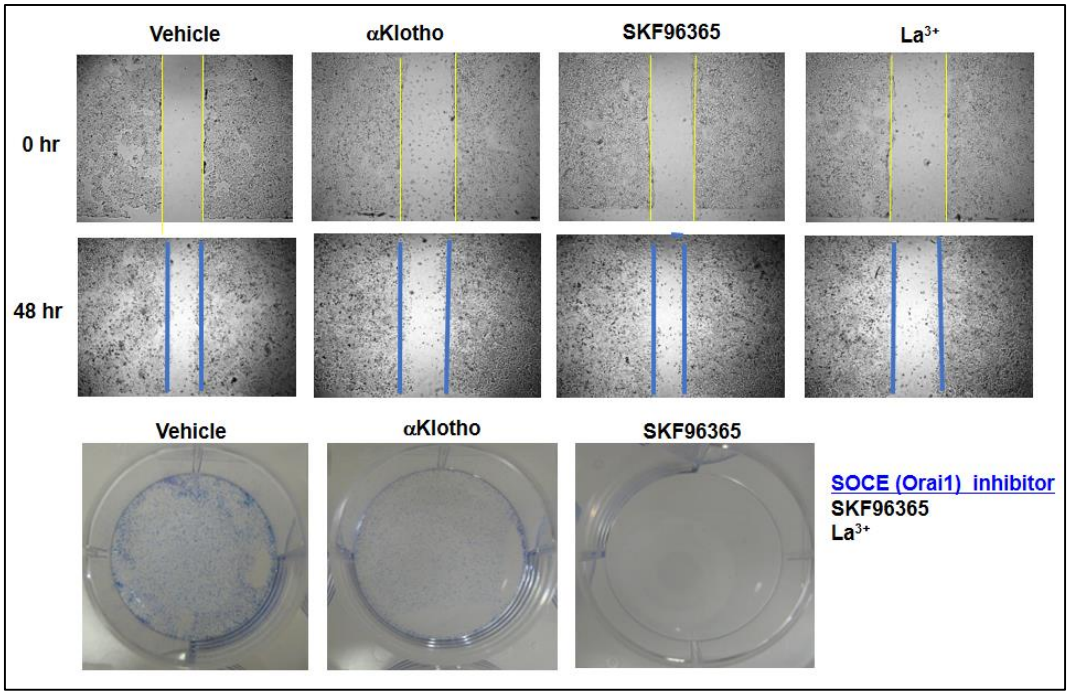

Fig. 8. αKlotho inhibits cell migration and proliferation of Ishikawa cells

4.1.3. Discussion

In this study, in ishikawacell, an endometrial cancer cell, Ca^{2+}entrance was increased by TNFα signaling, an inflammatory stimulation, and Orai1 was overexpressed. And klotho inhibited TNFα-induced overexpression of Orai1 and NFκb signaling during the phosporylation process. This fact is inconsistent with previous studies.

Klotho is as an antiinflammatory modulator, negatively regulating NFκB and, consequently, decreasing proinflammatory gene transduction. We have noted high expression of adhesion molecules, such as ICAM-1 (intercellular adhesion molecule 1) and VCAM-1 (vascular cell adhesion protein 1), in endothelial cells that have been exposed to inflammatory stimuli, such as TNF. Klotho suppresses TNF-induced ICAM-1 and VCAM-1

expression by attenuating NFκB activity, which has been associated with nitric oxide production. [119]. Klotho appears to modulate tissue inflammatory responses to injury or interact with inflammatory mediators [120,121]. Klotho has been found to regulate energy metabolism, exert anti-inflammatory and anti-oxidative effects, and modulate calcium and mineral homeostasis [122-124].

Endometriosis symptoms such as irregular menstruation, hypermenorrhea, and severe pain during menstruation can be caused by endometriosis. This phenomenon is thought to be related to the increased proliferation of endometrial cells, and specific changes in endometriosis are associated with endometriosis. There is also the view that it is a primary phenomenon [121]. In endometriosis, endometrial cell proliferation is increased, [122] abnormal aromatase apoptosis appears, and apoptosis is suppressed. Since it was observed that Klotho inhibits the migration and proliferation of endometrial cancer cells in this experiment, it can be expected to play a role in inhibiting adhesions caused by endometriosis and uterine bleeding, uterine pain and endometrial cancer caused by endometrial proliferation.

4.2. Adenomyosis and Endometrial Cancer

4.2.1. Background

Endometrial carcinoma (EC) has a worldwide incidence of 8.4 per 100,000 persons per year and is a leading cause of cancer mortality in women in developed in high-resource countries after ovarian and cervical cancer [125]. Both incidence and number of deaths per year have increased in recent years [125,127]. Increase in incidence appears

related to the increase in EC risk factors, such as obesity, metabolic syndrome, and causes of hyperestrogenic state [126]. The five most common histopathological subtypes of endometrial cancer are endometrioid, clear-cell, serous, mucinous endometrial cancer and adenocarcinoma not otherwise specified(NOS) [126].

This traditional classification is based on clinical, endocrinological, and histopathological features and serous subgroup II cancers have a more dismal prognosis than type I endometrial cancers (endometrioid, mucinous endometrial cancer and adenocarcinoma NOS). Adenomyosis is defined as ectopic endometriosis, which invades the endometrial glands into the uterine myometrium [128]. Hysterectomy is the treatment for the patient with persistent severe pain and not responding to hormone medication [129].

Adenomyosis appears to be one of the most frequent diseases associated with EC on hysterectomy specimens [132,133]. The association between EC and adenomyosis is still debated: on the one hand, adenomyosis is found with an incidence of 10 to 18% in EC specimen at final histology after hysterectomy [131]. On the other hand, accumulating evidence suggests that these two diseases share several altered molecular pathways, which lead to increased angiogenesis, abnormal tissue growth, and invasion. In details, adenomyosis and EC are both associated with a local microenvironment characterized by high level of vascular endothelial growth factor, platelet-derived growth factor, increased production of reactive species of oxygen and pro-inflammatory cytokines, KRAS mutations and, to a lesser extent, progesterone-resistance, epithelial mesenchymal transition(EMT) and fibroblast-to-myofibroblast trans-differentiation [130].

Insulin-like growth factor-1 (IGF-1) is a hormone, similar in structure to insulin, which regulates cell growth and metabolism and acts as a potent inhibitor of programmed cell death [134]. Its abnormal expression can induce carcinogenesis, such as ovarian

cancer, cervical cancer, endometrial cancer and other cancers [135,136]. Endometriosis and adenomyosis share characteristics with malignant tissue including tissue invasion, increased proliferative capability, induction of angiogenesis, the ability to evade apoptosis, and the ability to develop local and distantfoci [137,138]. Recently a large epidemiological dataset compared endometrioid EC co-existing with adenomyosis and endometrioid EC arising from adenomyosis with ECe without adenomyosis [138]. According to this analysis, EC arising from adenomyosis was associated with significantly younger onset ages and better survival than other cases where adenomyosis was just co-existing. It has been reported as a major factor with the potential for malignant transformation of endometriosis and adenomyosis. It has been reported as a major factor with the potential for malignant transformation of endometriosis and adenomyosis. Endometriosis and adenomyosis are both estrogen-dependent entities; additionally, type I EC are associated with increased estrogen levels [138,140].

In systematic review and meta-analysis to assess the prognostic value of coexistent adenomyosis in patients with EC, the risk of death is halved in EC patients with coexistent adenomyosis compared with EC patients without adenomyosis. However, this association needs to be assessed at multivariate analysis in future studies to assess the independence of the prognostic value. Furthermore, adenomyosis does not appear to affect the risk of EC recurrence [141].

To develop preventive strategies, future studies should focus on detection of women at risk for endometrial cancer in the group of women with endometriosis/adenomyosis. Although most women with endometriosis/adenomyosis do not develop endometrial cancer. But, it is important to identify specific risk factors for endometrial cancer in these women [142]. We want to see if Klotho can play this role.

4.2.2. Results

We used a modified Allred scoring system to evaluate positivity, with staining intensity and distribution being scored separately [143,144]. The staining intensity was scored as 0 points (negative), 1 point (weak), 2 points (intermediate), or 3 points (strong) and the distribution of positive-stained cells was assessed as 0 points (negative), 1 point (<1%), 2 points (1--10%), 3 points (11--33%), 4 points (34--67%), or 5 points (>67%). The total staining score was calculated as the sum of two parameters. Total staining scores from 0 to 3 points were considered negative, while scores from 4 to 8 points were considered positive. To overcome the limitations of this quantification method, we also compared the mean staining score as continuous variables in each group.

We compared the staining score of each IGF1, SGK1, Orai1 and Klotho in adenomyosis and endometrial cancer tissue with modified Allred scoring system in IHC assays. The mean staining score of IGF1, SGK1, Orai1 and Klotho were respectively statistically significant difference (Table 7, Fig. 9).

Table 7. Summary of Clinical and Pathological Findings

Score		Adenomyosis	Endometrial Cancer	p-value
	IGF IS	1.74±0.73	2.24±0.75	<.0001
	IGF PS	2.74±0.74	4.60±0.85	<.0001
	IGF SUM	**4.48±1.08**	**6.84±1.16**	**<.0001**
	SGK IS	1.45±0.61	2.08±0.62	<.0001
	SGK PS	2.94±0.75	4.67±0.71	<.0001
	SGK SUM	**4.39±1.00**	**6.75±0.96**	**<.0001**
	ORAI IS	2.08±0.66	2.66±0.48	<.0001
	ORAI PS	3.09±1.07	4.48±0.77	<.0001
	ORAI SUM	**5.18±1.49**	**7.14±1.01**	**<.0001**
	KL IS	1.89±0.66	2.48±0.59	<.0001
	KL PS	2.83±0.69	4.46±0.81	<.0001
	KL SUM	**4.72±0.88**	**6.94±0.97**	**<.0001**

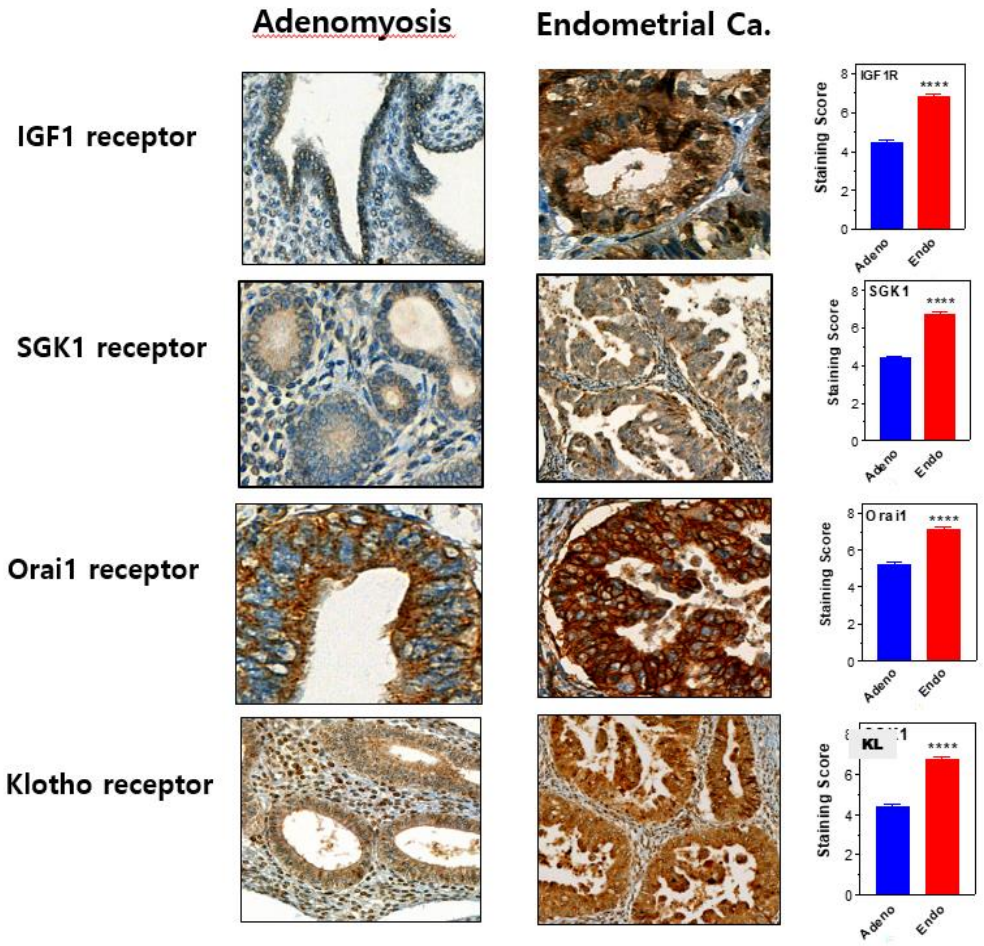

Fig. 9. Expression of IGF1, SGK1, Orai1 and Klotho in human adenomyosis and endometrial cancer tissue. They are not associated with tumor grade but staining score in adenomyosis **vs** endometrial cancer were statistically significant different

4.2.3. Discussion

IGF1, SGK1, and Orai1 were overexpressed in endometriotic tissue. Based on this, expression in human uterine tissue was confirmed through immunohistochemistry, and statistically significant overexpression in endometrial cancer was confirmed through staining score. Endometriosis and adenomyosis share characteristics with malignant tissue including tissue invasion, increased proliferative capability, induction of angiogenesis, the ability to evade apoptosis, and the ability to develop local and distant foci, Therefore, it it supports other existing studies that endometriosis and adenomyosis may be major factors for the potential for malignant transformation.

In this study, there are limitations in predicting progression from adenomyosis to endometrial cancer. For Klotho expression, the staining score was statistically significantly higher in endometrial cancer tissue than in adenomyosis tissue. This fact is inconsistent with previous studies.

In the case of klotho expression, it was not consistent with the previous results that the expression decreased in the malignant tissue of the uterus and ovaries. However, further research is needed to determine the possibility that Klotho was overexpressed for anti-tumor effect. Experiments were performed that supported anti-tumor effects by Klotho through targeting of IGF1/insulin signaling as well as other oncogenic pathways [146]. Klotho promoter is hypermethylated in tumor tissue and Klotho expression is consequently decreased. This was first demonstrated by Lee et al. who showed that the Klotho promoter is frequently hypermethylated in cervical carcinoma [145]. Klotho gene and protein expression have been shown to be decreased in cervical carcinoma [147], Klotho promoter

is hypermethylated in tumor tissue and Klotho expression is consequently decreased. This was first demonstrated by Lee et al. who showed that the Klotho promoter is frequently hypermethylated in cervical carcinoma [145]. Klotho gene and protein expression have been shown to be decreased in cervical carcinoma [147], reduced Klotho expression contributes to poor survival rates in human patients with ovarian cancer and overexpression of Klotho inhibits the progression of ovarian cancer partly via the inhibition of systemic inflammation in nude mice. [148-,149],

Klotho inhibits many pathways involved in carcinogenesis, including IGF1R (with downstream PI3K, Akt, and mTOR signaling), Wnt/β-catenin signaling, FGF2 signaling (generally affecting ERK1/2 signaling), and TGFβ1 and downstream Smad2/3 signaling.

Generally, in vitro anti-tumor effects were found to include the induction of apoptosis, inhibition of proliferation [149], induction of autophagy, inhibition of autophagy[150], and inhibition of migration[151]. Klotho-overexpressing mice, all of which resulted in fewer metastases after exposure to higher Klotho levels, likely through an effect on the TGFβ1 pathway [152].

4.3. Soluble αKlotho downregulates Orai1-mediated store-operated Ca^{2+} entry via PI3K-dependent signaling

4.3.1. Background

Soluble αKlotho can positively or negatively regulate transient receptor potential (TRP) superfamily of cation channels. αKlotho upregulates multiple TRPV channels including TRPV2, 5, and 6 [153], whereas several TRPC channels such as TRPC1, 3, and 6 are downregulated by αKlotho [154]. Additionally, αKlotho positively regulates multiple K+ channels such as ROMK, Kv1.3, KCNQ1/KCNE1, and hERG channels [155]. Soluble αKlotho increases the cell-surface abundance of TRPV and K+ channels by modifying their N-glycan through sialidase or β-glucuronidase activity of αKlotho [156]. This N-glycan modification by αKlotho increases the resident time of these channels at the plasma membrane by delaying their endocytosis [157]. Conversely, αKlotho downregulates TRPC channels with a distinct mechanism.

Soluble αKlotho inhibits TRPC1-mediated Ca^{2+} influx via binding directly to vascular endothelial growth factor receptor-2 (VEGFR2)/ TRPC1 complex to promote their co-internalization [158]. αKlotho decreases the cell-surface abundance of TRPC6 and TRPC3 via inhibiting PI3K-dependent exocytosis of these channels [159]. Recently, it is reported that soluble αKlotho targeting α2-3-sialyllactose binds to monosialogangliosides in lipid rafts to regulate TRPC6 [160]. Overall, these studies provide compelling evidence suggesting that soluble αKlotho can regulate multiple ion channels via distinct mechanisms. The ubiquitous second messenger Ca^{2+} regulates various cellular behaviors. Store-operated Ca^{2+}entry (SOCE) is vital for the maintenance of endoplasmic reticulum (ER) Ca^{2+}

stores at precise levels for signaling in both nonexcitable and excitable tissues to regulate a variety of cellular functions [161]. The molecular components of SOCE are Orai1 and STIM1 (stromal interaction molecule1), a pore-forming subunit, and an ER Ca^{2+} sensor, respectively. STIM1 is oligomerized and translocated to the plasma membrane during ER Ca^{2+} depletion that thereby triggers Ca^{2+}entry via Orai1, a Ca^{2+}-selective channel at the plasma membrane [162]. SOCE is a downstream effector of growth factor signaling. The explicit mechanism of Orai1 activation by PI3K-driven growth factor signaling in physiological conditions remains elusive. Moreover, soluble αKlotho suppresses aging and protects multiple disease progression by regulating growth factor signaling [163]. The mechanism linking αKlotho and SOCE by growth factor signaling has not yet been identified. Here, we examined the mechanism by which soluble αKlotho regulates Orai1-mediated SOCE by growth factor stimulation and its functional implications.

4.3.2. Results

4.3.2.1. Soluble αKlotho contributes to SOCE regulation

Orai1 and STIM1 couple are the canonical components of SOCE. There are three isoforms in the Klotho family: α, β, and γKlotho [166]. We firstly explored which Klotho isoform regulates Orai1-induced SOCE using HEK293FT cells heterologously expressing Klotho isoforms with Orai1 and STIM1. Overexpression of Orai1 and STIM1 increased SOCE (Fig. 110a–c). Full-length αKlotho inhibited SOCE whereas βKlotho or γKlotho did not affect it (Fig.10a–c). Among isoforms of Orai and STIM, Orai1 and STIM1 were predominantly expressed in HEK293 cells (Fig. 10d). In following SOCE and immunoblotting experiments throughout the paper, endogenous Orai1 and STIM1 were

evaluated. There are at least two types of functional αKlotho, membranous and soluble form [164]. We next examined which functional mode of αKlotho effectively regulates endogenous SOCE in HEK293FT cells. We found that both membranous and soluble αKlotho downregulate SOCE (Fig. 10e–f). The soluble form of αKlotho is more potent to suppress SOCE. Of note, overexpression of both membranous and secreted forms of αKlotho did not affect the expression of endogenous Orai1 and STIM1 in HEK293 cells (Fig. 10g). Together, soluble αKlotho is critical for SOCE regulation.

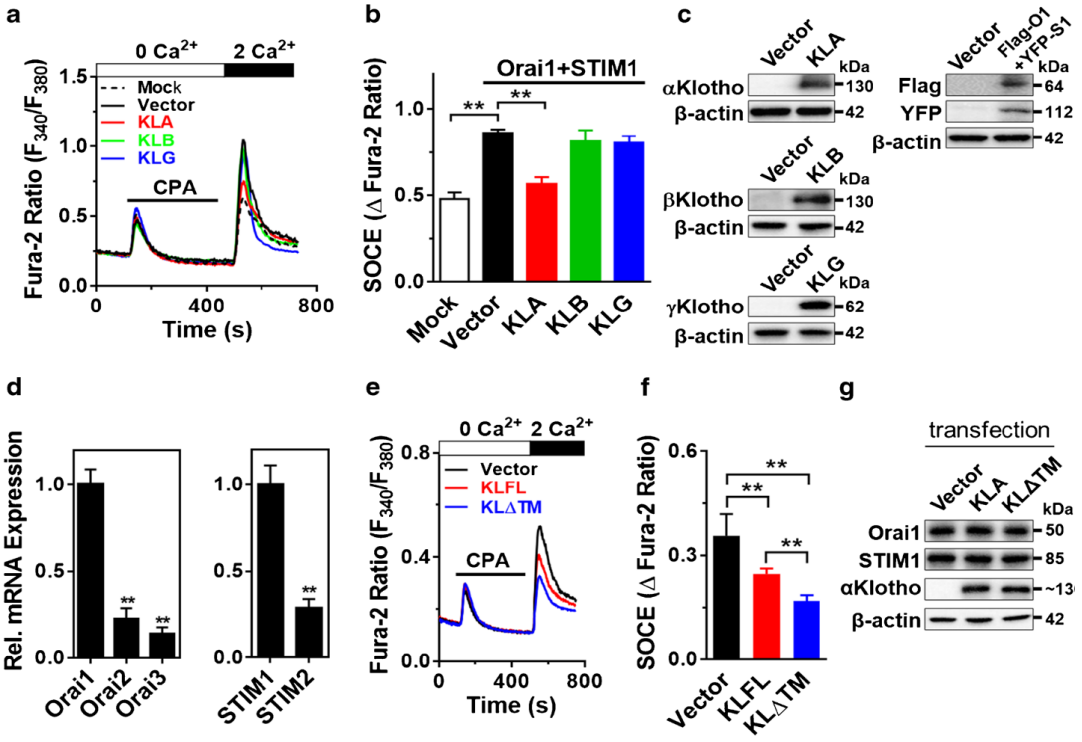

Fig. 10 A soluble form of αKlotho downregulates SOCE without affecting Orai1/STIM1. **a** Representative trace showing the effect of the Klotho family on SOCE. Flag-Orai1 and YFP-STIM1 co-transfected with Klotho isoforms: αKlotho (KLA), βKlotho (KLB), or γKlotho (KLG) in HEK293FT cells. Empty vector (pEF1 vector) used as transfection control. **b** Quantification of peak SOCE values is expressed as mean ± SEM (n = 55–173 each group). **c** Immunoblotting showing transfection of Klotho isoforms and Flag-tagged Orai1 and YFP-tagged STIM1. **d** Quantitative real-time PCR for relative mRNA expression of Orais and STIMs in HEK293FT cells. **e** Representative trace of endogenous SOCE in HEK293FT cells transiently expressing full-length (KLFL) and secreted (KLΔTM) form of αKlotho. Empty vector (pEF1 vector) was used as a transfection control (Vector). **f** Summary of the SOCE in panel e (n =123–186 each group). g Effect of αKlotho (KLFL and KLΔTM) on endogenous Orai1 and STIM1 protein expression in HEK293FT cells. **Denotes $p < 0.01$. Data were analyzed by one-way ANOVA (**b** left panel in **d** and **f)** and t test (right panel in **d**)

4.3.2.2. Soluble αKlotho downregulates serum-stimulated SOCE and CRAC current

Soluble αKlotho has pleiotropic cellular function including regulation of ion channels and growth factor signaling [163]. Here, we examined whether soluble αKlotho regulates serum-stimulated SOCE and Ca^{2+} release-activated Ca^{2+} (CRAC) channel current in HEK293FT cells. Endogenous SOCE was significantly increased in the application of serum compared with that in serum-deprived conditions, and this stimulation was attenuated by pretreatment with recombinant αKlotho protein (Fig. 11a and b). Orai1 is a principal pore subunit of the CRAC channel [161]. Next, CRAC channel current density was measured in HEK293FT cells overexpressing Orai1 and STIM1 by ruptured whole-cell patch-clamp recording. ER Ca^{2+} depletion evoked inward currents under dialyzed whole-cell configuration (Fig. 11c). Current-voltage (I-V) relationship curves showed characteristic inward rectifying CRAC currents (Fig.11d). Soluble αKlotho reduced Orai1 current density and SOCE but had no apparent effects on the general properties of whole-cell currents (Fig. 11c–e). These results support that soluble αKlotho downregulates serum-stimulated SOCE and Orai1 currents.

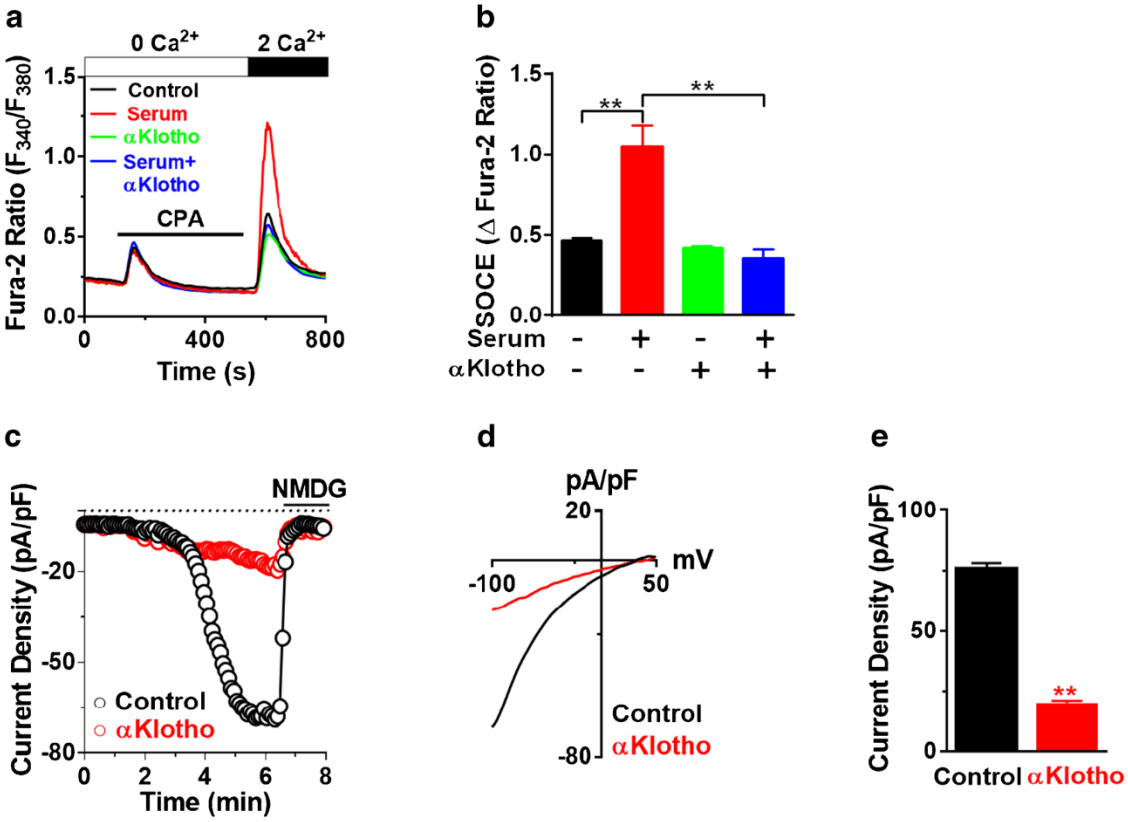

Fig. 11 Soluble αKlotho downregulates serum-stimulated SOCE and CRAC current. **a** Representative trace of SOCE showing the effect of soluble αKlotho protein on serum-stimulated native SOCE in HEK293FT. Serum was deprived (SD) for 16 h followed by incubation of serum (10%) with/without recombinant αKlotho protein (1 nM) for 1 h. **b** Summary of the SOCE in panel a (n = 58–86 each group). **c-e** Effect of soluble αKlotho on CRAC channel current density. Time course(**c**), the current-voltage (I-V) relationship **(d**), and current density (**e** n =13–24 each) of CRAC channel current measured under dialyzed whole cell patch-clamp configuration. All CRAC channel current was measured in HEK293FT cells heterologously expressing Orai1 and STIM1. Orai1 current density was at − 100 mV in **d** and **e.** **Denotes $p < 0.01$. Data were analyzed by one-way ANOVA in **b** and Student's t test in **e**

4.3.2.3. Serum increases the cell-surface abundance of Orai1 via stimulating its exocytosis

We examined the time course of SOCE stimulation by serum treatment. The stimulation of endogenous SOCE by serum was detected after 10 min incubation and reached a maximal effect at 1 h (Fig. 12a). We and others reported that serum growth factors promote transient translocation of TRPC5 and TRPC6 channels to the plasma membrane [165]. Similarly, serum treatment promoted the relocalization of GFP-tagged Orai1 to the plasma membrane (Fig. 12b). Moreover, biotinylation assay showed that incubation with serum increased the steady-state surface abundance of Orai1 but not in the total cell lysates (Fig.12c). Assessment of the cell-surface abundance of Orai1 was confirmed by no detection of intracellular protein at a biotinylated fraction (Fig. 12c). The growth factor stimulates a cell-surface abundance of TRPC channels via their SNARE-dependent vesicular exocytosis [167]. Thus, we examined whether a similar mechanism may involve the upregulation of Orai1 by serum. Brefeldin A (BFA)or tetanus toxin (TeNT) disrupt vesicular exocytosis. Serum-stimulated cell-surface abundance of Orai1 was blunted by preincubation with BFA or TeNT (Fig. 12c and d), indicating that steady-state vesicular exocytosis of Orai1 occurs in the presence of serum.

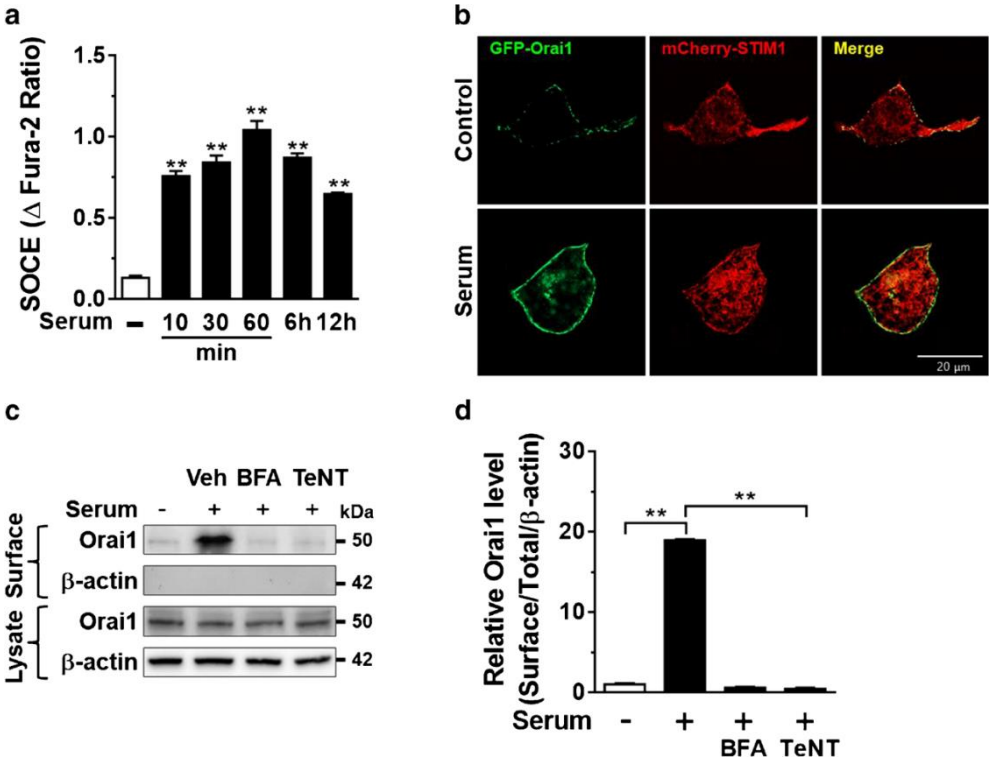

Fig. 12. Serum increases the cell surface abundance of Orai1 via stimulating exocytosis of the channel. **a** Time-dependent response of serum incubation on endogenous SOCE (n = 47–72 each point). **p < 0.01 vs. Serum deprivation (18 h). **b** Effect of serum (10%, 1 h)on plasma membrane localization of Orai1 in HEK293 cells with an inducible eGFP-Orai1 and mCherry-STIM1 protein. GFP and mCherry signals were measured using a confocal microscope. **c** Representative immunoblotting showing the effect of brefeldin A (BFA, 10 μM for 8 h)or tetanus toxin (TeNT, 60 nM, for 16 h) on the serum-stimulated cell-surface abundance of Orai1 analyzed by biotinylation assay. The lack of β-actin detection in the membrane fraction was used as a control for biotinylation. Surface and lysate denote biotinylated fraction and total cellular protein, respectively. **d** Densitometry of the surface abundance of Orai1 in panel c. **p < 0.01, data were analyzed by one-way ANOVA in **a** and **d.**

4.3.2.4. αKlotho reduces the cell-surface abundance of Orai1 via inhibiting exocytosis of the channel

We previously reported that soluble αKlotho downregulates cell-surface abundance of TRPC6 in cardiac myocyte and podocyte by inhibiting serum growth factor dependent exocytosis of the channel [167, 168]. We explored whether a similar mechanism may contribute to the suppression of Orai1 and SOCE. We next measured the effects of αKlotho on a cell-surface abundance of Orai1 using biotinylation assay. Preincubation of soluble αKlotho prevented steady-state and serum-stimulated surface abundance of Orai1 (Fig.13a and b), which supports the notion that αKlotho reduces the cell-surface abundance of Orai1. These findings of plasma membrane expression of Orai1 were confirmed by Ca^{2+} imaging showing that SOCE was inhibited by BFA or TeNT (Fig. 13c–f). The reduction in the cell-surface abundance of Orai1 by soluble αKlotho may result from decreased exocytosis and/or increased endocytosis of the channel. Moreover, inhibition of vesicular exocytosis of the channel by BFA or TeNT decreased SOCE and prevented further inhibition by soluble αKlotho (Fig. 13c–f). These results indicate that αKlotho reduces SOCE via downregulating vesicular exocytosis of the Orai1 channel.

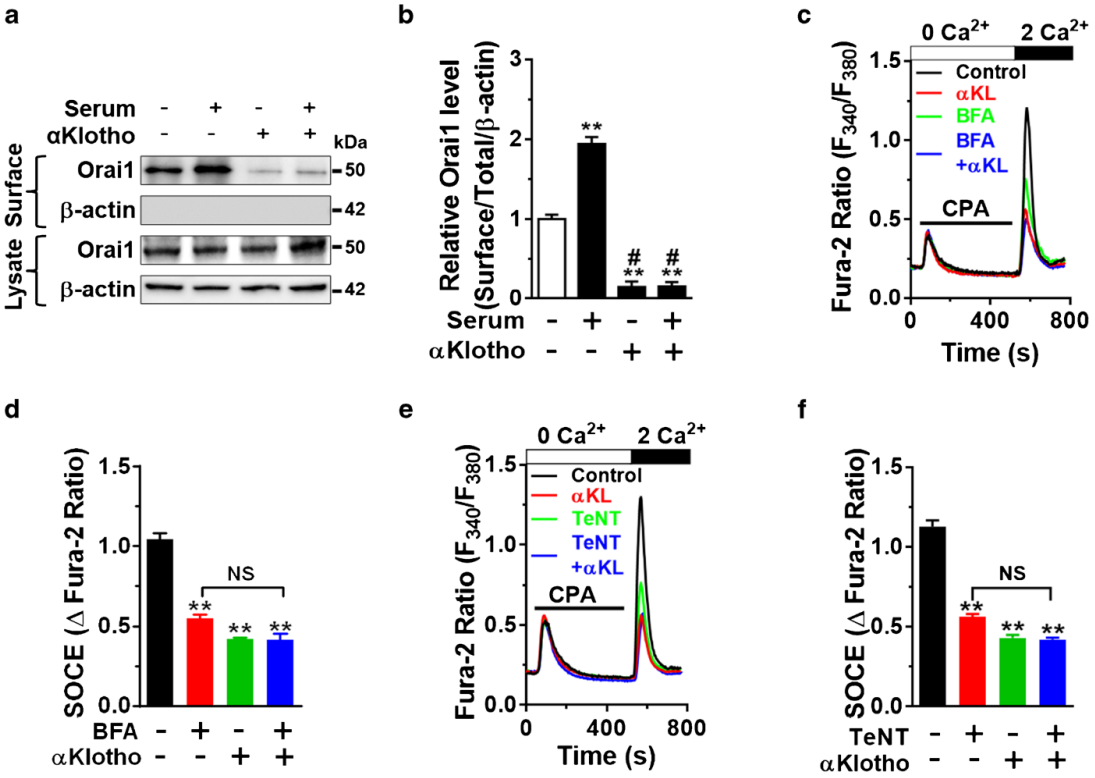

Fig. 13 αKlotho downregulates the serum-stimulated cell surface abundance of Orai1. a Cell-surface biotinylation assay showing the effect of αKlotho on the serum-stimulated cell-surface abundance of Orai1. **b** Quantification of the results in panel a. ***p* < *0*.01 vs. serum deprivation and **#p <** 0.01 vs. serum incubation. **c** Representative SOCE traces showing that αKlotho suppressed SOCE, and prevented the inhibition by Brefeldin A (BFA). Cells were preincubated with BFA (10 μM for 8 h) before αKlotho treatment (1 h). **d** Summary of SOCE in panel **c** (n = 53–252 for each). ***p* < 0.01 vs. vehicle control (no αKlotho). NS, not significant between each group. **e** Representative SOCE traces show that αKlotho reduced SOCE and prevented the suppression by tetanus toxin (TeNT, 60 nM, 16 h). f Summary of SOCE in panel e (n = 84–241 for each). ***p* < *0*.01 vs. vehicle control (no αKlotho). NS, not significant between each group. One-way ANOVA in **b, d,** and **f**

4.3.2.5. αKlotho inhibits SOCE and cell-surface abundance of Orai1 via PI3K-dependent pathway

Activation of the PI3K-Akt pathway by serum growth factors increases the plasma membrane abundance of TRPC channels by stimulating their exocytosis [167, 168]. Soluble αKlotho inhibits increased cell-surface abundance of TRPC3 and TRPC6 by inhibiting the PI3K-dependent pathway [167,169]. We next examined whether αKlotho suppresses SOCE and cell-surface abundance of Orai1 by inhibiting serum stimulated PI3K signaling. Inhibition of PI3K by preincubation with its blockers, wortmannin (WMN) or LY294002, reduced Akt phosphorylation (Fig. 14a–d). Accordingly, αKlotho also reduced serum-stimulated Akt phosphorylation (Fig. 14e and f). Blockade of PI3K by preincubation with WMN or LY294002 inhibited serum-stimulated cell-surface abundance of Orai1 (Fig. 14g and h). Moreover, inhibition of PI3K by WMN or LY294002 abrogated SOCE and prevented further αKlotho-induced inhibition (Fig. 14i and j). Collectively, these results support that soluble αKlotho suppresses SOCE via inhibiting PI3K dependent exocytosis of the Orai1 channel

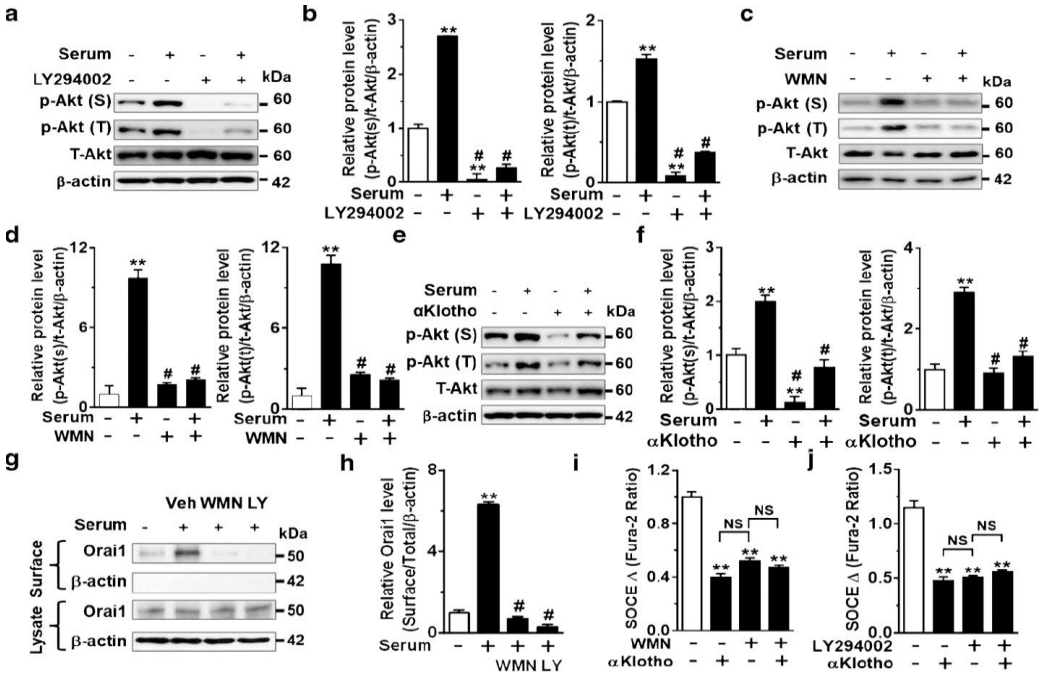

Fig. 14 αKlotho inhibits SOCE and cell membrane abundance of Orai1via the PI3K-dependent signaling pathway. a, c, and **e** Representative immunoblotting showing that effect of preincubation of PI3K inhibitors. **a** LY294002 (LY, 10 μM for 1 h) and **c** wortmannin (WMN, 50 nM for 1 h) and **e** recombinant αKlotho protein (1 nM for 1 h) on Akt phosphorylation at serine473 (p-Akt (S)) and threonine308 (p-Akt (T)) by serum stimulation (10%, 1 h). **b, d**, and **f** Quantification of Akt phosphorylation levels in panel **a, c,** and **e** respectively. **p < 0.01 vs. serum deprivation(SD) and #p < 0.01 vs. serum incubation. **g** Representative biotinylation assay showing the effect of PI3K inhibitors (WMN and LY) on the cell surface expression of Orai1 by serum stimulation. **h** Summary of the surface Orai1 level in panel g. **p < 0.01 vs. SD and #p < 0.01 vs. serum treated. **i** and **j** Summary of SOCE traces showing that αKlotho supressed SOCE and prevented the inhibition by preincubation of PI3K inhibitors, wortmannin (WMN, n = 40–186 each group) or LY294002(LY, n = 76–188 each group), respectively. **p < 0.01 vs. vehicle. NS, not significant between each group. Data were analyzed by one-way ANOVA in **b, d, f**, and **h–j**

4.3.3. Discussion

SOCE is essential for the maintenance of ER Ca^{2+} stores at a precise level for cellular signaling and functions [161,162]. Disturbed SOCE-mediated Ca^{2+} signaling and homeostasis of Ca^{2+} store have been implicated in the pathogenesis of multiple diseases [161]. Major downstream signaling effectors of growth factor receptors are PLCγ and PI3K-Akt pathways. The cellular mechanism of Orai1 activation can be mediated by serum and/or growth factors triggering PLCγ activation, IP3 generation, and Ca^{2+} release from the ER store. Depletion of ER Ca^{2+}store oligomerizes STIM1 to open the Orai1 channel at the plasma membrane [170].

PI3K-Akt pathway signaling contributes to the stimulation of exocytosis of multiple channels such as TRPC5 and TRPC6 [171, 169, 167, 168]. The underlying mechanism of Orai1 regulation by PI3K-derived growth factor signaling remains unsolved. Our data demonstrate that activation of the PI3K-dependent signaling pathway by serum increases the cell-surface abundance of Orai1 via enhancing forward trafficking of the channel to the plasma membrane. These findings support that a similar mechanism may contribute to the downregulation of Orai1-mediated SOCE. Notably, PI3K inhibitors have pleiotropic effects. Therefore, the underlying mechanism by downstream effectors of PI3K to regulate the cell surface expression of Orai1 awaits future study. The aging process is closely related to altered growth factor signaling and ion imbalance including $Ca^{2+}$and Pi[163,172].

The membrane-bound form of αKlotho and βKlotho forms a binary complex with FGFRs, which serves as the physiological receptors for FGF23 and FGF19/21, respectively [173, 163]. We found that membranous αKlotho but not βKlotho or γKlotho downregulates SOCE. Membranous αKlotho associated with FGF receptors functions as a coreceptor for FGF23 signaling to regulate Pi [173, 174]. Soluble αKlotho also regulates multiple ion channels [164]. Our data demonstrate that both types of αKlotho can downregulate SOCE. Soluble αKlotho is more potent to downregulate SOCE, supporting that soluble αKlotho is critical for Orai1-mediated SOCE.

Soluble αKlotho can up- or downregulate multiple channels via a distinct mechanism. αKlotho positively regulates several TRPV (TRPV2, 5, and 6) and K+ channels (ROMK, Kv1.3, KCNQ1/KCNE1, and hERG channels) through increasing cell-surface abundance of the channels by modification of their N-glycan through sialidase or β-glucuronidase activity of αKlotho [153,156,157]. Modifying N-glycans of the channel by αKlotho delays its endocytosis resulting in increased cell-surface abundance [157,175]. Conversely, αKlotho negatively regulates multiple TRPC channels with a distinct mechanism. αKlotho directly binds to the VEGFR2/TRPC1 complex to promote their cointernalization [158]. On the other hand, αKlotho downregulates the cell surface abundance of TRPC6 and TRPC3 via inhibiting their PI3K-dependent exocytosis [168, 176]. In the present study, αKlotho reduces the cell-surface abundance of Orai1 by inhibiting the serum-stimulated PI3K-dependent pathway. This supports the notion that the growth factor driven PI3K pathway is the downstream effector signaling of soluble αKlotho to regulate Orai1 as well as TRPC3 and TRPC6. Recently, the underlying mechanism of αKlotho on

the downregulation of TRPC6 by growth factor-mediated PI3K signaling is unraveled [160,169].

Soluble αKlotho specifically targets α2-3-sialyllactose of monosialogangliosides highly enriched in the lipid raft and particularly downregulates lipid raft-dependent PI3K-Akt signaling to suppress TRPC6 [160]. Orai1 is localized in the lipid raft and binds directly to caveolin-1 and cholesterol [177]. At a steady-state, Orai1 continuously recycles between the endosome and the plasma membrane [177]. In the present study, we show that soluble αKlotho suppresses Orai1 surface abundance via inhibiting PI3K-dependent exocytosis of the channel. Hence, future studies will explore the mechanism that specific lipid raft-dependent PI3K/Akt signaling may contribute to the downregulation of Orai1 by soluble αKlotho. Accumulating evidence demonstrates that the upregulation of Orai1/STIM1-mediated SOCE is associated with tumor progression and poor prognosis in multiple cancers including breast, lung, and renal cancer [178]. Hyperactivation of the PI3K/Akt signaling pathway promotes tumor cell migration. Currently, targeting growth factor receptor-driven PI3K signaling pathway with pharmaceutical agents have been suggested as a therapeutic solution for treating cancers and applied in clinical trials [179]. αKlotho suppresses growth factor-stimulated cell migration by inhibiting PI3K/Akt pathway in multiple tumors such as breast and renal cancers [180]. This study provides compelling evidence supporting αKlotho targeting PI3K-stimulated SOCE function as a tumor suppressor. SOCE is critical for finetuning ER Ca^{2+} stores for cellular signaling and function and its altered activity leads to pathologies [161,162]. Hence, αKlotho-based approaches may be attractive targets for treating SOCE-related pathologies including tumors.

4.4. Minimizes the use of bipolar coagulation and preserves ovarian reserve in endometriotic ovarian cystectomy : clinical randomized controlled trial

4.4.1. Background

Surgical laparoscopy is the most common technique for the treatment of benign ovarian cysts. Laparoscopic ovarian cystectomy has been demonstrated to improve postoperative fecundability and reduce the recurrence rate compared with fenestration and coagulation of the cyst wall. However, laparoscopic cystectomy is associated with a risk of surgical injury to the remnant normal ovarian tissue. The radical laparoscopic surgical approach resulted in an improvement of several disease-related symptoms and quality of life indices. Balancing the surgical radicality between the success of treatment (reduction of pain and recurrence) and the ovarian reserve is a challenging issue. Another crucial issue in laparoscopic ovarian cystectomy is how to reduce the damage and bleeding to preserve ovarian function. Many factors have been reported to affect the ovarian reserve, such as different surgical techniques for the excision (traditional stripping and combined excisional/ablative techniques)[181], mode of hemostasis (suture, compression, and cauterization)[182], operational method (cystectomy vs. wedge resection), and cyst characteristics (size, location, and pathologic types)[181,183], compression, and cauterization)[182], operational method (cystectomy vs. wedge resection), and cyst characteristics (size, location, and pathologic types)[181,184]. Notably, hemostasis by bipolar coagulation, which is commonly used, can result in thermal damage to healthy ovarian follicles and reduce the ovarian reserve [184-186]. Pharmacological intervention is an alternative hemostasis method for minimizing tissue damage. Vasopressin is

commonly used for hemostasis that occurs from the stripping of ovarian cysts and myomectomy [187]. However, intra-operative local injections of vasopressin frequently cause cardiopulmonary complications, such as cardiac arrest, hypotension, and pulmonary edema [188,189]. Therefore, we propose an alternative pharmacological intervention for hemostasis, epinephrine pledget compression (Epi-pledget). The Epi-pledget is widely used in endoscopic sinonasal surgery for local vasoconstriction and bleeding control [190], subsequently reducing blood leakage to the maxillary sinus and subcutaneous tissue after canine fossa puncture [191]. A recent study showed that topical epinephrine applied in prostate surgery facilitated post-operative hemostasis [192]. Intravenous low-dose epinephrine was used in total hip arthroplasty, resulting in a reduction of both bleeding and inflammatory reaction [193]. In addition, an epinephrine injection successfully produced hemostasis for bleeding of colonic diverticula in colonoscopy [194]. The present study evaluated the efficacy of the Epi-pledget on hemostasis to minimize subsequent complications including decreased ovarian reserve, tissue damage and bleeding in laparoscopic cystectomy.

4.4.2. Operative procedure of Participants and study protocols

According to the Consolidated Standards of Reporting Trials (CONSORT), we conducted a randomized controlled trial of patients who underwent laparoscopic ovarian cystectomy between January 2019 and September 2019 in the Department of Obstetrics and Gynecology at the Yonsei Wonju Severance Christian Hospital. The study was approved by the Institutional Review Board (IRB) of the Yonsei Wonju Severance Christian Hospital (CR118035). All procedures were conducted in adherence to the Declaration of

Helsinki and CONSORT. Inclusion criteria were as follows: unilateral ovarian cyst diagnosed by ultrasound examination; diameter of the ovarian cyst between 3 and 15 cm; and appropriate medical status for laparoscopic surgery (American Society of Anesthesiologists Physical Status classification 1 or 2). In detail, we included operative subjects with symptomatic huge size of ovarian cysts. We operated on patients with pathologic ovarian cysts (endometriotic cyst, dermoid cyst, serous cyst, mucinous cyst) that had multi-septated and heterogeneous features diagnosed by transvaginal ultrasonogram (US). Exclusion criteria were as follows: evidence of any other endocrine disorder such as diabetes mellitus, thyroid dysfunction, hyperprolactinemia, and Cushing´s syndrome; postmenopausal status; pregnancy; the use of any hormonal treatment (i.e., use of oral contraceptive pills in the three months before surgery); previous surgery due to adnexal pathology; any complication during operation; conversion to laparotomy; and loss of follow-up. We initially included 198 participants in this study, of which 179 participants completed the follow-up check. All patients provided written informed consent before participation (Fig. 15).

Operative procedure. Operative laparoscopy was performed in the usual manner described in several studies. After the entire cystic portion was separated from the ovarian cortex without vasopressin, a hemostatic material, bipolar coagulation, Epi-pledget compression, or Epi & Coagulation was applied for hemostasis. In the epinephrine groups, diluted epinephrine (0.5 mg/mL of epinephrine) in 50 mL of saline solution was used. In the Epi-pledget compression group, the bleeding sites were covered with the Epi-pledget and compressed directly with a laparoscopic applicator for 2 min to allow hemostasis (Fig. 16). In the Epi & Coagulation group, minimal electrical bipolar coagulation was performed after

compression with the Epi-pledget for 1 min. All operations in this study were performed by one surgeon experienced in laparoscopic ovarian cystectomy (Dr. E. Y. Park).

Study protocol. The primary outcome of the present study was the reserve of ovary tissue and the function after laparoscopic ovarian cyst removal. The ovarian reserve was mainly checked by AFC and AMH. US examination was performed with the 5–6-MHz transvaginal transducer (SSD-α10, Aloka, Japan) to determine the unilateral ovarian cyst size and antral follicle count (AFC) of both ovaries. All subjects underwent transvaginal and/or transrectal US examination twice, once preoperatively and once postoperatively. The preoperative examination was done in the early follicular phase of the menstrual cycle (days 3–7). The postoperative examination was done on a similar day during the second menstrual cycle at 6–8 weeks. Using the US, the AFC was determined as the total number of follicles with a diameter smaller than 10 mm, and both ovaries were counted using the largest cross-sectional sagittal view of the ovary. In all study groups, blood samples were taken within two weeks prior to and within 24 h after surgery. AMH levels were checked on the same day as US examinations. The decline rate of the serum AMH/AFC level (%) was calculated as follows: 100×([preoperative AMH/AFC level—postoperative AMH/AFC level] / preoperative AMH/AFC level).

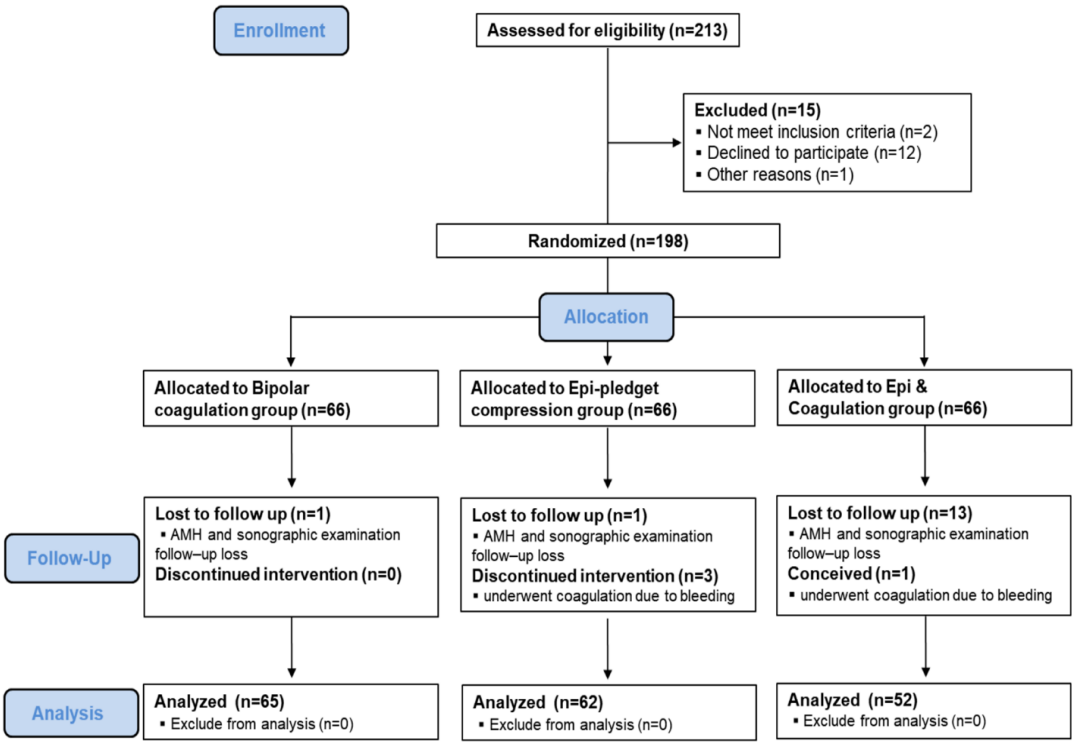

Fig. 15. Study flow diagram showing the study protocol and sequence of events in this study

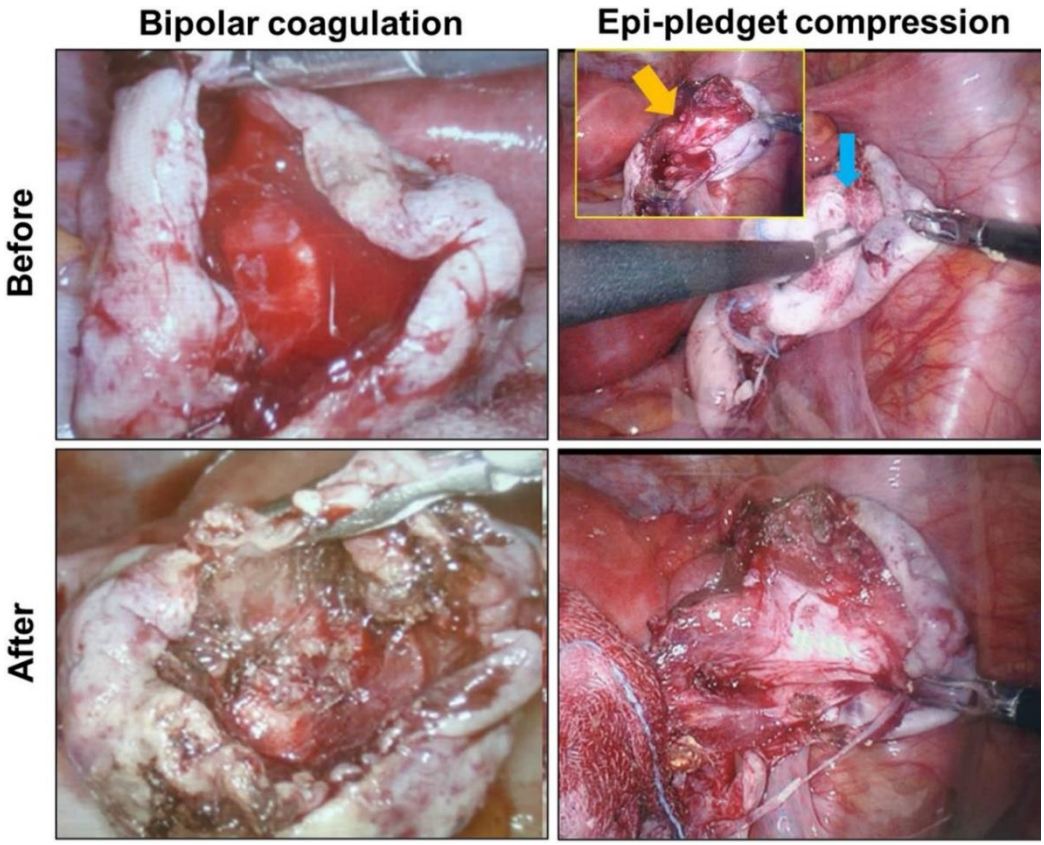

Fig. 16. An operative finding of hemostasis on a laparoscopic ovarian cystectomy lesion. Hemostasis with epinephrine-soaked pledget compression (Epi-pledget compression) and bipolar coagulation before and after laparoscopic stripping of ovarian cysts. Yellow and blue arrows indicate bleeding of the laparoscopic cystectomy lesion and Epi-pledget compression on the stripping lesion, respectively.

4.4.3. Animal model

Thirty female mice (C57B6/J) aged 12 weeks and around 21–23 g were randomly divided into three groups according to hemostasis techniques: the sham (no artificial incision injury and treatment to the ovary) group, the bipolar coagulation group, and the Epi-pledget compression group (n=10 per group). After an operation was performed to create an artificial incision injury to the ovary, bipolar coagulation, and Epi-pledget compression techniques were applied to the injured ovary. Five mice in each group were sacrificed at postoperative day 1. The remaining five mice were sacrificed at postoperative day 4. Except for the sham group, the ovary wounds in all other groups were treated with hemostasis. The hair on the backs of the mice was shaved 2 days before surgery. Mice were anesthetized via inhalation followed by a transverse incision at the lower back of the mouse (Fig. 4A) and entry into the retroperitoneal space to approach the ovary. A partial incision of the ovaries resulted in wound production and bleeding of the ovary. To stop the bleeding, bipolar coagulation and diluted epinephrine (0.5 mg/mL of epinephrine in 50 mL of saline solution)-soaked cotton pledget compression were introduced. When the stoppage of bleeding was confirmed in the damaged lesion, the skin was closed by suture and the mice were checked for vital signs and complications at 1 and 4 days. All animal research protocols were approved by the Institutional Animal Care and Use Committee, Yonsei University Wonju College of Medicine, Korea (YWC-170622–1).

4.4.4. Results

Figure 1. illustrates tissue changes in representative cases after hemostasis using different methods. We observed thermal injury in the bipolar coagulation group (Fig.

15). The Epi-pledget compression successfully stopped bleeding without burn injury in the stripped lesion of laparoscopic ovariectomy (Fig. 16). We measured the levels of AMH and AFC before and after surgery. Both the serum level of AMH and AFC count significantly decreased in the bipolar coagulation group compared with the Epi-pledget compression group and the Epi & Coagulation group (Table 8). Of note, no significant difference was observed regarding AMH and AFC between the Epi-pledget compression group and the Epi & Coagulation group (Table 8). We investigated the effect of hemostatic methods on AMH and AFC in patients stratified according to age. The group consisting of older subjects (age≥34) showed a significant difference in AMH according to different hemostatic methods (Table 8). Among the subgroups of the four types of ovarian cyst, the differences of AMH and AFC decline were insignificant.

	Types of hemostasis			P-value
	Bipolar Cauterization group	Epi-pledget group	Epi-pledget & Cauterization group	
All subjects	(n = 65)	(n = 62)	(n = 52)	
Rate of AMH decline (%)	24.5 (8.1–40.4)	0.8 (–18.7–39.2)[a]	10.34 (–17–30.0)[a]	0.005
Rate of AFC decline (%)	33.3 (– 41.7–60.0)[b]	25.0 (–100–50)	25.0 (–50–50)	0.026
Age < 34 years	(n = 42)	(n = 34)	(n = 31)	
Rate of AMH decline (%)	24.4 (7.5–40.6)	4.0 (–24.8–35.6)	20.5 (–11.1–43.3)	0.052
Rate of AFC decline (%)	33.3 (– 25.0–50.0)	29.2 (–50–45.8)	0 (–100–50)	0.125
Age ≥ 34 years	(n = 23)	(n = 28)	(n = 21)	
Rate of AMH decline (%)	27.1 (14.9–39.22)	– 2.4 (–8.5–40.5)	– 3.7 (–37.5–18.5)[a]	0.003
Rate of AFC decline (%)	50.0 (–41.7–63.3)	0 (–100–50)	33.3 (–50.0–50.0)	0.09

Table 8. Comparison of AMH and AFC decline rates between hemostatic groups. Data are presented as median (interquartile ranges). Decline rate was defined as 100 x [preoperative level (AMH or AFC) – postoperative level (AMH or AFC)] / preoperative level (AMH or AFC). AMH anti-Mullerian hormone, AFC antral follicle count, Tukey HSD Tukey honest significant differences.

Histopathological examination was conducted in all patient cases (n=198), yielding the following diagnoses: benign ovarian cyst, either mature cystic teratoma (n=75, 42%) or endometrioma (n=69, 39%); or serous and mucinous cystadenoma (n=35, 20%). No significant differences were evident in the mean maximum diameter of each ovarian cyst type [6.34±1.7 cm (mean±standard deviation) in the endometrioma group, 6.90±2.0 in the teratoma group, and 6.72±2.86 in the serous and mucinous cyst group]. Furthermore, the rates of decline in serum AMH and AFC did not differ among the subtypes of ovarian cyst. Next, we performed a subgroup analysis based on the endometrioma and non-endometrioma groups (Fig. 17). In the case of endometrioma, the Epi-pledget compression group showed less AMH decline than the coagulation group (Fig. 17A). When comparing the rate of serum AMH decline in the coagulation group, patients with endometrioma showed a greater AMH decline than those with non-endometrioma. The rate of AFC decline was not significantly different among the three hemostatic intervention groups and the ovarian pathologic cyst types. In hemoglobin changes and operative time based on the three hemostasis methods, we found that neither differed between the bipolar coagulation group and the Epi-pledget compression group (Fig. 18A–D). However, the coagulation group and the Epi & Coagulation group showed differences in operation time (Fig. 18C, D). Together these results indicate that Epi-pledget compression is a favorable hemostasis intervention for the follicle reserve in patients. To examine pathological changes after applying hemostatic interventions, we employed a mouse model and evaluated acute and chronic changes in the injured ovary tissues after hemostatic interventions (Fig. 19A). No cardiovascular or other complications were observed in the mice, although the same concentrations of epinephrine (0.1%) were used in human patients. Electrocoagulation

intervention exaggerated blood vessel collapse and fibrotic changes in mouse ovaries more so than epinephrine intervention (Fig. 19B, C). Moreover, electrocauterization caused a wide range of tissue injuries with eosinophilic granulomatous tissue formation around the necrotic area and a decrease in the number of growing follicles, while Epi-pledget intervention ameliorated the tissue and follicle injuries (Fig. 19D, E). Next, we examined the proliferative potential after hemostasis interventions using Ki-67, a proliferation marker. Ki-67 immunoreactivity in the granulosa cell layer of the growing follicle was markedly reduced in the electrocoagulation group, while the Epi-pledget intervention maintained Ki-67-positive cells (Fig. 20A). This was further confirmed by the Ki -67 index, which was significantly lower in the electrocoagulation group compared with the epinephrine group (Fig. 20B).

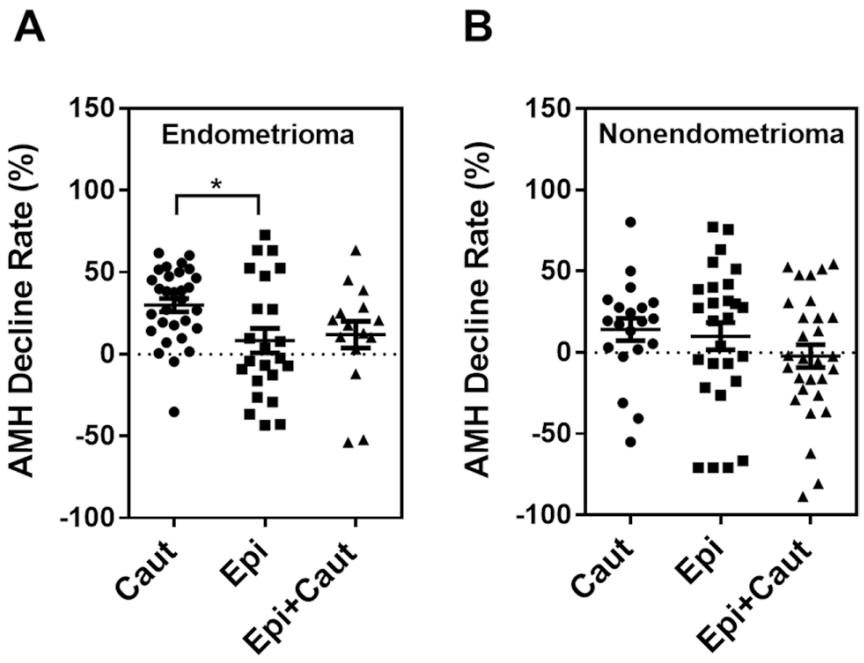

Figure 17. Comparison of AMH decline rates between hemostatic groups according to ovarian pathologic subtypes. AMH decline rates (%) in endometrioma (**A**) and non-endometrioma (**B**) groups. Decline rate was defned as 100 × [preoperative AMH level – postoperative AMH level] / preoperative AMH level. **p<0.05. Caut* cauterization, *Epi* epinephrine-pledget compression, *Epi+Caut* cauterization and epinephrine-pledget compression.

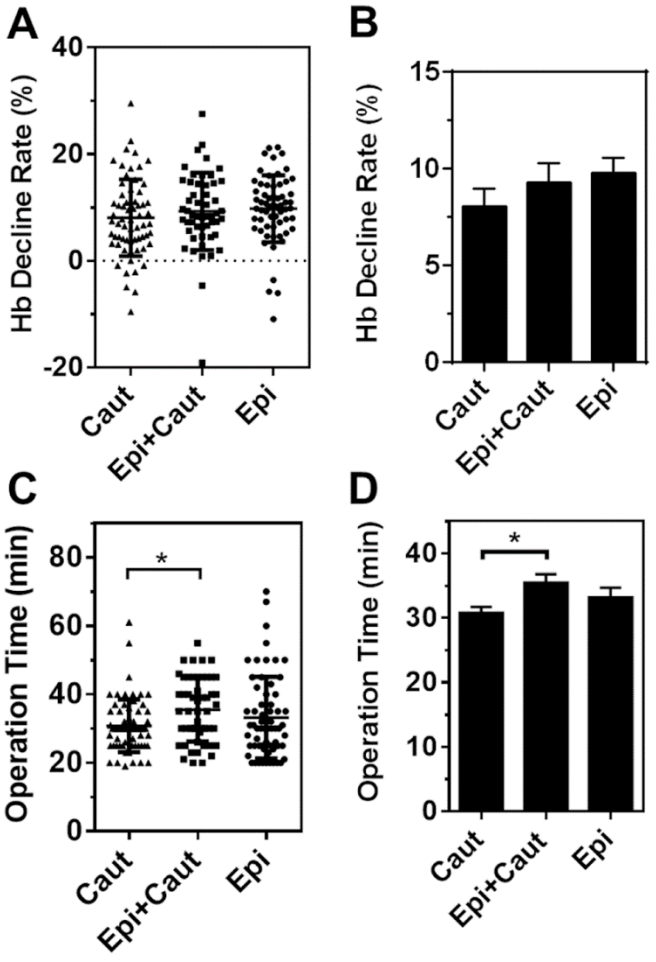

Figure 18. Postoperative change of hemoglobin and operation time. **(A)** Hemoglobin (Hb) decline rate in individual patients between hemostatic groups. Decline rate (%) was defined as 100 × [preoperative Hb level – postoperative Hb level] / preoperative Hb level. **(B)** Summary of panel **A. (C)** Operation time (min) of patients between hemostatic groups. **(D)** Summary of panel C. *p<0.05. Data are presented as mean±standard deviation. *Caut* cauterization, *Epi* epinephrine-pledget compressio*n, Epi*+*Caut* cauterization and epinephrinepledget compression, Hb hemoglobin.

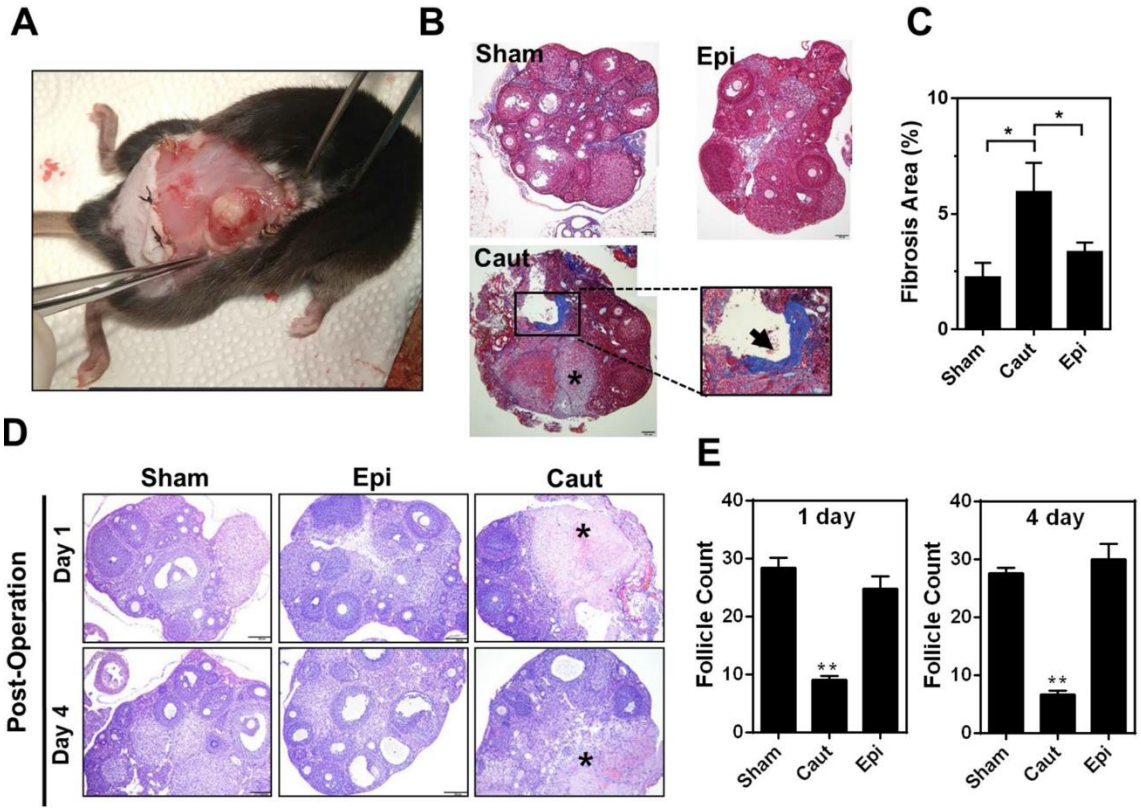

Figure 19. Quality of fibrosis and ovarian tissue reservation by hemostasis interventions in an injured ovary mice model. **(A)** Transverse incision at the lower back of the mouse and entry into the retroperitoneal space to approach the ovary. **(B)** Masson's trichrome stain of mouse ovaries of each group (magnification, 200×). Necrosis region (*) and fibrotic tissue region (arrow, magnification, 400×) were found in the electrocauterization group. **(C)** Quantification of fibrotic area (%). *p<0.05 **(D)** Histological evaluation of injured ovarian tissues after hemostasis with Epi-pledget compression and bipolar coagulation. The tissue of the Epi-pledget compression was almost similar to the sham group, whereas necrosis and fibrotic tissue change were observed in the coagulation group. An asterisk indicates necrotic regions. Magnification, 200×. (E) The follicles of each slide were counted to obtain the mean value. **p<0.01. *Sham* normal ovary, *Caut* electrocauterization group, *Epi* epinephrine-soaked cotton pledget compression.

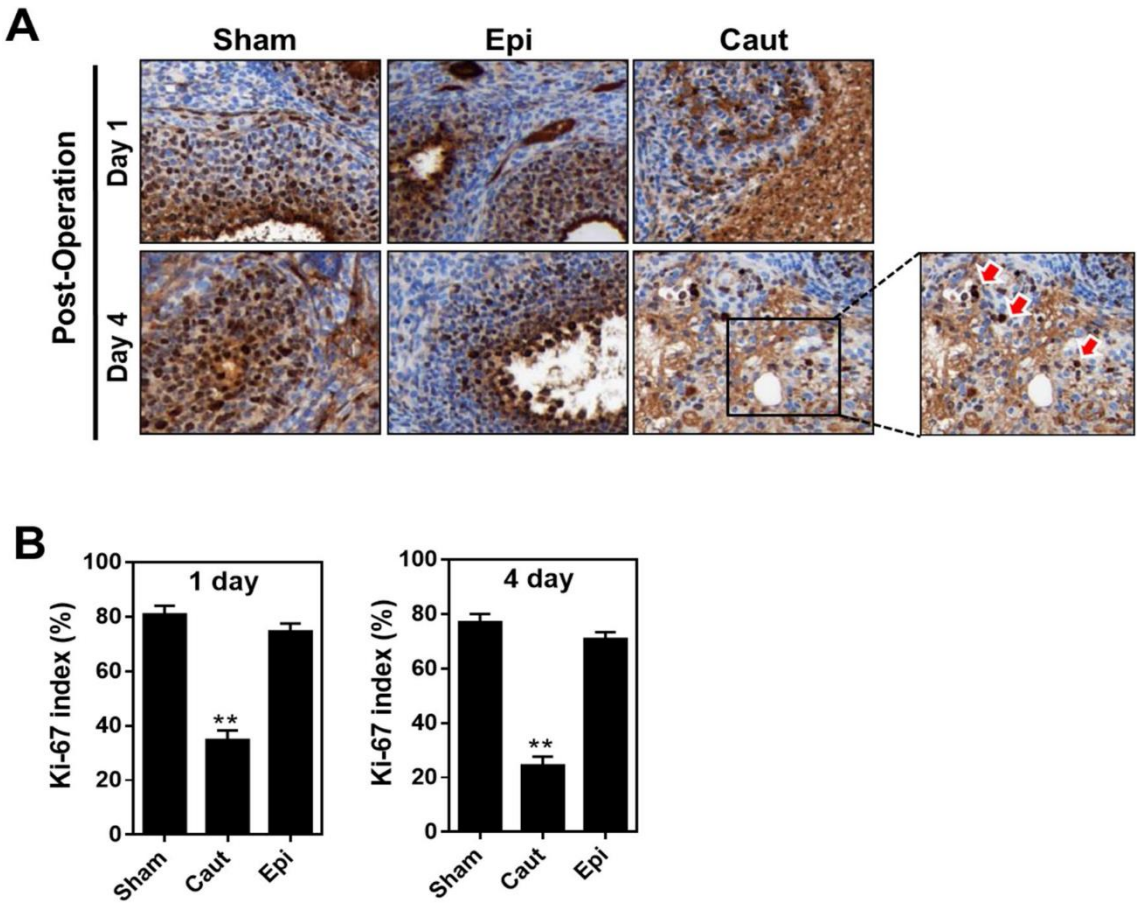

Figure 20. Proliferative capability by hemostasis interventions. **(A)** Proliferation capability was evaluated using Ki-67 immunoreactivity in mouse ovary tissue. Magnification, 200×. Stained nuclei (arrow) of the cauterization group ovarian tissue with Ki-67; the stained nuclei of normal growing follicular cells with Ki-67 (arrow) are easily identified. Magnification of inset, 400×. **(B)** Ki-67 index was calculated as the percentage of the number of stained nuclei at 1 and 4 days after the operation. ***p*<0.01 vs. Sham group

4.4.5. Discussion

The present study demonstrates the beneficial effects of epinephrine pledget compression on an ovarian cystectomy, especially endometrioma lesion preserving ovarian reserve. Additionally, we also provide evidence that the Epi-pledget causes less thermal damage to the ovarian parenchyma than bipolar coagulation in an animal model. Electrocoagulation was shown to cause a greater decline in serum AMH and/or AFC after surgery than suture[194]. The hemostatic method in group 3 included both Epi-pledget and bipolar cauterization. In detail, the major hemostatic method in group 3 was the Epi-pledget and the bipolar cauterization was the assistant hemostatic method for oozing site after the application of the Epi-pledget. The Epi-pledget reduced the time of application of the bipolar cauterization and might alleviate the ovarian damage by electronic power. In hemostatic interventions according to the pathologic subtype of ovarian cyst, the rate of serum AMH decline was lower in the endometrioma group with bipolar coagulation than in the endometrioma group with Epi-pledget compression or Epi & coagulation. Animal experiments supported the notion that bipolar coagulation aggravated the reduction of ovarian reserve after surgery and caused irreversible ovarian tissue damage compared with that of the Epi-pledget. The primary pathophysiological mechanisms in irreversible ovarian tissue injury were inflammation and fibrosis that were induced by the thermal damage by electrocauterization rather than vasoconstriction and ischemic change by pharmacological intervention. The Epi & Coagulation group had a significantly longer operation time than the coagulation group, but this did not affect the decline in AMH and hemoglobin levels. These results suggest that surgeons should consider the use of Epi-pledget compression alone or with minimal assistance of coagulation to preserve the

ovarian reserve after laparoscopic ovarian cystectomy. Similar to our study, the Epi-pledget or epinephrine-involved intervention was applied in other fields of surgery or procedures [190-192]. The effectiveness of hemostasis and the preservation of normal tissue function of the Epi-pledget indicated the potential application at the surgery for other hormone-secreting tissues [195,197]. The challenging issue in the surgical treatment of ovarian cysts is the possibility of encountering an unexpected ovarian malignancy. Approximately 0.9% and 3.0% of benign-appearing ovarian cysts were reported as unsuspected ovarian cysts in pre- and post-menopausal patients, respectively [196]. As another study recommended26, we strictly selected subjects via medical history, physical examination, serum CA125, and the transvaginal US. As a result, we did not report any patients diagnosed with ovarian malignancy. Our study reveals that hemostasis by Epi-pledget compression significantly increased ovarian reserve preservation compared with cauterization during laparoscopic benign ovarian cystectomy. Experiments using a mouse cystectomy model support the findings that the Epi-pledget intervention effectively ameliorates irreversible tissue damage causing the reduction of ovarian reserve. Based on the effectiveness and safety of bleeding control, the Epi-pledget showed similar effectiveness as bipolar coagulation. Our study suggests that Epi-pledget compression increases the preservation of the ovarian reserve by minimizing the use of coagulation.

V. CONCLUSIONS

In this study, in ishikawacell, an endometrial cancer cell, Ca^{2+}entrance was increased by TNFα signaling, an inflammatory stimulation, and Orai1 was overexpressed. And klotho inhibited TNFα-induced overexpression of Orai1 and NFκb signaling during the phosporylation process. This fact is inconsistent with previous studies. In addition to suppressing inflammatory cells, Klotho suppresses the proliferation and migration of cancer cells, confirming the role of klotho in malignant cells. In endometrial cancer cells, migration and proliferation were significantly reduced in klotho-treated cell. Since it was observed that Klotho inhibits the migration and proliferation of endometrial cancer cells in this experiment, it can be expected to play a role in inhibiting adhesions caused by endometriosis and uterine bleeding, uterine pain and endometrial cancer caused by endometrial proliferation.

We compared the staining score of each IGF1, SGK1, Orai1 and Klotho in adenomyosis and endometrial cancer tissue with modified Allred scoring system in IHC assays. The mean staining score of IGF1, SGK1, Orai1 and Klotho were respectively statistically significant difference (Table 7, Fig. 9).

We found that both membranous and soluble αKlotho downregulate SOCE (Fig. 10e–f). The soluble form of αKlotho is more potent to suppress SOCE. Of note, overexpression of both membranous and secreted forms of αKlotho did not affect the expression of endogenous Orai1 and STIM1 in HEK293 cells (Fig. 10g). Soluble αKlotho reduced Orai1 current density and SOCE but had no apparent effects on the general

properties of whole-cell currents (Fig. 11c–e). These results support that soluble αKlotho downregulates serum-stimulated SOCE and Orai1 currents.

we examined whether a similar mechanism may involve the upregulation of Orai1 by serum. Brefeldin A (BFA)or tetanus toxin (TeNT) disrupt vesicular exocytosis. Serum-stimulated cell-surface abundance of Orai1 was blunted by preincubation with BFA or TeNT (Fig. 12c and d), indicating that steady-state vesicular exocytosis of Orai1 occurs in the presence of serum. These findings of plasma membrane expression of Orai1 were confirmed by Ca^{2+} imaging showing that SOCE was inhibited by BFA or TeNT (Fig. 13c–f). The reduction in the cell-surface abundance of Orai1 by soluble αKlotho may result from decreased exocytosis and/or increased endocytosis of the channel. Moreover, inhibition of vesicular exocytosis of the channel by BFA or TeNT decreased SOCE and prevented further inhibition by soluble αKlotho (Fig. 13c–f). These results indicate that αKlotho reduces SOCE via downregulating vesicular exocytosis of the Orai1 channel. αKlotho also reduced serum-stimulated Akt phosphorylation (Fig. 14e and f). Blockade of PI3K by preincubation with WMN or LY294002 inhibited serum-stimulated cell-surface abundance of Orai1 (Fig. 14g and h). Moreover, inhibition of PI3K by WMN or LY294002 abrogated SOCE and prevented further αKlotho-induced inhibition (Fig. 14i and j). Collectively, these results support that soluble αKlotho suppresses SOCE via inhibiting PI3K dependent exocytosis of the Orai1 channel.

Therefore, the pathologic mediator signaling pathway of IGF1 and SGK1, it can be hypothesized that acute and chronic stimulation will lead to inflammation, fibrosis, and tumor progression through Orai1 activation and ca entry by IGF1 and SGK1.

VI. FUTURE DIRECTIONS

In this study, among the mechanisms of various clinical phenomena that endometrial cells exert on internal organs and women's lives, using pathways partially identified in the literature, the parts that can be applied to clinical practice were studied through experiments and demonstrations. I was thinking about strategy.

According to a literature review, three pathways were identified as pathogenic mediators involved in the causative mechanisms from endometriosis, a benign disease caused by expression of endometrial cells outside the uterus to endometrial cancer, according to literature review. The clinical output was confirmed by increasing Orai1 overexpression and ca2+ entry by each of these signaling through the PI3k/Akt signaling and SGK1 signaling pathways, and the process of Klotho inhibition was also confirmed. SGK1 and orai1 were actually increased in uterine adenomyosis and endometrial cancer tissue, so it was found that Orai1 was regulated by SGK1.

Further details on the precise signaling pathway and the mechanism of inhibition by Klotho. In addition, Klotho is known to have anti-tumor effect because it has the ability to inhibit cancer proliferation and migration in cancer. In the process of progressing from endometriosis and adenomyosis, which are diagnosed in many women of childbearing age, to endometrial cancer, klotho inhibits tissue invasion and proliferation, weakens the induction of angiogenesis, and If the ability to suppress the developmental ability of the distal lesion is discovered, it can be a useful early detection marker.

The extension of this study is in the area of endometriosis for which the current

treatment is not clear. It could be an important contribution to laying the academic foundation for the development of therapeutics.

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VIII. ABSTRACT IN KOREAN

자궁내막세포 질환에 대한 Klotho 의 방어적 역할

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자궁내막증은 일반적으로 자궁 내강 내층에 위치하는 자궁내막 조직이 자궁 내강 외부에 존재하는 상태이다. 가임기 여성의 10~15%에서 발생하지만 원인, 병태생리, 진단, 및 치료와 예방 방법은 아직까지 정확하게 알려져 있지 않으면서 여성이 폐경에 이르기 전까지 심각하고 반복적인 골반통과 불임등 다양한 증상을 유발하고 여성의 삶을 황폐하게 만드는 진행성 질환 중 하나이다. 자궁내막증의 원인에 대해서는 다양한 가설이 보고되고 있고, 여러 문헌들에 따르면 inflammation을 비롯하여 SGK1, IGF1 및 Orai1 등이 자궁내막증, 자궁선근증 및 자궁내막암을 유발하는 pathologic mediators로 제시되고 있다.

본 연구는 이러한 mediators를 이용하여 자궁내막증의 병원성 경로를 밝히고자 하였다. 염증 신호는 여러 보고서에서 NFκB를 통한 Ca^{2+}진입(SOCE)을 증가시켜 자궁내막증을 유도하는 것을 확인하였다. IGF1은 PI3K/Akt 경로를 통해 Ca^{2+} 진입(SOCE)을 증가시켜 Orai1을 활성화시키는 것을 확인하였다. SGK1의 경우는 자궁선근증 및 자궁내막암에서 높게 발현되고 Orai 1을 활성화시키는 것을 확인하였다.

αKlotho 유전자는 hypomorphic klotho 대립 유전자 마우스에서는 성장 지연, 혈관 석회화, 골다공증 및 조기 사망을 포함한 다양한 수명 관련 표현형을 보였으나, 반대로 klotho 유전자가 과발현된 쥐는 노화 방지 유전자라는 증거를 뒷받침하는 수명 연장을 보여주었다. αKlotho는 유형 1 막횡단 항노화 단백질입니다. αklotho 결핍 마우스는 조기 노화 표현형과 Ca^{2+} 및 인산염을 포함한 이온 항상성의 불균형을 보입니다. Soluble αKlotho는 다중 이온 채널 및 성장 인자 매개 PI3K 신호 전달을 조절하는 것으로 알려져 있다. Klotho 계열 중에서 αKlotho는 SOCE를 하향 조절하는 반면 β Klotho 또는 γKlotho는 SOCE에 영향을 미치지 않는 것으로 알려져 있다.

Soluble αKlotho는 혈청 자극 SOCE 및 Ca^{2+} 방출 활성화 Ca^{2+} (CRAC) 채널 전류를 억제하며, serum 채널의 vesicular exocytosis를 자극하여 Orai1의 세포 표면 풍부함을 증가시킨다. 혈청 자극 SOCE 및 Orai1의 세포 표면 풍부는 αKlotho 단백질 또는 PI3K 억제제의 사전 배양에 의해 억제됩니다. Brefeldin A 또는 tetanus toxin 또는 PI3K 억제제의 전처리 실험에서 SOCE 및 Orai1가 세포 표면 위에 많아지는 현상을 억제하는데 αKlotho에 의해 이러한 현상이 방해 되는 것을 확인하였다.

이상의 결과들은 soluble αKlotho가 Orai1 채널의 PI3K 구동 vesicular exocytosis를 억제함으로써 SOCE를 하향 조절하고 SOCE 매개 종양 세포 이동의 억제에 기여한다는 것을 입증하였다. 다음 실험에서 Klotho 발현은 종양 형성 및 혈청 또는 IGF-1 매개 자궁내막증 진행과 음의 상관관계가 있었으며, 이는 Klotho가 자궁 내막세포 뿐만 아니라 관 세포에서도 보호 역할을 할 수 있음을 설명할 수 있었다. 이 모든 결과를 통해 Klotho와 성장 인자 및 호르몬 수용체 매개 Ca^{2+} 채널 활성화를 연결하는 메커니즘에 대한 새로운 관점을 제공하고 Ca^{2+} 신호 조절 장애로 인한 질병 치료를 위한 새로운 치료 전략을 제공할 수 있을 것이다.

핵심되는 말: 자궁내막증, Klotho, Orai1, IGF1, SGK1, Ca^{2+} signaling, Ishikawa cell

IX. ACKNOWLEDGEMENTS IN KOREAN

'하나님과 사람 앞에서 성실하고 겸손하게 노력 하거라.' 늘 집을 나서기 전 부모님이 등 뒤에서 축복해 주는 말씀입니다. 이 말씀처럼 조금씩 착실하게 배워가며 좀 긴 시간이었지만 "중요한 것은 계속해 나아가는 용기 (It's the courage to continue that counts)라는 처칠의 말처럼 한 걸음씩 떼어놓다 보니, 마침내 학위 논문의 마지막 페이지 앞에 있습니다. 모든 순간이 감사한 분들과 고마운 시간이었습니다. 연구하는 동안 혼자 있게 하지 않으시고, 막막할 때마다 순간순간 훌륭한 선생님들의 도움을 통해 부족함 없이 가르침을 받도록 인도하신 하나님께 감사드립니다. 빠듯한 삶 속에서도 지치지 않도록 저의 호흡을 지키시고 이끄신 하나님을 찬양합니다.

새벽 3시면 어김없이 제일 먼저 예배당의 불을 밝히고 나라와, 교회와 자녀들을 위해 평생 무릎으로 사신 어머니, 아버지. 이 자리에서 부르지 않아도 이미 가슴 속에 커다란 사랑으로 와 계시는 두 분을 존경하고 사랑합니다. 그리고 큰 누이를 부모님처럼 섬겨주는 항상 멋지고 착한 동생들 은경, 대영, 대현에게 고마움을 전하고 싶습니다.

긴 시간 동안 보이지 않게 특유의 꼼꼼하심으로 지지해 주시면서 이 논문의 완성을 볼 수 있도록 해 주신 최성진 교수님께 감사드립니다.

소중한 친구이자 멘토인 차승규 교수님, 연구에 대한 진정한 개념이 없는 저에게 꾸준하고 변함없는 인내와 격려로 저의 학위과정을 의미 있는 시간으로 만들어 주었습니다. 진료와 많은 수술들 속에서도 실험결과를 얻기 위해 홀로 실험실에서 수많은 새벽을 맞이하면서 지쳐서 저의 처음 의지를 내려놓고 싶었을 때 나눠준 실질적인 조언은 단순한 학위취득보다 연구 과정 중에 더 값진 열매들을 경험할 수 있도록 해주었습니다. 이 경험은 향후 제가 지속적으로 연구에 대해 새로운 이해와 관심을 가지고 매진할 수 있도록 하는 지원의 원천이 되었습니다.

많은 시간 동안 의학관 4층의 실험실에서 함께하며, 수많은 실수와 허점 투성이인 내가 귀찮고 힘들었을 텐데도 아낌없이 자신의 시간들을 나눠주며, 인내를 가

지고 모든 실험을 기초부터 완성까지 저의 곁을 떠나지 않고 끊임없이 응원하고 가르쳐 주며 조언과 피드백을 준 고마운 생리학교실 김지희 박사와 황규희 박사 그리고 생리학교실 모든 연구원들 한 사람 한 사람에게 마음 깊은 감사와 사랑을 드립니다. 또한 실험실을 아무 제약 없이 마음대로 사용할 수 있도록 해주시면서 볼 때마다 용기와 사기를 북돋아 주신 박규상교수님, 공인덕교수님. 평생 연구와 교육에 매진하신 두 분을 보면서 학문이 참 성실 해야 함을 배울 수 있었습니다.
이 학위 과정에서 저의 가장 큰 열매와 감사는 학위논문 보다 하나님이 저를 위해 예비해 주신 많은 인연들 이었다고 고백합니다.

힘든 일이 있을 때 언제든지 투정하고 맘껏 속마음을 내려놓을 수 있도록 넉넉한 나무 그늘이 되어 주신 문애경-윤대규목사님, 조성민 선생님. 하나님이 형님과 아우로 맺어주신 사랑으로 그저 고맙습니다. 덕분에 힘내서 견딜 수 있었습니다.

수련하는 과정 중에 같이 일하는 동기가 없어 힘들었던 시간이었지만 제가 그 시간 동안 견딜 수 있었던 것은 산부인과 교실 은사님들의 사랑과 격려 그리고 아껴주심 이었습니다. 죄송하게도 제가 학위를 마치기 전에 은퇴하신 차동수, 한혁동, 이영진, 정인배 교수님께 이 논문을 바칩니다. 그리고 수많은 교실의 살림과 복잡한 문제를 지혜롭고 차분하게 해결해 주시면서도 성급하고 미숙한 저의 부담과 부족함을 항상 걱정해 주신 한경희 교수님, 선생님께서 참고 배려해 주신 덕분에 제가 저의 일에 집중하고 여기까지 올 수 있었음에 깊이 감사드립니다. 또한 서툴고 부족한 논문이지만 세심하게 검토해 주시고 시간 될 때마다 많은 경험과 훌륭한 영감을 나눠 주신 정약식, 엄민섭, 손준형 교수님께 감사의 말씀을 올립니다.

마지막으로, 저의 환자들에게 감사합니다. 환자분들은 제가 학위과정을 시작하고 연구하는 의사가 되겠다고 결심한 이유입니다. 환자들이 저에게 몸을 믿고 맡기셨을 때 저의 무지함과 지혜 없음과 무관심이 환자들과 그 가족의 삶에 일생 동안 아픈 상처를 남겨 줄 수 있다는 것을 깨닫고 제가 올 곧게 일하는 의사가 되도록 하셨기 때문입니다.

우리 수련의들에게는 항상 환자를 보며 고민 하고 연구하며 멋지게 성장하는 슬기로운 의사들이 되는데 작은 자극이라도 될 수 있기를 기대합니다.

X. PUBLICATION LISTS

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XI. VITAE

Park Eun-young was born on January 4, 1970 in Hae-do, Hampyeong-gun, Jeollanam-do, the daughter of Park Hyung-soo and Lee Young-jae. She has a younger sister Eun-kyung and younger brothers Dae-yeong and Dae-hyeon. After graduating from Songwon Girls' High School in Gwangju in 1988, and graduating from the Department of Mathematics at Chosun University in 1992, she obtained a Bachelor of Science, and entered the Department of Rehabilitation at Yonsei University's College of Health Sciences in 1994 and obtained a bachelor's degree in occupational therapy in February 1997. In March 1997, he entered Yonsei University College of Medicine, obtained a Bachelor of Medicine in 2004, and passed the medical examination. In March 2004, he completed an internship at Wonju Severance Christian Hospital, and in March 2006, he started obstetrics and gynecology residency training. He served as a fellowship in the same hospital from 2010 to February 2011, as an assistant clinical professor from 2011 to February 2016, and as an associate clinical professor from 2016-2021.

XII.