Master's Thesis of Science in Agriculture

The effect of ETV2 on the reversal of endothelial to mesenchymal transition in endothelial cells

ETV2에 의한 내피-중간엽 전환 회복에 대한 연구

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Abstract

ETV2, endothelial transcription factor, can function as a therapeutic agent against kidney fibrosis selectively in endothelial cells

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Various types of damage such as toxins, infections, ischemia, mutation, or autoimmune reactions induce inflammatory reactions in multiple organs including the kidney. Renal fibrosis, defined as the deposition of extracellular matrixes (ECMs) in the interstitial tissue structure, is one of the outcomes of renal inflammatory reactions. Fibrosis develops the pathway of highly activated inflammatory reactions at the locally damaged tissue structure, ending in turning parenchymal/subordinary cells into ECM-producing cells, for example, epithelial cells to myofibroblasts, or fibroblasts to myofibroblasts. Among the original type of cells of the cellular transition to ECM-producing cells, an innermost layer of blood vessels, the endothelium can be highlighted for its contributing roles due to angiogenetic behavior to developing fibrotic niche by supplying blood cells, leukocytes from lymphatic vessels to the local area, thus, accelerates/develop inflammatory reactions. Therefore, Endothelial cells (ECs) itself have crucial roles in the progression of inflammation, as well as the hyperactive-sites cell signaling reactions initiate, and mediate from. Recently, ECs had lots of attention as anti-fibrotic targets for the

development of renal fibrosis. Hence, fibrosis develops by the cellular crosstalk signals from resident fibroblasts, epithelial cells, and infiltrated macrophages from newly synthesized lymphatic vessels by the angiogenesis and differentiated fibroblasts during the formation of the fibrotic niche.

This study examines whether the transition of human renal glomerular endothelial cells (GEnCs) can be inhibited by the overexpression of an embryonic transcription factor ETV2. ETV2 is an essential transcription factor for early hematopoietic/endothelial development and has features of temporary activation. Gene expression analysis and immunoblotting show that overexpression of ETV2 can inhibit fibrotic changes in renal glomerular endothelial cells undergoing EndMT. Specifically, ETV2 induces a prolonged expression of an endothelial gene (CD31), while the expression of mesenchymal (pSmad2/3 and fibronectin, α-SMA) protein is reduced within cells. This result may provide important information required to understand the transcription factor-mediated reversal of EndMT in renal glomerular diseases, as well as develop novel strategies for fibrosis-driven pathologies.

Keywords: ETV2, Endothelial to mesenchymal transition (EndMT), Fibrosis, Inflammation, α-SMA, Fibronectin, CD31

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List of acronyms

BF	Bright-field Microscopy
BRG1	Brahma-Related Gene-1
BSA	Bovine serum albumin
CD	Cluster of Differentiation
CKD	Chronic Kidney Disease
COL1A1	Collagen type 1 alpha 1 chain
EC	Endothelial Cells
ECM	Extra Cellular Matrix
EGFL7	EGF Like Domain Multiple 7
EGM-2	Endothelial Growth Medium-2
ERG	ETS Related Gene
Etsrp	ETS-related protein
ETV2	E26 transformation specific (ETS) variant transcription factor 2
FBS	Fetal bovine serum
FLI1	Friend leukemia integration 1 transcription factor
FLT1	Fms Related Receptor Tyrosine Kinase 1
FOXC1	Forkhead Box C1
FSP1	Fibroblast specific protein 1
GA	Gentamicin/amphotericin-B
GEnC	Glomerular endothelial cells

GFP	Green fluorescent protein
HUVEC	Human Umbilical Vein Endothelial Cells
IL-1β	Interleukin-1 beta
JMJD2A	Jumonji domain-containing protein 2A
LMO	LIM only protein
mRNA	Messenger RNA
OVOL2	Ovo Like Zinc Finger 2
PAEC	Pulmonary Arterial Endothelial Cell
PTH	Parathyroid hormone
ROS	Reactive oxygen species
RT-qPCR	Real Time-Quantitative Polymerase Chain Reaction
SCL	Stem Cell Leukemia
SOX	SRY-Box Transcription Factor
SMAD	Mothers against decapentaplegic homolog
TAL1	T-cell acute lymphocytic leukemia protein 1
TGF-β2	Transforming growth factor – beta 2
TIE1	Tyrosine Kinase With Immunoglobulin Like And EGF Like Domains 1

1. Introduction

1.1. Diagnosis of the health of kidneys

In humans, the kidney receives 20 to 25% of total cardiac output from the heart [1]. Mammalian kidneys play multiple functions, such as removal of metabolic waste, nutrient, and water resorption, and regulating electrolytes and body fluids [2, 3]. Decreased kidney functions are a huge burden in most developing countries, such as Nicaragua, and El Salvador have a death rate of over 70% from chronic kidney diseases (CKD to renal transplantation) per 100,000 population in 2019 according to World Health Organization (WHO) [4]. In addition, the number of renal patients in the Republic of Korea reached more than 100,000 in 2019, an increase of 100% compared to 2010 according to the Korean Society of Nephrology [5, 6].

The common marker of the health of kidney functions, estimated glomerular filtration rates (eGFR) show under 60mL/min/unit in renal patients, while healthy patients show a range from 90 to 120 mL/min/unit [7, 8].

Damages to kidney units from the usage of multiple drugs, antibiotics, infections, or diuretics induce the destruction of parenchymal cells such as the glomerulus. These create inflammatory reactions such as high concentrations of interleukin18 (IL-18) and plasma neutrophil gelatinase-associated lipocalin (NGAL) excreted to urines in renal patients [9, 10].

Inflammation in local tissue develops into wound healing or fibrosis. However, the specific mechanism of developing fibrosis has not been fully understood.

1.2. Cell Biology of Fibrosis

The definition of cell fibrosis is depicted as the accumulation of excess extracellular matrix (ECM) within a single cell [11]. Fibrosis is often compared to or further defined as an abnormal wound healing program. For example, acute injury in the skin reacts as the recruitment of platelets and erythrocytes form fibrin clots. Next, macrophages are recruited by chemo-attractant, clean the debris, and sterilize the injury sites, also release a secretum of chemokines and growth factors to activate the differentiation of fibroblasts to fill the empty spots of tissue structures [12].

Specifically, pathogen-associated molecular patterns (PAMPs), and damage-associated molecular patterns (DAMPs) are recognized through pattern recognition receptors (PRRs) of T cells, macrophages, or other antigen-presenting cells (APCs) to mediate deeper inflammatory behavior by recruiting immune cells [12, 13]. In addition, released platelet-derived growth factor (PDGF)/Transforming growth factor (TGF) by macrophages function as signaling factors in endothelial cells nearby, activating differentiation into phenotypically different (mesenchymal cell, no longer a unit member of the endothelial cell (EC) layer) type of cells (EndMT) proliferate.

These several processes remodel tissue architecture into an inflammatory/highly reacted immunological environment, thus, the architecture becomes vulnerable to developing fibrosis by each cell differentiated from the functional (normal) to non-functional (abnormal) cells.

In tissue re-modeling, endothelial progenitors (non-ECs) are recruited to the injury sites due to the limited proliferative capacity of mature ECs [14]. The endothelial layer is often considered a subtype of epithelium that shares similar

phenotypes, but however, is specialized by its stratification and permeability [15]. The endothelial layer has the most active and dynamic sites of signaling activity in such roles of vascular permeability, including endothelial nitrogen oxide synthase (eNOS), angiogenesis, blood circulation maintenance, and inflammatory response properties [16].

EC activation is also directly involved in perivascular disease, stroke, heart disease, chronic renal failure, thrombosis, tumor growth, and viral infection [16]. Until recent decades, the endothelium was thought to have no special function other than selective permeability to water and electrolytes. However, ECs are widespread throughout the circulatory system, from the heart to the smallest capillaries. ECs function as fluid filtration, vascular tone, hemostasis, neutrophil recruitment, compose of lymphatic vessels, and hormone transport even up to in the renal glomerulus [17]. The endothelium plays an important role in ensuring proper hemostatic balance [18]. One of the functions of ECs is to provide a non-thrombogenic lining of the vessel wall that helps maintain blood fluidity [16]. In hereditary or acquired diseases, the endothelium plays a major role in maintaining the procoagulant system by switching from the mode of anti-coagulant to the mode of pro-coagulant in the injury site [16].

Bleeding and thrombosis occur when the complex balance between the anticoagulant systems is disrupted in the absence of external injury, for example, in the epithelium around the surface of the tissues. This abnormal interaction causes an inflammatory reaction to prevent bleeding [19], however, platelet-leukocyte interactions (P-selectin, promoter of the adhesion) and aggregation on the action of the thrombus ultimately lead to vascular occlusion [20].

By playing this negative effect on the thrombus and hemostasis, leukocyte becomes an inactive state, which is possible to worsen the transition to pro-fibrotic environments [16, 18, 20]. Moreover, the existence of collagen can also worsen the environment by directly binding to glycoproteins on platelets and releasing thrombin to activate G protein-coupled receptors (GPCR) [21].

1.3. Biology of Endothelial Cells

The development of vascular networks forms via coordination between different cell types changing and is precisely dependent on signaling exchanges [16]. Antioxidants from food such as phytochemicals help neutralize free radicals in bodies. However, when antioxidants lack or have too many oxidizing elements, damage occurs to the endothelium, leading to NO (Nitric Oxide) imbalance [22]. When the endothelium is damaged and NO levels are unbalanced, VEGF-NO-induced angiogenesis and vascular permeability become flawed [22, 23]. Also, cells that should remain in the blood can enter nearby body tissues via blood vessels. Some of these proteins reach the liver and are involved in inflammation [16]. This is because endothelium covers the high length of blood vessels in the body approximately, 100,000 kilometers.

Obesity, smoking, lack of sleep, acute pathogenic infections, high glucose, exposure to metals, and as well as air pollutants increase the number of free radicals. There is a high possibility of a relationship between the congenital decrease in nephron number and glomerular endothelial dysfunction and hypertension [24]. Atherosclerosis, a condition of thickened or/and hardened arteries caused by an accumulation of plaque in the inner lining of an artery can result from systemic dysfunction of the vascular endothelium [25].

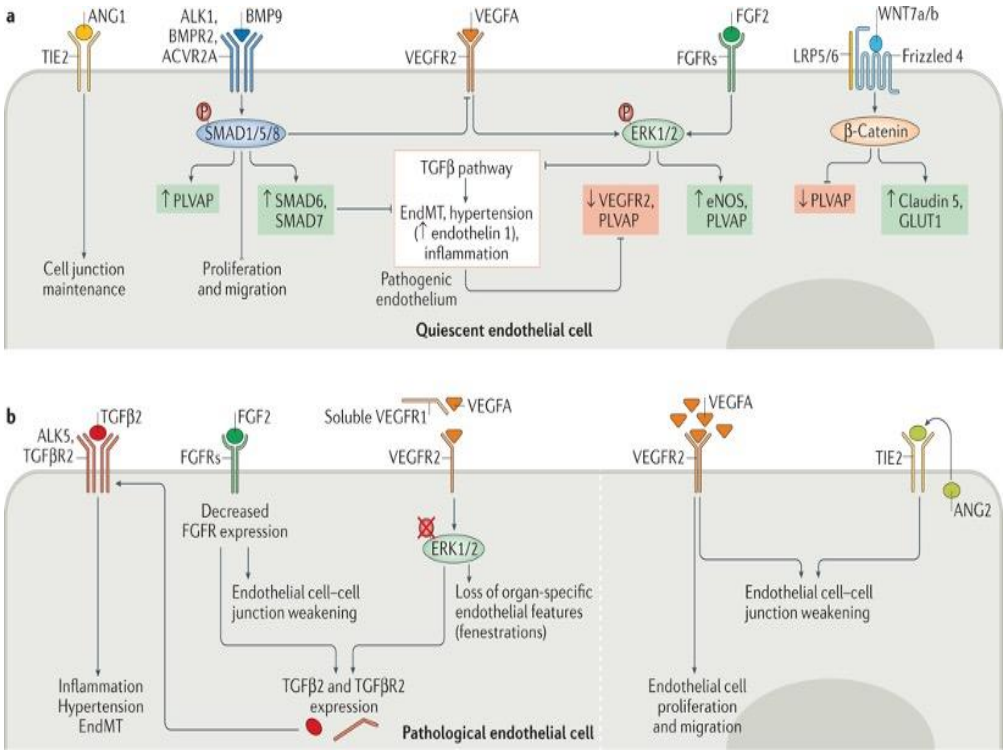


Figure 1. Illustration of endothelial cell signaling of normal and pathological ECs. Reproduced from Ricard et al. (Nat Rev Cardiol 2021;18:565–580) (a) Regulation of endothelial homeostasis is achieved by a set of signaling pathways. Vascular endothelial growth factor receptor 1/2 (VEGFR1/2) has synergistic roles with fibroblast growth factor (FGF) in proliferation, adhesion, angiogenesis, and tight junction of EC layers with the simultaneous activity of suppressing the Transforming growth factor-β (TGFβ) signaling. In quiescent ECs, FGF induces phosphorylation of Extracellular signal-regulated kinase 1/2 (ERK1/2), as well as VEGFR2 expression (not shown in the figure), VEGF expression via MAPK pathway [26]. TGFβ signal regulates inflammation, migration, and angiogenesis positively via Smad2/3, and negatively via Smad1/5/8 (not shown in this figure) [26]. ERK phosphorylation induces cell proliferation through up-regulation of eNOS, plasmalemma vesicle-associated protein (PLVAP) activity. ERK pathway is subject to change easily. Inhibition of the TGFβ signaling is controlled by several signaling pathways including bone

morphogenetic protein (BMP), FGF, and VEGF signaling orbits. (b) Pathological endothelium. Damaged or decreased FGF or VEGF signaling activity leads to activation of a self-secretion (autocrine) TGFβ signaling conduit that, in return, induces inflammatory reactions, hypertension via contracting activity, and endothelial-to-mesenchymal transition (EndMT) from cell-fate changes to having mesenchymal phenotypes upon reaction to cytokines. Extra levels of VEGF signaling and autocrine angiopoietin 2 (ANG2) signaling induce pathological angiogenesis. ACVR2A, activin A receptor type 2A; ALK, activin receptor-like kinase; eNOS, endothelial nitric oxide synthase; GLUT1, glucose transporter 1; LRP, lipoprotein receptor-related protein; SMAD, mothers against decapentaplegic homologue; TIE2, angiopoietin 1 receptor [27]

1.4. Endothelial-to-Mesenchymal transition (EndMT)

Endothelial-to-mesenchymal transition (EndMT) was first reported by Leonard M Eisenberg in 1995, in the early heart development of the cardiovascular system during embryo development when the acquisition of migratory characteristics in a mesodermal layer by activating EndMT to form myocardium [28-30]. As unusual, however, EndMT in past decades has been reported pathologically to occur in adult cells contributing to the progression of diseases by producing ECMs after the mesenchymal transition to such diseases, as fibrosis [31]. Epithelial cells, instead of endothelial cells, EMT (Epithelial-to-mesenchymal transition) contributes to fibrosis 15% in Le Bleu's study in renal fibrosis [32].

It is commonly acknowledged that endothelial to mesenchymal transition occurs under two axis, TGFβ dependent or non-TGFβ dependent (Notch, Wnt, and PI3K/Akt) pathways. In the TGF pathway, the biologically active form of TGFβ via

catalyzation during immune responses such as the surface of macrophages, or in cancers, makes the complex with latency-associated peptide (LAP) and latent transforming growth factor-beta binding protein (LTBP). These three subunits-complex binds to the TGF-β receptor in the transmembrane of the recipient cell, activating the phosphorylation of SMAD2/3. By the signaling pathway, it activates transcription factors (Twist1, Snail1, Slug) endeavoring mesenchymal phenotype of the cells [33, 34]. Thus, cells in response, translate cytoskeletal proteins such as α-smooth muscle actin, or smooth muscle 22 alpha actins, (Alpha smooth muscle actin; α-SMA, Smooth Muscle-22 alpha; SM-22α/TAGLN) decrease the original endothelial-specific proteins, VE-cadherin, which is critically modulating endothelial characteristics, VEGF receptor activities.

Blood carries all significant lineages of immune cells, as well as circulating antibodies and excreted cytokines. Therefore, the chance of endothelial cells is more likely TGFβ pathway to activate within cells than others. Knockdown of Smad2/3 resulted in decreased EndMT in endothelial cells [33]. Inflammatory cytokines such as Interleukin-1β, commonly found in the microenvironments at the activated immune response are reported to have the synergistic effects of accelerating EndMT by activating the additional endogenous interleukin transcription factor and by switching the expression ratio of endogenous TGF-β isotypes in vitro results [35]. It is not only true that endothelial cells only behave pathological by TGFβ activation, but EndMT occurs in response to the Fibroblast growth factor (FGF), interferon-gamma (IFN-γ), and metabolic factors, such as oxidized low-density lipoproteins(oxLDL) as well as disturbed flow, oxidative stress, radiations, histone demethylase, PTH, 27-Hydroxycholesterol (27HC) [26, 36-39]. Therefore, it can be said that endothelial susceptibility is high.

1.5. Mechanisms of EndMT

During EndMT, the endothelial-specific proteins are assumed as are going to decrease such as VE-cadherin, CD31/Platelet Endothelial Cell Adhesion Molecule 1 (PECAM-1). it has been shown by studies using disease models of renal fibrosis [33, 40-43]. However, it is not yet shown that in vitro experiments of solely isolated renal ECs. The advantage of isolated EC experiments is that its implications represent solely of ECs as the variable-controlled experiments without the existence of exosomes produced from tubular epithelial cells *in vivo,* known as cargos of signaling mediators in kidney tissue environments [44]. Thus, it can strictly provide reliable outputs.

Endothelial proteins: PECAM-1/CD31, VE-cadherin gradually decrease over the experiments, which transforms by disposing of the character of the layer of endothelium. Concurrently, the level of expression of skeletal actin proteins (α-SMA; SM-22α/TAGLN) increases, known to be providing motility of the cells. In addition, the expression of ECM proteins such as Fibronectin (FN) and Collagens (Type I – IV, depending on what type of ECs) also increase, furthermore, secrets into interstitial tissue by the activity of exocytosis [45-47]. Due to the morphological changes of ‘fibroblast-like', this cell type is termed a ‘myofibroblast’ [35].

Heart valve formation is completely dependent on EndMT. Wang *et al.* showed that inhibition of Notch1 or Jagged1 by using Cre in the endocardium led to malformed heart valves and vessels by staining PECAM-1 in mouse embryos at E9.5 [48]. Mesenchymal transition doesn’t occur after the completion of development in normal conditions. ECs, however, are exposed to numerous physical, inflammatory, and chemical stimuli (LDL) in their cell lifecycle, making them the most dynamic

groups with a high chance of active changes in cell fates. In addition to that, ECs are divided into groups of cells during the cardiovascular development from lymphatic vessels, originating from mesoderm. Afterward, differentiated ECs express the tissue-specific characteristic proteins to maintain their functions such as fenestration in different functional units.

1.6. Endothelial-Specific Transcription Factor, ETV2

As EndMT originated from its embryo developments, signaling activators and pathways from previous studies have been well known. ETS factors, one of the transcription factors transiently expresses, exclusively regulating FLK1+ mesoderm during embryogenesis have been highlighted for the past decades. Among them, ETV2/ER71, a member of the ETS domain family proteins, has been intensively highlighted.

In Mice, blood island and vascular plexus during embryo development share expression receptor tyrosine kinase (RTKs), flk1, flt1, tie1, tie2 and vascular endothelial growth factor (VEGF), the ligand of flk1, implies the existence of common hematopoietic/endothelial progenitors [49-52]. Additionally, given the facts that embryonic stem cells (ESCs) differentiate into blood cell production and ECs by the form of forming colonies *in vitro* experiment, it ascertains the existence of endothelial development occurs by the mechanism of single progenitor [53].

To closer look, in Zebrafish embryo development, vascular endothelial and hematopoietic cells commonly express helix group helix transcription factors such as scl/tal17 and ETS domain Fli1 [54]. Overexpression of scl induced overproduction of blood progenitor cells (haemangioblast), and the increased

expression of Flk1 [55], whereas a knock-down study of Scl showed the failure of hematopoietic and endothelial development [55]. This presents the relation of ETV2 and Scl in endothelial development.

In addition, the Single-cell labeling technique of zebrafish embryos showed individual cells develop both to erythroid and ECs [56]. It implies blood and endothelial development are closely connected, however, separated by the certain protein expression on the cells, for example, receiving the ligand activation signals through receptor molecules on the surface of the cells.

Ets1-related protein (Etsrp) RNA is known to be closely interplaying with Scl. In the overexpression of Etsrp RNA in Zebrafish embryos, the expression of Scl was induced ectopically in different parts dependent on the location of Etsrp activation [57]. This information brings that Etsrp is a necessary and sufficient condition for starting myeloid cell formation, and ECs are separated from the myeloid line during embryo development, and it is maintained only in EC precursors and does not express in myeloid cells [58].

ETS transcription factors bind the biologically conserved part of the core GGA (A/T), forming the winged helix-turn-helix motif of the 85-amino acid sequence to regulate the core gene [59]. In both deletions of ETS1/2, the blood vessel formation is defective, while a single deletion of neither ETS1 nor ETS2 does not induce the defect, and the role of ETV2 is alternatively replaced with ETS1 [60, 61]. Furthermore, the mechanism of regulating signaling pathways of endothelial differentiation was also veiled: FLI1, ERG, and TAL1 as well as EGFL7 and von Willebrand factor [62].

ETV2 critically regulates BRG1 and OVOL2 [63, 64]. Deletion of ETV2 in mouse embryos reduced the expression of FLK1, a key factor involved in VEGF

[65, 66], meaning its action as an upstream of VEGFR/Flk1. Plus, ETV2 is reported to play a key role in angiogenesis, interplaying with Flt1, Lmo2, Scl, Tie2, GATA2, FOXC1/2, JMJD2A/2D, and Sox7 [67-71]. Thus, all results above show that Ets Variant Transcription Factor 2 (ETV2) has the potential to differentiate and maintain endothelial lineages, possibly even to reverse the adult pathological endothelial cell to mesenchymal transition.

In the previous clinical studies, ETV2 alone successfully induced the phenotypes of endothelial cells from adult human dermal fibroblasts at the >20 days of cultures [62, 72]. It is assumed ETV2 directly activated the expression of VEGFRII/Flk1 [73]. Similarly, ETV2 in iPSCs induced morphologies of endothelial cells in culture conditions [74]. Moreover, a recent study showed ETV2 locates to bind nucleosomal DNA and recruits BRG1. BRG1 remodels chromatin structure around endothelial genes and leads to an increase in H3K27ac deposition [63].

There exist successful transcriptional activations to order cell fate changes. Reverse reprogramming factors OCT4, SOX2, KLF4, and c-MYC (OKSM factors) from adult fibroblasts to stem cell-like phenotypes(iPSCs) [75]. Also, a study of heart fibrosis (Myocardial infarction; MI model) injected with Lenti-ETV2 showed increased capillary densities, anti-fibrotic gene profiles, transited fibrotic cells to EC-like cells expressing endothelial markers such as CD31 with co-expression of ETV2 in vivo [76, 77]. In addition, Park et al. showed that after injury, where neo-angiogenesis is crucial, Flk1(VEGFR2) is upregulated by ETV2, whereas VEGF response is blunted in ETV2-deficient ECs [72]. Likewise, endothelial sprouting from the embryo bodies increased by the enforced ETV2 expression through VEGF response [72].

Given altogether, the research question of this thesis is to find out if the endothelial transcription factor ETV2 can reverse endothelial to mesenchymal transition.

2. Materials and Methods

2.1. Cell Cultures

Primary Human Glomerular Microvascular Endothelial Cells (GMVECs, or GEnCs, Cat#. ACBRI-128, Cell Systems, Kirkland, WA, USA), Human Umbilical Vein Endothelial Cells (HUVECs, Cat#. 2519A, Lonza, Walkersville, MD, USA) and Human Pulmonary Artery Endothelial Cells (HPAEC, Cat#. C-12241, PromoCell, Heidelberg, Germany) were cultured in EGM-2 (Cat#. CC-3162, Lonza, Walkersville, MD, USA) 6 % Fetal bovine serum (FBS, atlas Biologicals, Fort Collins, USA) and 0.1 % Gentamycin (GA-1000) from EGM-2 kit. After cell growth reached 80 % confluence, cells were split into 1:3 using Trypsin-EDTA (Welgene, Cat#. LS 015-08, Gyeongsan-si, Gyeongsangbuk-do, Republic of Korea). EGM-2 medium was used in all experiments. Cells were obtained at passages 1-2, used between passages 2 and 8. Human recombinant interleukin-1β (hIL-1β), and human recombinant transforming growth factor β-2 (TGFβ-2) were purchased from PeproTech (NJ, USA), R&D systems (Cat#. 302-B2-010, Minneapolis, MN, USA) and prepared following manufacturer's protocols. TGFβ-2 was used at the concentration of 10ng/ml, IL-1β at 5ng/ml in all experiments. Media was refreshed every 24 h.

2.2. Viral Vector System

Adenoviral/lentiviral construct containing ETV2 was used in this study,

pAAV(Exp)-CMV>hETV2(NM_014209.4) (ns):T2A:EGFP: WPRE(VB220203-1143vcz), pLV(Exp)-CMV>hETV2(NM_014209.4)-CBA>EGFP(VB220711-1132jvc), were constructed and packaged by VectorBuilder. and frozen at -80°C until use. The serotype of AAV2 and AAV9 were used. For transduction, 30,000 cells/cm^2 were seeded. The following day, the medium was refreshed and lentiviral vectors with either EGFP or EGFP-ETV2 expression construct were added to the medium at the dose of 20 multiplicity of infection (MOI). After 17 h, cells were washed twice with DPBS and refreshed with a complete medium (EGM-2 containing 6% FBS), stabilized for 3 days before the appropriate treatments. The vector ID can be used to retrieve detailed information about the vector on vectorbuilder.com.

2.3. RT-qPCR Assay

ECs were harvested and then lysed using Trizol (Invitrogen, Carlsbad, CA, USA). The concentration of the total RNA was measured using DeNovix DS-11 (Denovix, Wilmington, DE, USA). All extracted RNA was incubated with DNase I (Cat#. EN0521, Thermo Fischer Scientific, MA, USA) according to the manufacturer's protocols. RNA was reverse transcribed either with a cDNA synthesis Kit (Philekorea, Daejeon-si, Korea), or Maxima First Strand cDNA Synthesis Kit (Cat#. K1641, Thermofischer Scientific, MA, USA). An amount of cDNA equivalent to 1μg of RNA input into a cDNA synthesis reaction was used per single RT-qPCR reaction. PCR was conducted using the Accupower 2X GreenStar qPCR Master Mix (Bioneer, Daejon-si, Korea), in the CFX96 Touch Real-Time PCR Detection System (Bio RAD, Hercules, California). All reactions were performed in duplicate. Subsequent calculations were performed in MS Office Excel and GraphPad Prism. All Ct values were normalized to geometrical means of gapdh and 18srRNA Ct

values. Data are presented as fold change vs. indicated control, obtained using the 2-ΔΔCt method. The list of primers (Bioneer, Daejon-si, Korea) is enclosed in Table 1.

Table 1. The sequences of primers were used for all experiments.

Target gene	**Sequences**
GAPDH	F:5'-GAGTCAACGGATTTGGTCGT-3'
	R:5'-TTGATTTTGGAGGGATCTCG-3'
RNA18S1	F:5'-CGGCGACGACCCATTCGAAC-3'
	R:5'-GAATCGAACCCTGATTCCCCGTC-3'
SNAI1	F:5'-GGCAATTTAACAATGTCTGAAAAGG -3'
	R:5'-GAATAGTTCTGGGAGACACATCG-3'
TWIST1	F:5'-CTCAAGAGGTCGTGCCAATC -3'
	R:5'-CCCAGTATTTTTATTTCTAAAGGTGTT -3'
CD31/PECAM1	F:5'-GACGTGCTGTTTTACAACATCTC -3'
	R:5'-CCTCACGATCCCACCTTGG-3'
CDH5	F:5'-GACCGGGAGAATATCTCAGAGT-3'
	R:5'-CATTGAACAACCGATGCGTGA-3'
VWF	F:5'-CGACTTCCTTACCCCCTCTG-3'
	R:5'-GCAGGAGCACACGTCGTAG-3'
ETV2	F:5'-CCGACGGCGATACCTACTG-3'
	R:5'-CGGTGGTTAGTTTTGGGGCAT-3'
COL1A1	F:5'-GAGGGCCAAGACGAAGACATC-3'
	R:5'-CAGATCACGTCATCGCACAAC-3'
SNAI2	F:5'-CGAACTGGACACACATACAGTG-3'
	R:5'-CTGAGGATCTCTGGTTGTGGT-3'
ACTA2	F:5'-GTGTTGCCCCTGAAGAGCAT-3'
	R:5'-GCTGGGACATTGAAAGTCTCA-3'

FN1	F:5'-CGGTGGCTGTCAGTCAAAG-3'
	R:5'-AAACCTCGGCTTCCTCCATAA-3'
S100A4/FSP1	F:5'-GATGAGCAACTTGGACAGCAA-3'
	R:5'-CTGGGCTGCTTATCTGGGAAG-3'
TAGLN	F:5'-AGTGCAGTCCAAAATCGAGAAG-3'
	R:5'-CTTGCTCAGAATCACGCCAT-3'
EGFP	F:5'-ACGTAAACGGCCACAAGTTC-3'
	R:5'-AAGTCGTGCTGCTTCATGTG-3'
SMAD2	F:5'-CCGACACACCGAGATCCTAAC-3'
	R:5'-GAGGTGGCGTTTCTGGAATATAA-3'
SMAD3	F:5'-CCATCTCCTACTACGAGCTGAA-3'
	R:5'-CACTGCTGCATTCCTGTTGAC-3'

2.4. Immunofluorescence

10,000 cells were seeded in each Poly-D-lysine-coated 8 wells cell culture slide (SPL) and cultured until reaching appropriate confluency to progress EndMT. The following day, the medium was exposed to EndMT treatments. After washing 3 times with ice-cold PBS, fixed cells using 4 % paraformaldehyde in DPBS (pH 7.4) for 20 mins at room temperature. For permeabilization, all cells were incubated for 10-15 mins by DPBS containing 0.03% Triton X-100, followed by 3-time washes by DPBS. Thereafter, cells were blocked by PBST (PBS + 0.03 % Tween20) containing 1% BSA and 5% Normal goat serum (NGS) for 30 mins. After these preparations, samples then were incubated with primary antibodies overnight at 4°C, next morning were washed 3 times with DPBS. Next, primary antibody-labeled cells were incubated with secondary antibodies for 2 hours at RT. After 3-time washes by DPBS, samples were stained for nuclei staining with VECTASHIELD® Antifade Mounting

Medium with DAPI until hardening. The images were analyzed and recorded under either a confocal microscope (Leica TCS SP8 STED, Wentzler, Germany) or measured by Cytation™ 5 Cell Imaging Multi-Mode Reader (Biotek, USA). The antibodies used for Immunofluorescence (IF) and Immunoblotting analysis are listed in Table 2.

2.5. Flow Cytometry

ECs were harvested at 80 % confluency. Then, cells were resuspended $1x10^6$ in 200uL of DPBS containing 4 % FBS (FACS buffer). For the cell surface labeling, cell suspensions were incubated at 4 °C for 1 hr. with the following specific primary antibodies PE-CD31 Mouse anti-Human (10:1) (Clone WM59, RUO, BD Biosciences, CA, USA), or isotype control Mouse normal IgG at the same dilution ratio (Cat. no. sc-2025, Santacruz, TX, USA). After incubation, the samples were washed two times in 1 mL FACS buffer. the acquisition was set to include 10,000 cell flow to calculate the PE^+ cell count. Collected data were analyzed by the BD FACS Canto™ II Cytometer and FACS DIVA software (Ver 6.1.3, BD Bioscience, Franklin Lakes, NJ, USA), FlowJo software (Ashland, OR, USA)

2.6. Western Blot Analysis.

Cells were washed three times with ice-cold PBS, lysed by using RIPA lysis buffer containing proteinase inhibitor, phosphatase inhibitor (sodium orthovanadate, sodium fluoride), and EDTA and centrifuged at 15,000 x g for 15min at 4°C. The protein concentration was evaluated by the Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA). Equal amounts of whole cell lysates of total protein (15-30μg) were loaded and separated by 6-15% gels

depending on the target of protein sizes. After sodium dodecyl, sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), gels were transferred to nitrocellulose membranes by using the Power Blotter-Semi-Transfer system (Thermo Fisher Scientific, Waltham, MA, USA). For blocking, 5% non-fat dry milk (normal forms) or 3% BSA (phosphorylated forms) were used in Tris-buffered saline and 0.03% Tween-20 buffer (TBST), The primary antibodies for anti-(p)-Smad2/3, Smad2/3, Fibronectin, Transgelin, Collagen type1 (Abcam, Cambridge, UK, 1:1000), anti-vimentin, (Santa Cruz, Dallas, TX, USA), S100A4, CD31 (Thermo Fisher Scientific, Waltham, MA, USA), VECAM, α-SMA (Cell Signaling Technology, Denver, MI, USA), anti-ETV2 (Biorbyt, Cambridge, UK), β-Actin (Santa Cruz, Dallas, TX, USA; 1:5000) were incubated with the membrane at 4 °C overnight. After the membrane was washed six times with TBST (0.05 % Tween 20) the membranes were incubated for 2 h at room temperature with horseradish peroxidase-conjugated anti-rabbit or mouse secondary antibody (Santa Cruz, Dallas, TX, USA; 1:5000). After being washed six times with TBST, the reactivity was examined by an enhanced chemiluminescence kit (Thermo Fisher Scientific, Waltham, MA, USA). The image of the membrane was taken using chemiluminescence on an Azure 280 chemiluminescent imaging system (Azure Biosystems, CA, USA). The blots were quantified using Microsoft Excel (WA, USA) and Image J software (Version 1.53, National Institutes of Health, Bethesda, MD, USA).

Table 2. The antibody list was used in this study.

Target Protein	Primary antibody	Secondary antibody
CD31	CD31 / PECAM-1 Antibody: Invitrogen (1:100)	Mouse anti-rabbit IgG-HRP (sc2357), Santacuz (1:5000)
β-ACTN	Beta Actin antibody (C4) (sc2357), Santacruz (1:5000)	Goat Anti-mouse IgG H&L (HRP) (sc2005), Santacruz (1:5000)
SNAI1/2	Anti-SNAIL + SLUG antibody (ab180714) abcam (1:1000)	Goat Anti-mouse IgG H&L (HRP) (sc2005), Santacruz (1:5000)
FN	Anti-Fibronectin (ab2413), abcam (1:1-2000)	Mouse anti-rabbit IgG-HRP (sc2357), Santacruz (1:5000)
TWIST1	Anti-Twist1 antibody(2C1a) (ab50887) abcam (1:500)	Mouse anti-rabbit IgG-HRP (sc2357), Santacruz (1:5000)
SM-22α	Anti-TAGLN/Transgelin antibody, abcam (ab14106)	Mouse anti-rabbit IgG-HRP (sc2357), Santacruz (1:5000)
CDH5	VE-Cadherin Antibody (#2158), CST (1:1000)	Mouse anti-rabbit IgG-HRP (sc2357), Santacruz (1:5000)
VIM	Vimentin antibody, Santa cruz (sc373717) (1:1000)	Goat Anti-mouse IgG H&L (HRP) (sc2005), Santacruz (1:5000)
COL1A1	Recombinant Anti-Collagen I antibody (EPR7785) (ab138492), abcam (1:1000)	Mouse anti-rabbit IgG-HRP (sc2357), Santacruz (1:5000)
S100A4	S100A4 antibody (NBP2-67740), Novus biologicals (1:1000)	Mouse anti-rabbit IgG-HRP (sc2357), Santacruz (1:2000)
SMAD2/3	Anti-Smad2 + Smad3 antibody (ab63672), abcam (1:2500)	Mouse anti-rabbit IgG-HRP (sc2357), Santacruz (1:2000)
pSMAD2/3	Recombinant Anti-Smad2 (phospho T8) + Smad3 (phospho T8) antibody (EPR23682-64) (ab254407), abcam (1:2500)	Mouse anti-rabbit IgG-HRP (sc2357), Santacruz (1:2000)
ETV2	Anti-ETV2 antibody (orb156791), Biorbyt (1:1000)	Mouse anti-rabbit IgG-HRP (sc2357), Santacruz (1:2000)

2.7. Statistical Analysis

Statistical analyses were performed using various analyses of variation (ANOVA). Where statistical significance was found, an unpaired Student's t-test was conducted between two groups. All analysis was performed by using GraphPad Prism (GraphPad, San Diego, CA, USA). Significance was defined as $p < 0.05$.

3. Results

3.1. TGFβ2 and IL-1β induced EndMT in cultured GEnCs

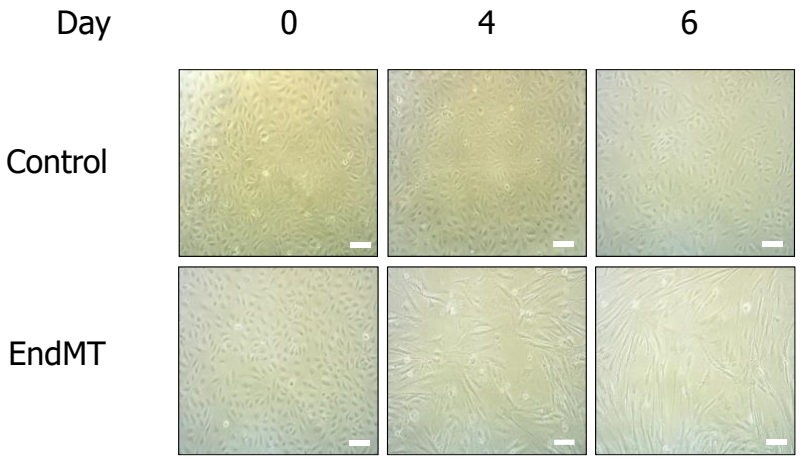
A method of EndMT induction by TGFβ2 and IL-1β (Maleszewska *et al.*) is applied [35]. However, due to the severe cytotoxicity of IL-1β (10 ng/ml), the concentration was decreased by half, 5ng/ml. TGFβ2 is known to sustain the progression of EndMT, although IL-1β is reported to induct EndMT [35]. Distinct cell morphologies from cobblestone to fibroblast-like were observed from day 2 (not shown).

The upregulation of ECM component expression level supports strengthening a change in cell phenotype. The shapes of multiple spikes throughout the cell membranes were specifically characterized on day 4 of EndMT. In addition, the length of cells was enlarged on day 6 as shown in Fig. 2A. Severe cell deaths were observed in about 50% of cell densities on day 5.

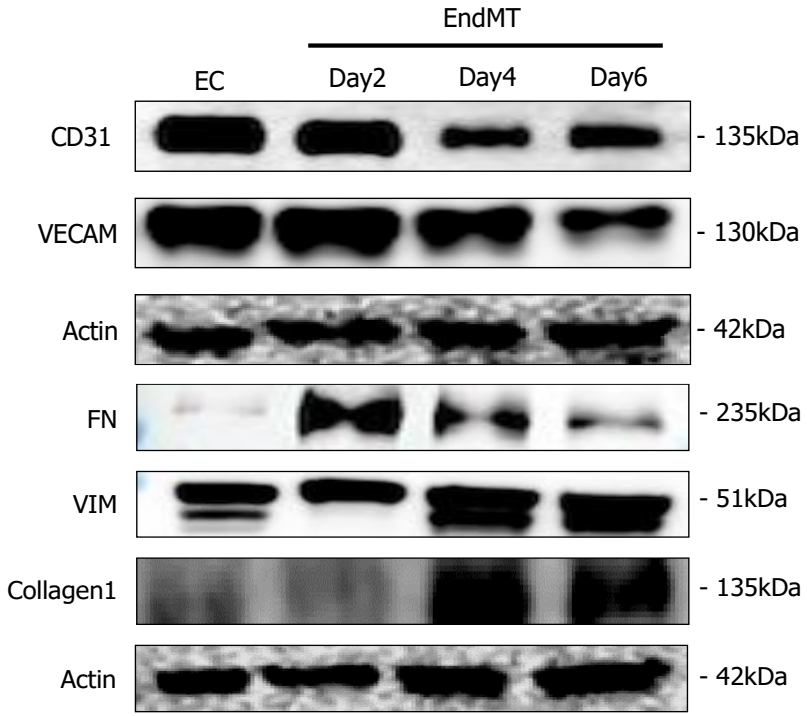
As expected, after day 2, mesenchymal protein (Fibronectin) was significantly upregulated, appearing ribbon shape of the protein band. The level of protein bands slowly diminished up to day 6, the thickest at day 2 (Fig 2B). TGFβ2 and IL-1β treatments for 4 days in GEnCs resulted in the earlier upregulation of ECM components, Collagen type I, even though Col1 in HUVECs is upregulated after 7 days [35]. Vimentin, intermediate filament did not change, therefore remained lower than the control group (Fig. 2C; d2-6).

The protein expression levels of endothelial protein CD31 and VE-cadherin after day 4 of stimulation were lower in cells treated with TGFβ2 and IL-1β than in cells not treated (Fig. 2B; EC).

A.


Day
0
4
6
Control
EndMT

B.


EndMT
EC
Day2
Day4
Day6
CD31
- 135kDa
VECAM
- 130kDa
Actin
- 42kDa
FN
- 235kDa
VIM
- 51kDa
Collagen1
- 135kDa
Actin
- 42kDa

C.

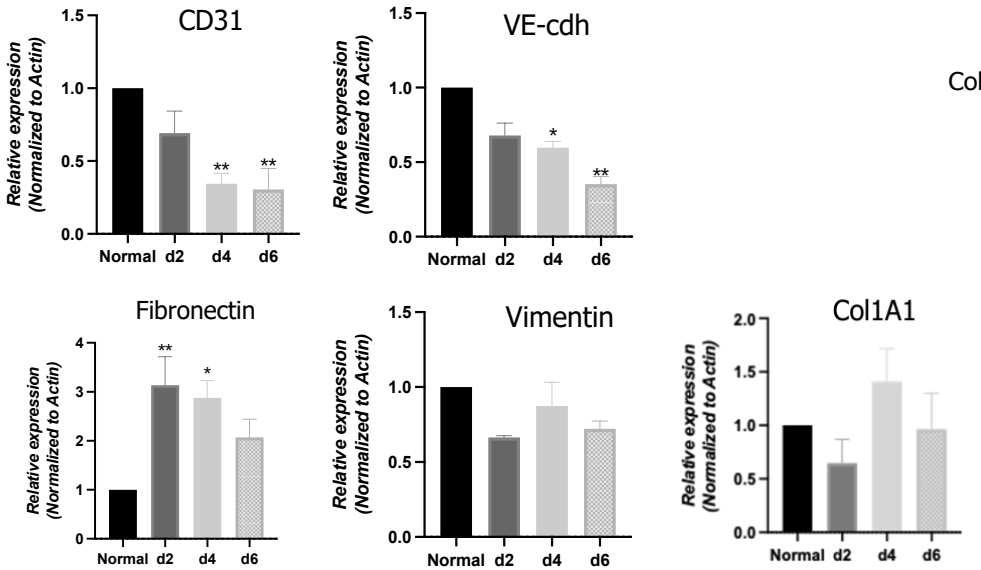

CD31
VE-cdh
Fibronectin
Vimentin
Col1A1
Relative expression (Normalized to Actin)
Normal
d2
d4
d6

Figure. 2. TGFβ2 and IL-1β induced EndMT in cultured GEnCs (A) Morphology of GEnC cultured for marked time in control conditions (upper row) or with TGFβ2 and IL-1β (lower row). Representative bright field micrographs at 10× original magnification are shown. Scale bar: 100μm. (B and C) Representative Western blots of endothelial (CD31, VE-cadherin) and mesenchymal (Fibronectin, Vimentin, Collagen type I) proteins from day 2 to 6 of culture in either control (EC) or stimulation conditions, showing the increase in Fibronectin, Vimentin, and Collagen type I protein levels and complementary decrease in CD31 and VE-cadherin protein expression during EndMT. β-actin served as a loading control.

3.2 The expression of pro-fibrotic Smad2 and SM22α are upregulated in TGFβ2 and IL-1β induced EndMT

From the previously published study, pro-fibrotic transcription factor Smad2 is upregulated by TGFβ2 and IL-1β stimulation and activated nuclear translocation on day 1 and day 5 in HUVECs [35]. Thus, it was further investigated whether costimulation of TGFβ2 and IL-1β can upregulate pro-fibrotic Smad2 in GEnCs.

TGFβ2 phosphorylates Smad2 and IL-1β releases NFκB from complexes with its inhibitory protein 1IκBα, through IκBα phosphorylation by IKK, then enable NFκB subunits to cross locate to the nucleus to transcribe target genes. NFκB and Smad2 are reported to cooperate at molecular levels and proven as mediators of EndMT [35, 78].

After 2 days of costimulation, phosphorylation of Smad3 (Upper; 60kDa) and Smad2 (Lower; 55kDa) significantly increased in cells treated by TGFβ2 and IL-1β than in cells not treated (Fig. 4A). Smad3 is reported to be a less sensitive transducer of TGFβ signaling than Smad2 in these cell types (human ESCs, HEK 293 cells, and human skin fibroblast) [79].

SM22α upregulation is the result of NFκB activation via inflammatory stimulation mediated by IL-1β [35]. By reports, NFκB and Smad2 cooperate, therefore, SM22α upregulation and pro-fibrotic Smad2 in GEnCs were further set up as the signaling activation markers.

After 2 days of stimulation, the protein expression level of SM22α was significantly upregulated up to day 6, in a time-dependent manner (Fig. 4A). The protein expression level of phosphorylation of Smad2 increased in cells treated with TGFβ2 and IL-1β at day 2 (Fig. 4A; lower band; 55kDa). However, it is hard to say that the costimulation of TGFβ2 and IL-1β downregulated the level of Smad2 and Smad3 expression at day 4 and day 6 (Fig. 4A)

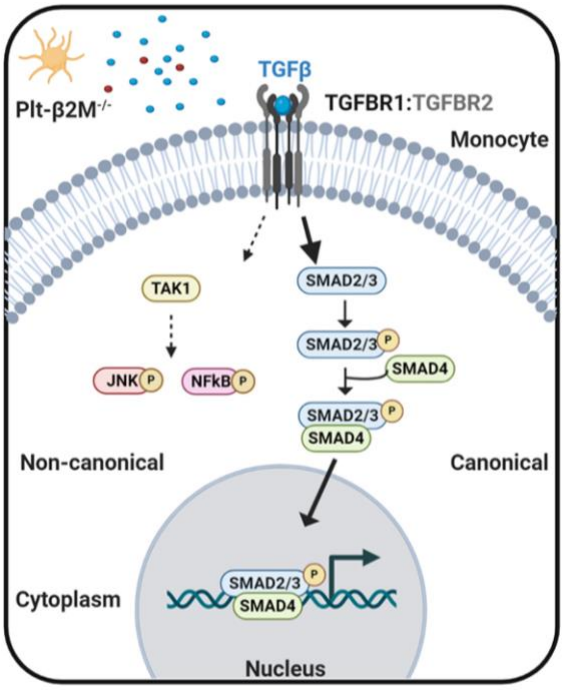
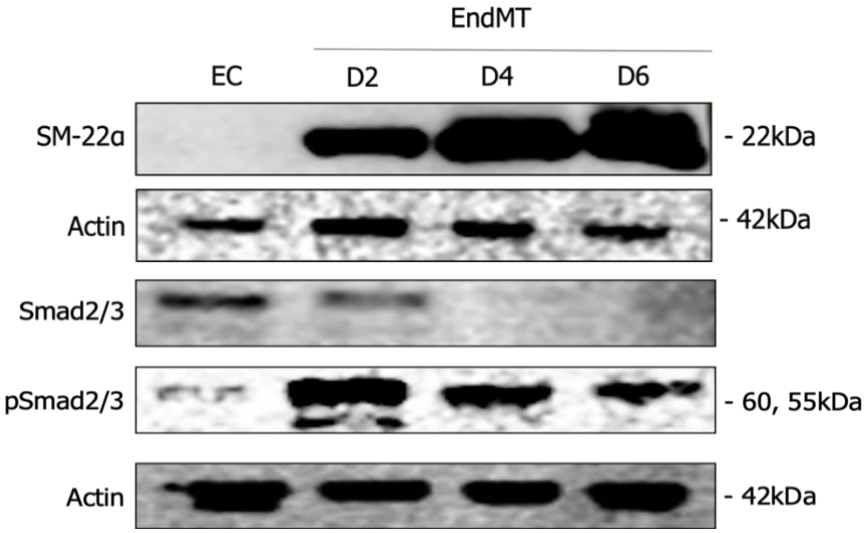


Figure 3. TGF-β/Smad pathways in mammalian cells. Reproduced from Zachary *et al.* (Cir Res 2021;128:5). TGFβR is one of the Receptor serine/threonine protein kinase (RSTKs) family members which is composed of hydroxyl amino acids (serine/threonine/tyrosine) for effector sites. Extracellular domains respond to ligands, and cytoplasmic domains respond to kinase activity. TGFβR responds specifically to TGFβ as ligand-receptor models. TGFβ pathways are divided into canonical (dependent on the transcription factor, Smad2/3) and non-canonical (independently of Smad2/3) for its transcription of genes.

A.



B.

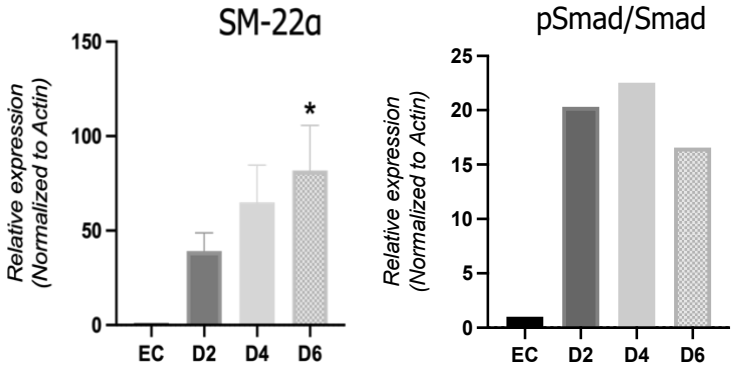


Figure 4. TGFβ2 and IL-1β induced upregulation of SM-22α and pSmad2/3 in cultured GEnCs. (A-B) Representative of Western blots of mesenchymal (SM22α, pSmad2/3) proteins in GEnCs cultured for 6 days under control conditions (EC), or with IL-1β and TGFβ2. High expression of SM22α and pSmad2/3, as well as loss of Smad2/3 expression, was observed under the co-stimulation conditions, as compared to control (EC). β-actin served as a loading control. Graph data is presented as three independent experiments. Data are presented as the mean with SEM. *p<0.05, **p<0.01, ***p<0.001, unpaired one-way ANOVA assays.

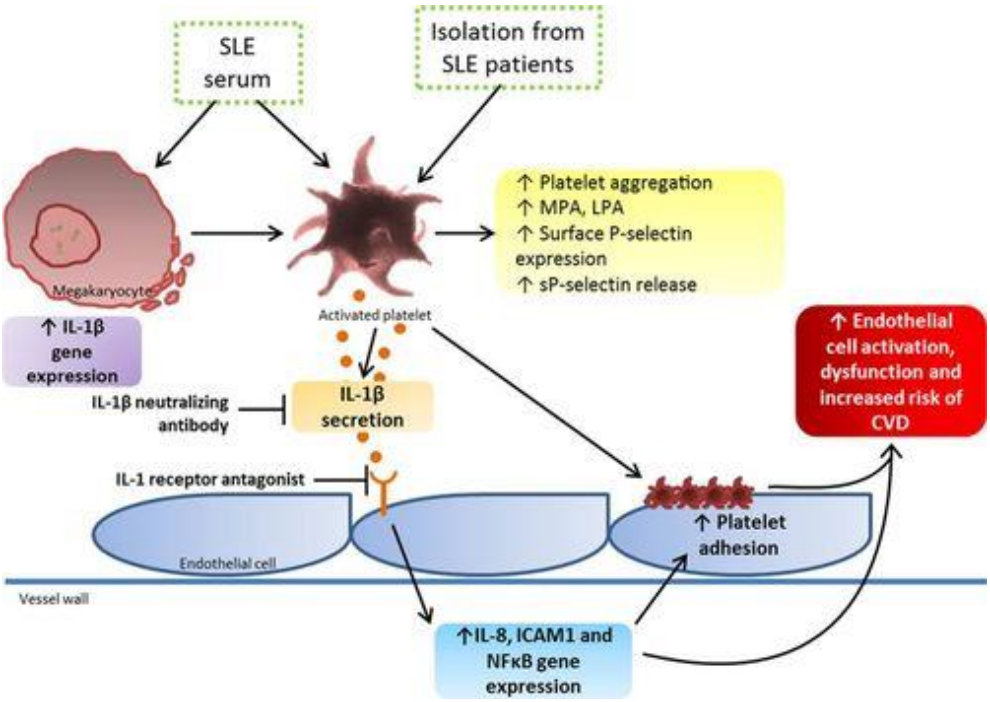


Figure 5. The action of signaling IL-1β in endothelial cells. Reproduced from Nhek *et al.* (ATVB 2017;37:4). IL-1β secreted by activated platelets, dendritic cells (DC), and macrophages, as well as B lymphocytes, neutrophils, and natural killer cells (NK) cells, for example, under the situation of immunological attack, bind to IL-1 receptor expressed on the surface of endothelial cells in vessel walls lead to NFκB gene, IL-8, and ICAM1 gene expression that increases endothelial dysfunction, activation, thus increase the risk of cardiovascular disease.

3.3 Comparison vector transduction by different virus types

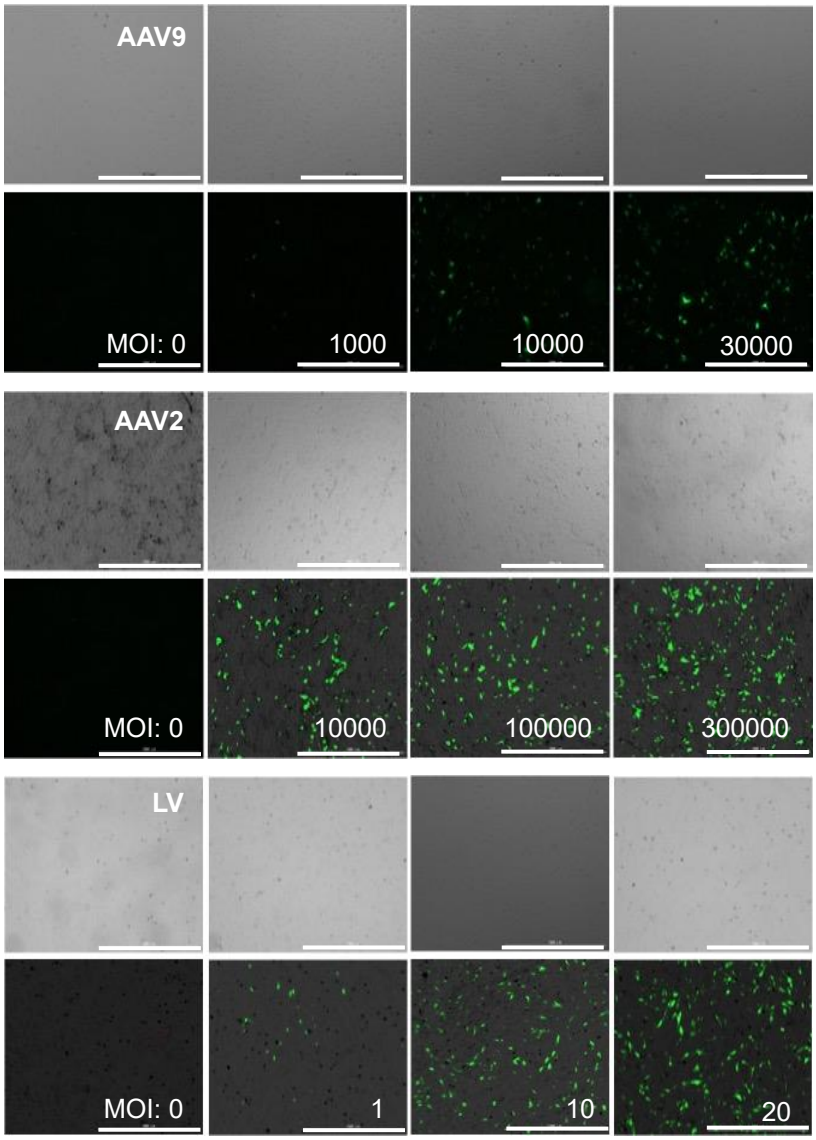
Adeno-Associated Viruses (AAV) and Lentivirus (LV) are prominent viral vectors to introduce nucleic acids into mammalian cells. Both systems are known to be amenable to research applications such as protein overexpression. Lentiviruses, in particular, the carrying genome integrate into the genome of the host cells, therefore, it is able to express the protein stably in both dividing and non-dividing cells.

Unlike the RNA genome of lentiviruses, AAVs carry single-strand DNAs. AAV has commercially available 18 different types including AAV1-9, AAV6.2, AAVrh10, AAV-DJ, AAV-DJ/8, and AAV-PHP.eB, AAV-PHP.S, AAV2-retro, AAV2.7m8, AAV2-QuadYF; technically mutated in capsid structures that have different tissue tropisms. While AAV9 is known to be more robust on endothelial cells, AAV2 was reported to have the highest efficiencies in kidney tissues with the reported experiment results of successful 89% efficiency in HUVECs [80].

Given these, three types of viruses AAV9, AAV2, and lentiviral vectors expressing eGFP were used to be compared to decide the best efficient type of vector virus (Fig. 5). According to the manufacturer's protocols, a multiplicity of infection (defines here as genome copies per a single cell) of lentivirus, 1 to 20, and AAV9, 1000 to 30000; AAV2, 10000 to 300000 were tested. After infections in primary GEn cells, all cells were exposed to eGFP channels after two days from the infection.

Lentivirus was the best vector virus that stably express eGFP proteins further 6 days of culture condition. As for the most efficient method, a lentiviral vector (MOI:20) was decided as the transfection technology.

A.


AAV9
MOI: 0
1000
10000
30000
AAV2
MOI: 0
10000
100000
300000
LV
MOI: 0
1
10
20

B.

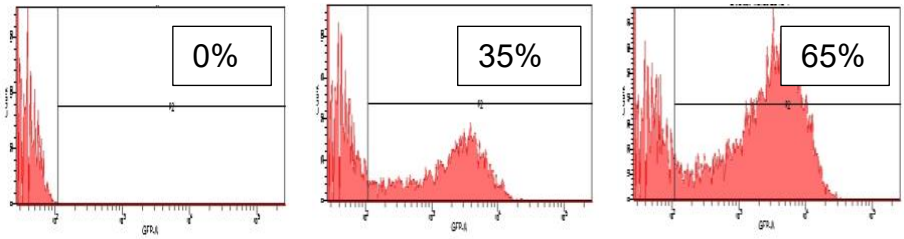

LV MOI:0
MOI: 10
MOI: 20
0%
35%
65%

Figure 6. Transduction of two ssAAV serotypes or lentivirus on primary human glomerular microvascular endothelial (GEn) cells. Subconfluent GEn cells in 6-well plates were infected in parallel with multiple multiplicities of infection (MOI) 1×10^3 - 3×10^6 viral particles of ss forms of AAV.GFP or 1 – 20 lentiviral particles of LV.GFP. Images were captured at the same exposure with an internal BioTek Cytation 5 camera and Gen5 software at 2 days post-infection. The total integrated intensity of fluorescence was quantified on an average of 10,000 cells using BD FACSAria software**.** (A) Representative fluorescent images of AAV serotype 9, serotype 2, and lentivirus transduction (B) Fluorescence-activated cell sorting (FACS) analysis FACS analysis of GFP positive GEn cells transfected with lentivirus vector eGFP (Left to right; MOI:0, 10, and 20). Original magnification: ×100. Scale bar: 1 mm

3.4 Activation of ETV2 inhibited TGFβ2 and IL-1β induced EndMT

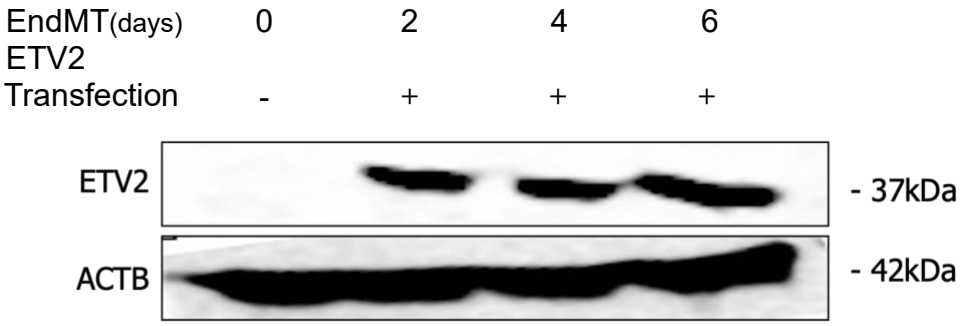
To validate the stable expression level of ETV2 in cells transfected ETV2, Western blot of ETV2 was performed (Fig. 7A). All cells were transfected by lentivirus vector, carrying EGFP-ETV2 48h before stimulation of TGFβ2 and IL-1β (Fig. 7A). The stable expression of ETV2 in primary GEnCs was sufficient to suppress TGFβ2 and IL-1β induced EndMT.

As shown in Figure 7B, the protein expression levels of pSmad2/3, Fibronectin, and α-SMA were significantly reduced. Meanwhile, EGFP sham infection caused a significant increase of pSmad2/3, Fibronectin, and α-SMA in cells treated with TGFβ2 and IL-1β. The results were further proved by RT-qPCR analysis.

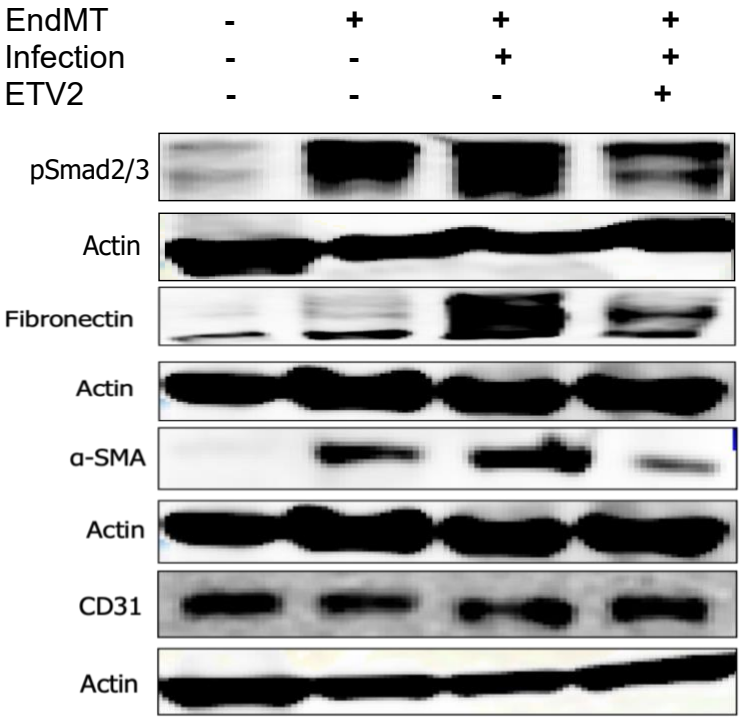
As indicated in Figure 7D, the forced protein expression of ETV2 restored endothelial marker CD31, which was downregulated in cells treated by TGFβ2 and IL-1β. Furthermore, ETV2 decreased the mesenchymal marker proteins a-SMA, that was significantly upregulated in cells treated by TGFβ2 and IL-1β.

Interestingly, overexpression of ETV2 maintained CD31protein expression levels, which were downregulated in cells treated by TGFβ2 and IL-1β (Fig 7B). But, ETV2 abolished the ability to upregulate SM22α protein expression levels in ETV2-transfected GEnCs compared to eGFP-transfected GEnCs measured by Immunocytochemistry assay (Fig. 7E).

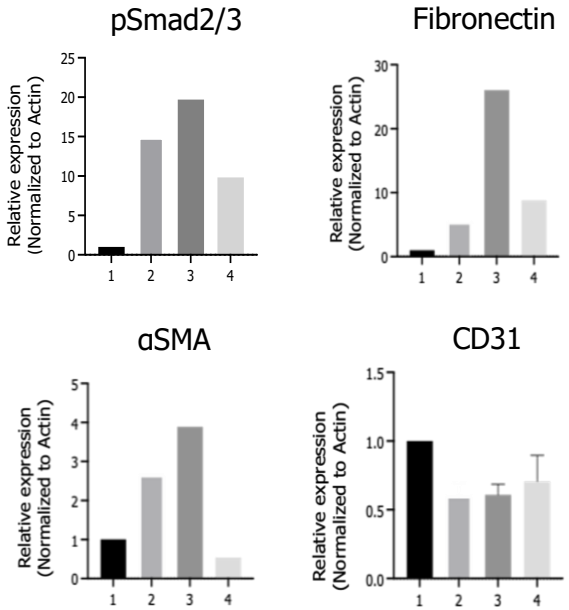
A.



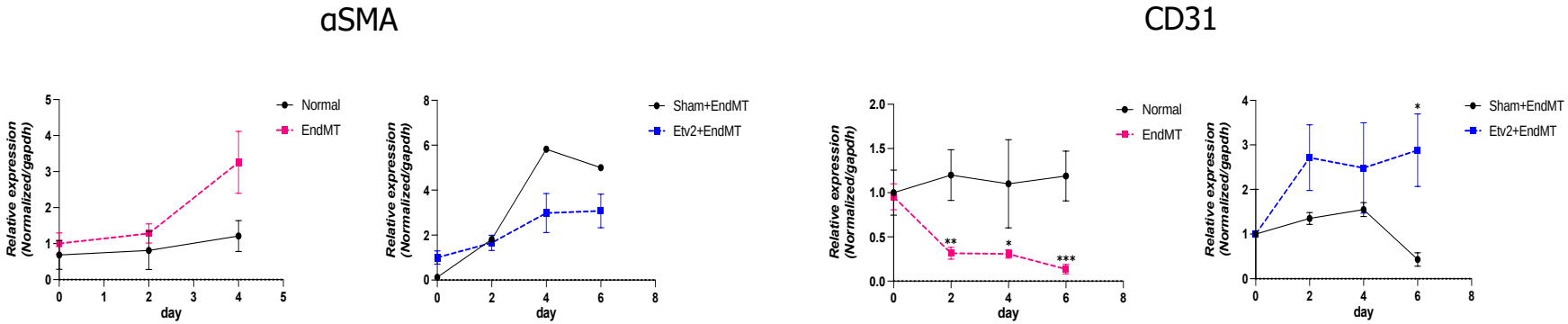
B.


EndMT - + + +
Infection - - + +
ETV2 - - - +
pSmad2/3
Actin
Fibronectin
Actin
α-SMA
Actin
CD31
Actin

C.


pSmad2/3
Fibronectin
αSMA
CD31
Relative expression (Normalized to Actin)

D.


αSMA
CD31
Normal
EndMT
Sham+EndMT
Etv2+EndMT
Relative expression (Normalized/gapdh)
day

E.

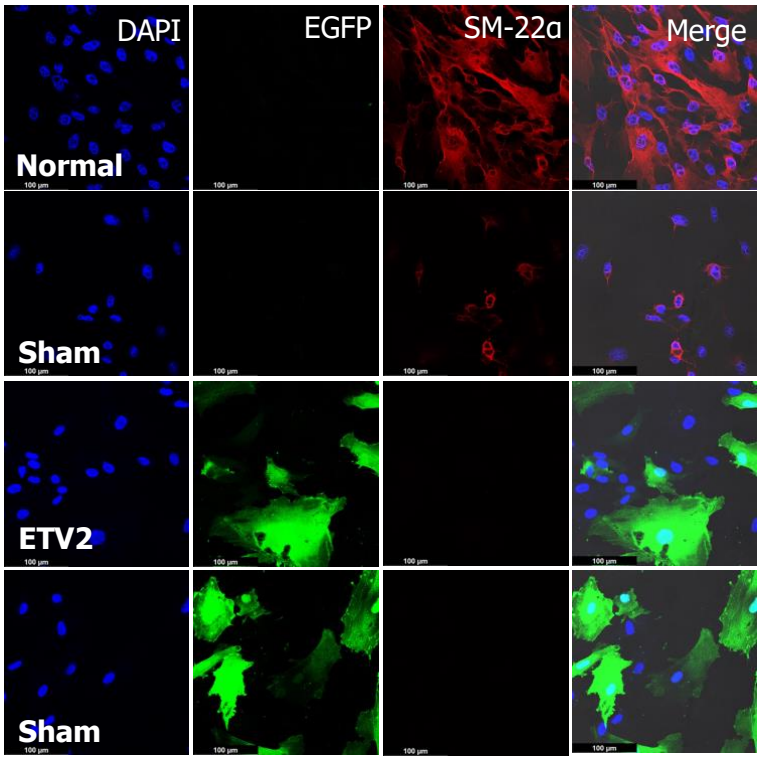


Figure 7. ETV2 inhibited TGFβ2 and IL-1β induced EndMT. (A) Representative of Western blots of Etv2 expression underwent stimulation of TGFβ2 and IL-1β after lentivirus infection carrying ETV2. β-actin was used as a loading control. (B-C) Representative of Western blots of expression levels of mesenchymal (pSmad2/3, Fibronectin, Alpha-SMA) and endothelial (CD31) proteins in primary GEnCs between cells with nothing treatment and cells treated TGFβ2 and IL-1β only and cells sham-transfected eGFP or cells transfected ETV2. Cells were infected 2 days before the EndMT stimulation, followed by the stabilization. β-actin served as a loading control. Graph data are presented as mean (D) Gene expression levels of mesenchymal (Alpha-SMA) and endothelial (CD31) mRNAs assessed by RT-qPCR assay. Graph data is presented as three independent experiments. All mRNA expression levels were normalized to those of the housekeeping gene Gapdh. Data are presented as the mean with SEM. (E) Representative image of immunofluorescence microscopy analysis of mesenchymal (SM-22α) protein. The expression of the SM-22α (red), nuclei (blue), and infection marker (green) in GEnCs labeled with SM-22α antibodies either in cells, nothing transfected (Normal) or in cells transfected (ETV2) followed by TGFβ2 and IL-1β for 4 consecutive days were assessed by immunofluorescent staining. Normal rabbit IgG was used for sham immuno-labeling (second row, nothing transfected; fourth row, ETV2 transfected). Scale bar: 100 μm. *$p<0.05$, **$p<0.01$, ***$p<0.001$, unpaired one-way ANOVA assays.

4. Discussion

Epithelial/Endothelial to mesenchymal transition is known to contribute to kidney fibrosis by numerous studies [81]. Profiling of the patterns of protein expression showed upregulation of mesenchymal (α-SMA, SM-22α, Collagen, FSP1, pSmad2/3) and downregulation of endothelial (CD31, VE-cadherin, von Willebrand Factor, eNOS) protein expressions. The transited cells are named after 'myofibroblast' [82-85].

Kidney fibrosis is mediated by diverse signaling molecules, thus unique sources of development are reported such as extravesicles (EVs) and exosomes that are secreted and produced by tubular epithelial cells, podocytes, and mesangial cells [42, 44].

In normal conditions, some wounds result in healing via inflammation, however, some wounds do not. Reasons vary, but, in the end, it is the result of the lost potency of proliferation. Generally, wound healing progresses through new blood vessel formation (angiogenesis), especially by recruiting macrophages, neutrophils, and lymphocytes to the local tissue of the injury site [86]. Therefore, endothelial cells have significant roles in the mediation of wound healing by angiogenetic activity.

The reports regarding fibrosis study the activation of transcription factors. Notably, inhibition of Smad3 showed fewer macrophage infiltration to the inflammatory sites and less deposition of collagens in the lesion studied by db/db mice [87]. In combination with Smad2, conditional knock-out and disruption of TGFβRII in endothelial cells led to downregulated Smad2/3 activation, ameliorated cisplatin-induced AKI, as well as the decreased expression of inflammatory cytokine factors, tumor necrosis factor α (TNFα), p65, and NFκB phosphorylation [88].

Current medications for fibrosis are alternative methods to reduce the driver of fibrosis, inflammation organ-specifically such as anti-reflux of acids in the esophagus in eosinophilic esophagitis (EoE) patients. Corticosteroids, azathioprine, cyclophosphamide, and mycophenolate mofetil (Cellcept®, Myfortic®) also inhibit the inflammations, commonly prescribed for idiopathic pulmonary fibrosis (IPF) [89, 90].

The kidney has a certain histological feature specifically where the density of blood

vessels increased. Kidneys generally represent the health status of the circulatory system of the body. Kidney malfunction can bring disastrous outcomes to vessel networks, including heart attack, thrombosis, and inflammation.

ETV2, which has a role as a master transcription factor in endothelial development during embryogenesis has been highlighted in recent decades due to the potency of reprogramming non-endothelial cells into endothelial cells by Lee *et al.* 2017. In this study, it is demonstrated that ETV2 suppresses the TGFβ2 and IL-1β-induced EndMT in glomerular ECs. It has been demonstrated that EndMT significantly contributes to multiple pathological positions, such as myocardial infarction or fibrosis [28, 81]. TGFβ is the powerful and important cytokine that regulates EndMT [91]. These results confirm that TGFβ2 and IL-1β-exposed GEnCs undergo EndMT. After the treatment of TGFβ2 and IL-1β, the morphology changed, EC markers decreased, and fibroblast markers increased.

The antifibrotic function of ETV2 has been reported in cardiac fibrosis, and vascular diseases [77]. This study shows that the activation of ETV2 could inhibit TGFβ2 and IL-1β-induced EndMT. The potential mechanism of pro-fibrotic Smad2/3 and ETV2 are not fully understood.

Interestingly, endothelial marker protein (VE-cadherin/CDH5) is reported to be directly activated by VEGFR2 [92]. VEGFR2/Flk1 function downstream of ETV2 in embryogenesis [92, 93]. VEGF-A, a ligand molecule of VEGFR2 was contained in a culture medium (EGM-2™, Lonza). Therefore, one of the two methods should solve the problem. One is that VE-cadherin is not a proper marker protein of endothelial cells in the ETV2 study. The other is that the culture medium should eliminate VEGF-A during EndMT due to the upregulation of continuous VE-cadherin as opposed to the hypothesis.

In this study, VE-cadherin/CDH5 was significantly upregulated during 6 days of TGFβ2 and IL-1β costimulation by RT-qPCR and Western blot analyses which can explain the upregulation of VEGFR2 by ETV2 transfection, continuous binding of VEGF-A supplied by everyday refreshed endothelial medium upregulated VE-cadherin.

Transcription factors such as Slug, Snail, Twist-1 and Twist-2 are marker proteins of early mesodermal lineage. During this study, after 2-4 days of costimulation of TGFβ2 and IL-1β

in GEnCs, the protein expression level of Slug, Snail, and Twist1 were dramatically upregulated, analyzed by RT-qPCR (data not presented). The phase of early vertebrate development is specified as the three germ layers, endoderm, mesoderm, and ectoderm. ETV2 has an important role in the mesodermal development of muscles and blood including endothelial lineages. The mesodermal differentiation to tissues and organs needs the specified and delicate integration of signaling pathways such as BMP, FGF-5, TGFβ and Wnt.

Among these mesodermal secretion marker proteins, Activin and Nodal (TGFβ superfamily) are known to be essential for mesodermal induction, while FGF and Wnt are only responsible for its maintenance and BMP oversees its patterning [94, 95]. Stimulation of TGFβ2 that is bound to TGFβ receptor II, and also recognized by receptor I, phosphorylated, propagate to downstream substrates, for example, Smad2/3. Activated transcription factor Smad upregulates mesenchymal protein expression within cells.

Fibroblasts and their mesenchymal lineages have key functions including extracellular matrix (ECM) secretion and remodeling, secretion of signaling factors for surrounding cells, mechanical force generation, and regulation of tissue metabolism and metabolite secretion. Fibroblast can convert by fibroblast to endothelial transition through a p53-dependent pathway. However, further studies showed that pre-existed ECs exclusively oversee neovascularization instead of ECs that transited from fibroblasts [96]. Based on numerous published articles, it is difficult to differentiate between fibroblasts and endothelial cells because the marker protein of fibroblast FSP-1 commonly expresses on myeloid cell types or endothelial cells. Furthermore, FSP-1+ cells express marker proteins of hematopoietic or endothelial cells in normal infarcted, and remodeling hearts.

In summary, GEnCs in this study were stimulated along by two factors: TGFβ2 and IL-1β. It is not sufficient to use TGFβ alone to induce EndMT, to accelerate the mesenchymal gene activation by NFκB activated by IL-1β.

Given all together, this study confirmed costimulation of TGFβ2 and IL-1β upregulated mesenchymal genes SM-22α, Fibronectin, pSmad2/3, Collagen I, downregulated endothelial marker genes CD31, which suggest successful EndMT to fibroblast-like cells. ETV2 overexpression during EndMT decreased mesenchymal (pSmad2/3, α-SMA, Fibronectin) protein

expression levels assessed by Western blotting, recovered endothelial (CD31) mRNA expression levels proven by RT-qPCR, ultimately blocked mesenchymal (SM-22α) protein expression levels shown by immunofluorescence.

In conclusion, even though EndMT has other non-TGFβ/Smad dependent signaling pathways such as Wnt, Notch, it is assessed that costimulation of TGFβ2 and IL-1β could induce EndMT in GEnCs, and ETV2 could inhibit this process. Therefore, it can be suggested that ETV2 is a possible treatment for kidney fibrosis.

감사의 글

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국문 초록

독소, 외상, 감염, 허혈, 돌연변이 또는 자가면역반응에 의한 다양한 손상은 신장을 포함한 여러 장기에서 염증 반응을 유발한다. 신장 중간 조직에서의 세포외기질물질 (ECM)의 퇴적으로 정의되는 섬유증은 신장 손상의 결과 중 하나이다. 섬유증의 시작은 손상된 국소 조직 구조에서 고도로 활성화된 염증 반응을 시작으로 다양한 세포에서 세포외기질물질을 생성하는 세포로의 전환 (중간엽 전이) 함으로써 발전한다. 중간엽 전이세포의 기원이 되는 세포는 조직의 상피세포, 섬유아세포, 대식세포 등 다양하나 혈관 가장 안쪽에 위치한 혈관 내피 세포 (EC)는 최근 새로운 중간엽 전이세포로의 전환 (EndMT) 뿐만 아니라 섬유화 전반에 걸친 그 역할이 규명되었다. 제브라피쉬 (Zebrafish) 및 생쥐 등 동물 실험을 통해 중요성 및 필수적 역할이 확인된 ETV2는 배아발달단계에서 심혈관계 구성을 지시하는 전사인자로, ETV2가 배아 형성 단계에서 결실된 개체는 심혈관계 형성에 실패하는 결과를 보인다. 이에, 기이학적 병리적 현상으로 정의되는 성인 내피-중간엽 전이 현상에서 초기 심혈관계 계통 발달에서 필수적인 전사인자 ETV2를 발현시켜 성인 내피-중간엽 전이가 억제될 수 있는지를 연구하였다. 실시간 중합효소연쇄반응 및 면역 형광 분석 기법을 활용해 나타낸 결과는 ETV2의 강제 발현이 성인 내피-중간엽 전이를 억제할 수 있음을 혈관내피세포 고유 단백질 (CD31) mRNA의 장기 발현을 유도하며, 중간엽 전환의 지표 (pSmad2/3, Fibronectin, and α-SMA) 단백질 발현을 현저하게 감소시킴으로 확인하였다. 본 연구의 결과는 섬유증 질환에서 내피-중간엽 전이의 세포 운명 변화를 이해하는 데 필요한 중요한 정보를 제공할 뿐만 아니라 섬유증 치료에 대한 새로운 전략을 개발할 것이다.