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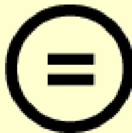

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2021년 2월
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Role of low density lipoprotein receptor-related protein 6 in vascular smooth muscle cells migration

조선대학교 대학원

의 과 학 과

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혈관평활근세포 이동에서 LRP6의 역할 및 기전 규명

2021년 2월 25일

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초 록

혈관평활근세포 이동에서 LRP6의 역할 및 기전 규명

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죽상동맥경화증은 전 세계적으로 높은 사망률을 유발하는 주요 원인 중 하나로, 심비대증, 심부전, 심근경색 등과 같은 대표적인 심혈관계 질환에 속한다. 심혈관계 질환의 일차적인 원인으로 지목되는 죽상동맥경화증의 초기 진행은 혈관평활근세포의 이동으로부터 시작되는 것으로 잘 알려져 있다. LRP6 (Low-density lipoprotein receptor-associated protein 6)는 LDLR family에 속하며, Wnt ligand에 결합하여 Wnt/β-catenin 신호 전달을 활성화하는 공동 수용체로 알려져 있다. LRP6가 과발현되면 Tcf-4 (T cell factor 4)의 활성화를 증가시켜서 혈관평활근세포의 증식을 유도한다는 보고가 있으며, 반대의 경우로 LRP6(R611C) 모델에서 LRP6의 활성이 감소될 경우에 PDGF (Platelet-derived growth factor)에 의해 유도된 혈관평활근세포의 증식을 촉진시키거나, 분화를 억제함으로써 경동맥 손상모델의 신생 내막 증식

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(neointimal hyperplasia)을 일으킨다는 보고가 있다. 그러나 LRP6 조절에 의한 혈관평활근세포의 이동 억제에 대한 기전 연구는 알려진 바가 없다. 그러므로 본 연구에서는 동맥 경화의 원인이 되는 혈관평활근세포의 이동성에서 LRP6의 과발현 및 저해에 의해 미치는 영향 및 기전을 밝히고자 하였다. 먼저 혈관평활근세포는 쥐의 흉부 대동맥에서 획득하였고, LRP6의 역할 규명을 위해 LRP6 과발현 및 저해 아데노바이러스를 제작하여 western blot을 통해 농도의존적으로 잘 발현되는 것을 확인한 바, 이후 결정된 농도를 실험에 사용하였다. 2D와 3D 이동성 분석에서 LRP6의 과발현시 혈관평활근세포 이동성에 유의적인 변화가 없었으나, LRP6 저해는 혈관평활근세포의 이동성을 현저히 촉진시키는 것으로 확인되었다. 그러나 BrdU assay를 통한 증식성에서는 유의적인 변화가 없었다. 이를 바탕으로 LRP6의 발현을 저해한 실험 군에서 세포 이동의 지표인 MMP2, 9, 13의 발현 변화를 western blot, q-PCR analysis를 통해 확인한 결과, MMP2와 13에서 유의적인 증가를 확인하였다. zymography, luciferase assay 분석을 통해 MMP2의 유의적인 증가를 추가로 보여줌으로써 LRP6 저해에 의한 혈관평활근세포 이동 증가 효과가 MMP2에 특이적이라는 것을 확인할 수 있었다. 또한 세포의 이동, 성장, 분화, 생존, 염증반응 등 다양한 생물학적 과정에서 형성되어 세포를 세포 외 기질에 연결시키는 구조적인 역할뿐만 아니라, 신호전달의 시작점으로써 신호 조절 역할을 수행하는 것으로 잘 알려진 FAK (Focal Adhesion Kinase)가 LRP6의 공동 수용체인 Wnt와 관련되어 있다는 논문들을 바탕으로 FAK가 LRP6와도 연관이 있을 것이라 생각하였다. 그래서 본 연구는 FAK가 LRP6의 저해로 증가한 혈관평활근세포의 이동에서 어떠한 역할을 하는지 알아보고자 하였다. FAK의 인산화 부위인 Y397과 Y925를 western blot을 통해 확인한 결과, Y397에서는 유의하게 증가하였고, Y925에서는 대조적으로 감소하는 것을 확인하였다. 더 나아가 Y397의 기능이 억제되는 아데노바이러스 제작 및 FAK inhibitor인 Y15와 focal adhesion kinase-related non-

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kinase (FRNK)를 사용해 추가적인 연구를 진행하였다. LRP6를 저해시킨 혈관평활근세포에서 돌연변이 Y397, Y15 및 FRNK를 처리한 바, 2D와 3D 이동성 분석에서 LRP6의 저해에 의해 증가된 혈관평활근세포 이동성이 FAK의 인산화 억제 시 감소하는 것을 확인할 수 있었다. 이를 바탕으로 immunofluorescence staining, zymography 분석을 시행한 결과에서 세포 이동의 중요한 지표인 MMP2 활성이 유의적으로 감소되는 것을 다시 확인할 수 있었다. 추가적으로 western blot을 통해 Akt가 MMP2의 신호전달 체계임을 확인하였다. 이와 같은 결과들을 통해, 본 연구에서는 LRP6 저해 시 FAK 및 Akt 신호전달을 통한 MMP2, 13의 증가에 따른 혈관평활근세포의 이동성 증가를 확인함으로써, LRP6가 혈관평활근세포의 이동성을 조절할 수 있는 새로운 표적 단백질이 될 수 있는 것으로 생각된다.

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1. Introduction

Cardiovascular diseases (CVDs) are a group of disorders of the heart and blood vessels and they include coronary heart diseases such as myocardial infarction, atherosclerosis, stroke, cardiac hypertrophy, and heart failure [1]. Among them, in particular, Atherosclerosis is one of the diseases that cause high mortality worldwide [2] and is caused by a number of risk factors, including macrophage accumulation, pro-inflammatory cytokine production, and dysfunction of endothelial and vascular smooth muscle cells (VSMCs) [3]. Atherosclerotic lesions begin when the lipoprotein LDL cholesterol is deposited in the endothelium (EC) and transformed, causing endothelial dysfunction. EC-induced damage causes increased lipid permeability, macrophage recruitment, foam cell formation, and T-lymphocyte recruitment, and releases various growth factors, cytokines, chemokines, etc. As a result, VSMC migrates and proliferates from the medium to the inner membrane, synthesizes and they secrete the extracellular matrix (ECM), and forms plaques through a complex action with macrophages, leading to atherosclerosis [4] [5]. Matrix metallopeptidases (MMPs) function to degrade extracellular matrix (ECM) proteins and are known to play an important role in cell behaviors [6]. It has been reported that MMP can catalyze and remove the basement membrane around VSMCs, and promote contact with the interstitial matrix to promote the transition from contractile VSMCs to active synthetic cells capable of migrating and proliferating [4]. Also, nesfatin-1 stimulates the proliferation, migration, and neointimal proliferation of VSMCs by raising the levels of MMP2 and MMP9 [7]. In VSMC of Cav-1-deficient (Cav-1 KO) mice, mRNA expression of MMP3 and MMP13 were downregulated and MMP14 was upregulated, pyrogallol showed the result of inhibiting migration by inhibiting the activity of MMP2 in Cav-1 wild-type (WT) VSMC [8]. Among them, MMP related to migration of VSMC is MMP2 and membrane-type MMP [9].

Low-density lipoprotein receptor-associated protein 6 (LRP6) is a member of the family of low-density lipoprotein receptors (LDLR). LRP6 is a co-receptor that binds to Wnt ligands and

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activates Wnt/β-catenin signaling with LRP5 and Frizzled protein family members [10]. LRP6 is well known for its role in standard signalings such as lipid metabolism, blood pressure, atherosclerosis, and tumor formation, and inhibiting Wnt signaling, which promotes arterial smooth muscle cell proliferation and vascular calcification [11] [12]. Mutations in LRP6 signaling raise serum LDL, triglyceride, and glucose levels, which are associated with several risk factors for cardiovascular disease [13] [14]. LRP6 has been shown to act as a scaffold protein that regulates cardiac gap junction assembly in the adult heart, and downregulation of LRP6 plays an important role in cardiac arrhythmias [15]. Also, cardiac LRP6 deletion exacerbated cardiac dysfunction by inhibiting autophagy and fatty acid utilization simultaneously with activation of dynamin-associated protein 1 (Drp1) and downregulation of nuclear transcription factor EB (TFEB) [16] [17]. Wnt/β-catenin signaling is an early developmental pathway that plays an important role in the development of vascular smooth muscle cells [18] [19], and it was confirmed that LRP6 regulates VSMC proliferation and survival through Wnt/β-catenin signaling [20]. Importance of LRP6 in atherogenesis came to light by a discovery of a missense mutation in LRP6 gene in humans [21]. In human atherosclerotic coronary arteries, colocalization of LRP6 expression and Platelet-derived growth factor receptor β (PDGFR-β) was detected in the subintimal layer, and the proliferation of vascular smooth muscle cells was promoted by platelet-derived growth factor (PDGF) in the LRP6 (R611C) mouse model [22]. Also, in the LRP6 mutant knockdown mouse model, LRP6 activity decreased, promoting the loss of VSMC differentiation, leading to medial aortic proliferation, resulting in total vascular occlusion [23]. However, the role of LRP6 in migration is not known.

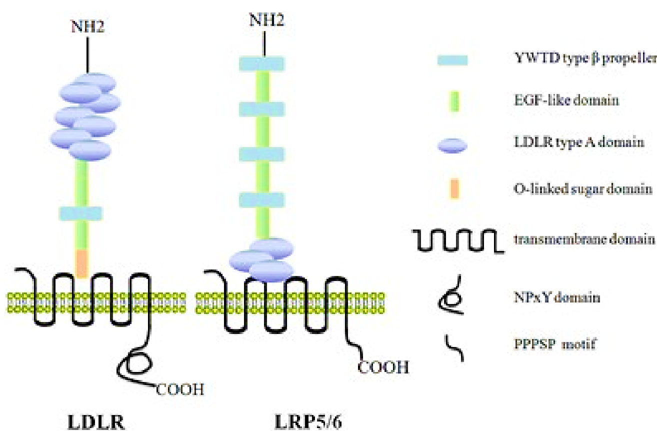

Figure 1. The structure of LRP6 [24]

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Focal adhesion kinase (FAK) is a cytoplasmic protein-tyrosine kinase first identified at the site of extracellular matrix and integrin receptor cell adhesion and is a major regulator of cell migration and plays a role in motility and cell survival, and is activated by a variety of stimuli [25]. FAK plays an important role in promoting invasive properties in tumor cells and the tumor environment that controls tumor metastasis [26] [27], and is important in promoting the proliferation of SMCs when blood vessels are damaged [28]. Moreover, it has been reported that vascular ECM regulates SMC proliferation through FAK regulation of the Skp-2 protein [29], and inhibition of FAK inhibits SMC proliferation and neointimal hyperplasia through GATA-binding protein 4 (GATA4)-cyclin D1 signaling [30]. It is also known that FAK expression modulates MMP2 and MMP9 [31].

In recent years, many studies have shown the association of FAK with Wnt/β-catenin signaling. In breast and pancreatic cancer cells, FAK acted upstream of the Wnt/β-catenin pathway [32], and in mouse osteoclasts, FAK activation stabilized the β-catenin protein, promoting nuclear translocation [33]. In contrast, FAK acts downstream of Wnt in the mouse intestine to promote intestinal regeneration [33], and the elevated expression of FAK in APC mice and human colorectal cancer tissues leads to β-Catenin accumulation and intestinal tumor formation through GSK3β (Y216) [34]. In addition, FAK knockdown plays a pivotal role in promoting the bone formation of synovial mesenchymal stem cells by BMP9 through Wnt and MAPK signaling pathways [35]. The interaction between LRP6 and Wnt/β-Catenin signaling is a well-known fact [36] [37] [38]. Therefore, it was speculated that FAK may have a direct relationship with LRP6 as well as Wnt/β-Catenin signaling. In this study, we sought to determine the effect of LRP6 on the mobility of VSMC and the effect of FAK-related signaling on its developmental mechanism. It was confirmed that silencing of LRP6 promotes migration of VSMC and affects FAK phosphorylation and mitogen-activated protein kinase (MAPK). This study demonstrates the role of LRP6 and FAK-related mechanisms in VSMC migration.

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2. Materials and Methods

2.1. Isolation and culture of vascular smooth muscle cells

Vascular smooth muscle cells (VSMCs) were isolated from the thoracic aorta of 6-week-old male Sprague-Dawley rats. Surrounding connective and adipose tissue and blood were removed in DMEM containing 0.1% penicillin-streptomycin. After incubation for 30 min at 37 °C in DMEM containing 1 mg/ml collagenase type I and 0.5 mg/ml elastase, the outer cell membrane was removed and fragmented, and It was reacted for 2 hours in new DMEM containing collagenase type I and elastase. The separated VSMC was precipitated by centrifugation at 1,600 rpm, and the obtained cells were dispersed in DMEM containing 0.1% penicillin-streptomycin and 10% FBS and incubated in a CO2 incubator. Subculture was carried out by treatment with 0.05% trypsin-EDTA, and cells used in all experiments were cultured in DMEM containing 10% FBS, 5% SMGS, and 1% penicillin-streptomycin in a 37°C incubator under the same conditions.

2.2. Preparation of adenovirus

Adenovirus overexpressing LRP6 was created using ViraPower™ adenovirus expression system (Invitrogen) (Ad-LRP6). VSVG-tagged LRP6 was used in pcDNA construct by cell signaling cloned from pENTR1A dual selection vector. Then, the above LRP6 was subjected to an LR recombination reaction with pAd/CMV/V5/DEST gateway vector to produce an adenovirus clone. Then, the prepared adenovirus vector was cut with a PacI restriction enzyme and transfected into 293A cells using Lipofectamine 3000 reagent (Invitrogen life Technologies). The multiplicity of infection (MOI) was confirmed using an Adeno-X™ qPCR titration kit (Takara, Clontech). As a control, an adenovirus vector expressing lacZ-β-galactosidase was used (Ad-lacZ).

The adenovirus silencing LRP6 expresses rat LRP6-targeting shRNA and was constructed using the BLOCK-iT™ adenoviral RNAi expression system (Invitrogen) (shLRP6). The target

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nucleotide sequence according to the oligo duplexes is as follows: 5'-GGU UGU UCC CAU UUG UGU U-3'. The double-stranded oligonucleotide was cloned using the BLOCK-iT™ U6 entry vector, and an adenovirus clone was produced by LR recombination reaction with the pAd/BLOCK-iT™-DEST vector. As a control, a pAd-GW/U6-laminshRNA adenoviral vector was used (lamin).

2.3. Cell migration assay

The migration capability was determined using the scratch-wound healing assay and boyden chamber assay. First, incubate VSMC in a 6 well plate at $2x10^5$ cells/2ml in DMEM containing 10% FBS, 5% SMGS for 12 h. LRP6 Ad and shLRP6 Ad were each treated and incubated for 72 h. From here on, the scratch-wound healing assay lines within the VSMCs were scraped using a sterile plastic pipette tip in each cultured well. The cells were washed twice in warm PBS to remove cellular debris. Cells were then further cultured in 1% FBS, 5% SMGS DMEM and photographed at 0 and 24 hours. In the boyden chamber assay, 1% gelatin was first coated on a polycarbonate filter in the trans well for 1 h. Cells cultured in 6 wells are treated with 0.5X trypsin-EDTA and the quantity is measured in serum-free medium. 600ul of DMEM containing 1% FBS was added to a 24-well plate, and $4x10^4$ cells/400ul were carefully seeded inside the insert of the trans well. cells were cultured for 24 hours at 37°C. After fixing the cells and staining with HEMA 3, the cells that did not migrate across the trans well membrane were then removed by gently wiping with a cotton swab. Migrated cells were viewed with an optical microscope, imaged with a camera using.

2.4. Cell proliferation assay

BrdU proliferation assay was performed using a cell proliferation colorimetric ELISA system (Promega) according to the manufacturer's protocol. Cells were inoculated into a 96-well plate at $3x10^3$ and cultured for 24 hours, and then adenovirus was treated for 24 hours. Then, the cell was treated with a fixing/denaturing solution for 30 minutes at room temperature. Cells were treated

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with detection antibody solution, HRP-conjugated secondary antibody solution, and TMB substrate, and absorbance was measured at 450 nm.

2.5. Western blotting

VSMCs were washed with PBS (Phosphate buffered saline) were lysed in RIPA buffer (phosphate-buffered saline containing 1% Triton X-100, protease inhibitor cocktail, and 1mM phenylmethylsulfonyl fluoride (PMSF)). Protein concentrations were determined using a Bradford protein assay kit (Bio-Rad, Hercules, CA). Equal quantities of protein were separated on SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel via electrophoresis) and transferred to a PVDF (polyvinylidene difluoride, Bio-Rad) membrane. After being blocked, the membranes were incubated with primary antibodies then, the membranes were washed and incubated with an HRP-conjugated secondary antibody for 1 h at room temperature. Immunoreactive proteins were detected using an enhanced chemiluminescence (ECL) system (Intron). The Image J software was used for quantification

2.6. Quantitative real-time PCR analysis

Total RNA was isolated from cells using TRIzol reagent (Invitrogen) according to the protocol. cDNAs were synthesized using 1μg total RNA and the 1st Strand cDNA Synthesis kit (Takara). The quantitative real-time polymerase chain reaction (qPCR) analysis was performed with the RealHelixTM SYBR premix qPCR kit (NanoHelix) in a Roter-Gene 3000 (Corbett Research). The relative mRNA expression levels were determined by the $2^{-\Delta\Delta Ct}$ method using GAPDH as an internal control. The list of primers is as follows:

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Table 1. The sequence of primers used for real-time quantitative PCR

Gene	Primer sequence
GAPDH	Forward: 5’-CAG TGC CAG CCT CGT CTC AT-3’ Reverse: 5’-TGG TAA CCA GGC GTC CGA TA-3’
LRP6	Forward: 5’-CGG AGT CTC AGT TCC AGT GT-3’ Reverse: 5’-CTG TAG GAT GGT GGT GGG TT-3’
MMP2	Forward: 5’-ACA GGG CAG TGG GAT ACA GGT-3’ Reverse: 5’-AAA CAG CAA AGG GCA ACA AAG-3’
MMP9	Forward: 5’-AC AGG ACT CCC CTC TGC AT-3’ Reverse: 5’-AGG CCT TGG GTC AGG TTT AGA-3’
MMP13	Forward: 5’-TCT GCA CCC TCA GCA GGT TG-3’ Reverse: 5’-CAT GAG GTC TCG GGA TGG ATG-3’

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2.7. Gelatin zymography

VSMC was treated with shLRP6 for 24h, replaced with Serum-starved medium, and incubated for 48h. The conditioned medium was collected, mixed with SDS sample buffer, and electrophoresed on 10% SDS-PAGE with 0.8% gelatin added. Separated gels were renatured by washing in 2.5% Triton X-100 solution for 1 h to remove SDS in the gel and then incubated in incubation buffer (50mM Tris-HCl (pH 7.5), 5mM CaCl2, 1μm ZnCl2, 0.02% sodium azide, 1% Triton X-100) at 37°C. After staining with 1% Coomassie brilliant blue R-250, the stained gel was destained in a destaining buffer (4% methanol, 0.01% acetic acid). The activity of MMPs was detected as a clear band on the blue background of the gel.

2.8. Luciferase reporter assay

Control, LRP6-Ad, and shLRP6-Ad were treated for 24h, then MMP2-luciferase reporter plasmid (Addgene) and renilla luciferase vector (pRL-TK; Promega) were transfected for 24h. The cell lysate was harvested for firefly and Renilla luciferase assay analysis according to the protocol using the Dual-Luciferase Reporter Assay System (Promega). MMP2 Luciferase activity was measured using a Micro-Lumat Plus LB96V Luminometer (EG & G Berthold). Firefly luciferase activity was normalized to Renilla luciferase activity.

2.9. Immunofluorescence staining

Cover glass (BD Biosciences) was placed on a 24-well plate, and the cells were seeded at $2x10^4$/well and incubated at 37°C for 24 hours. Cells were treated with adenovirus for 24 hours. For the immunofluorescent labeling, cells were washed with cold 1X PBS and fixed with cold 2% paraformaldehyde in PBS for 15 minutes at room temperature. Washed 3 times with PBS, and permeabilized for 15 minutes at room temperature with 0.5% Triton-X-100 in PBS. The primary

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antibody was overnight at 4°C with 2% bovine serum albumin (BSA) in PBS for 1 hour at room temperature, and then the secondary antibody was incubated for 1 hour at room temperature in the dark. After washing with PBS, the cell nuclei were stained with 0.2μg / ml DAPI (Sigma-Aldrich) for 1 minute, and the cover glass was mounted on the slide. Finally, cells were visualized with a laser scanning confocal microscope (Fluoview FV1000, Olympus).

2.10. Statistical analysis

All quantified data were analyzed as an average of at least 3 samples. Data are expressed as mean ± SD. Statistical significance between groups was performed using Student's t-test. Two-sided $P < 0.05$ was considered statistically significant.

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3. Results

3.1. Adenovirus expressing LRP6

We constructed adenovirus to determine if the expression of LRP6 affects vascular smooth muscle cells. We constructed an adenovirus vector that is tagged with VSVG and overexpresses LRP6 (Ad-LRP6) and an adenovirus vector that expresses LRP6 (shLRP6) targeting shRNA to silence endogenous expression. After transforming the pAd/CMV/V5-DEST vector, which is a plasmid vector that makes adenovirus to the plasmid vector targeted by LRP6, through enzymatic treatment and ligation, the pellet obtained by digestion with pacI was infected with 293A cells to constructed adenovirus (Figure 2A). Next, vascular smooth muscle cells were infected with Ad-LRP6 and shLRP6 for 48 hours or 72 hours, respectively. These results indicate that overexpression of LRP6 and silencing of LRP6 were successfully established in a concentration-dependent manner. (Figure 2B and 2C)

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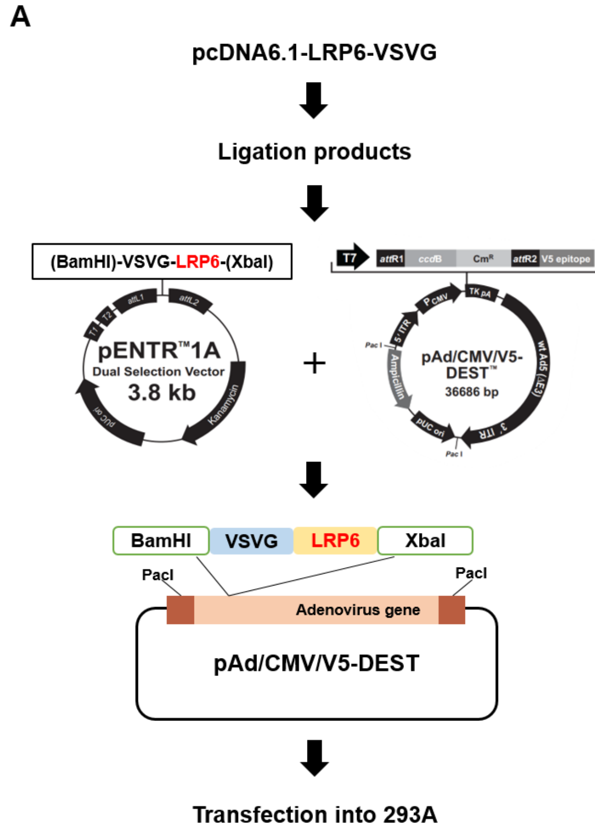

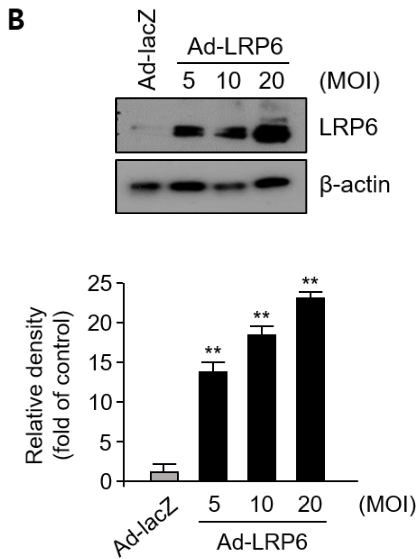

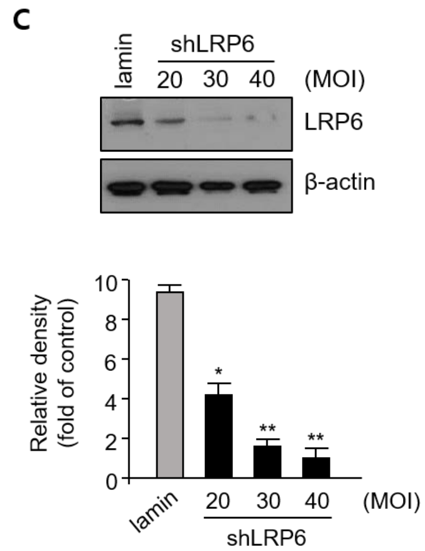

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Figure 2. Construction of LRP6 or shLRP6 adenoviral vectors for gene delivery

(A) This is a simple schematic example of adenovirus construction. (B)(C) Rat vascular smooth muscle cells were infected with adenovirus that overexpresses LRP6 or silences LRP6 at the indicated MOI values. The effect was confirmed by western blotting. Results represent the results of three independent experiments. Relative LRP6 expression levels were normalized to b-actin. Values are expressed as mean ± SD.

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3.2. Silencing of LRP6 promotes VSMCs migration

VSMC migration also significantly contributes to vascular disease development. Thus, to investigate whether the LRP6 and shLRP6 affect VSMCs migration, two-dimensional wound healing, and three-dimensional Boyden chamber cell migration assays were conducted. In the 2-D migration analysis of VSMC, the migration distance was not changed in LRP6 overexpression compared to the control, whereas in LRP6 silencing, it was significantly increased compared to the control (Figure 3A). To further confirm this result, a three-dimensional movement analysis using a trans well chamber was performed. Cells that migrated through the membrane of VSMC increased significantly compared to control cells in LRP6 silencing (Figure 3B). BrdU analysis was performed to measure the proliferation of VSMCs and there was no change in proliferation (Figure 3C). Based on the results, we focused on increasing the migration of VSMC due to LRP6 silencing.

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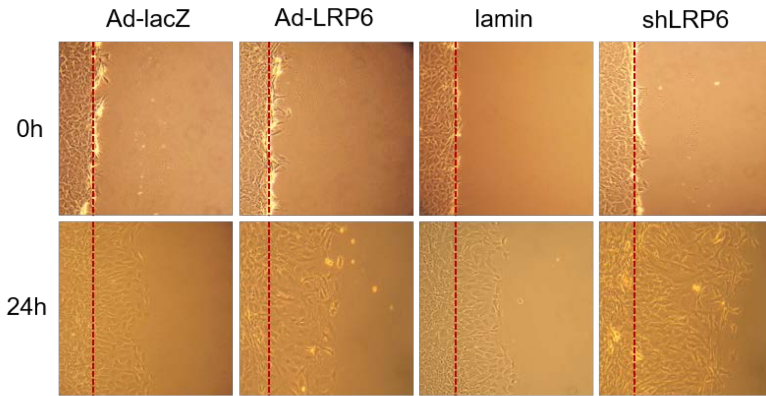

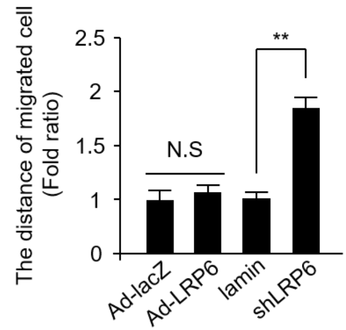

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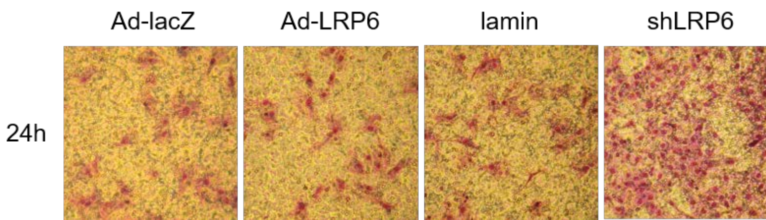

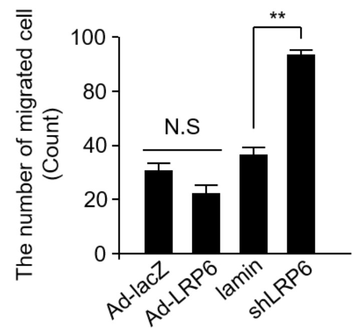

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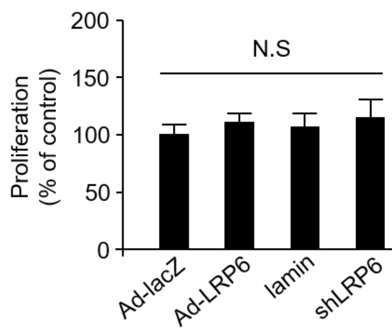

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Figure 3. Effect of shLRP6 on migration and proliferation of VSMCs

Effects of LRP6 overexpression and invasion on VSMC migration and proliferation. The migration verification experiment of VSMC was determined by both 2-D wound healing assay (A)-up and 3-D Boyden chamber assay (B)-down. Proliferativeness was determined by BrdU analysis (C). Vascular smooth muscle cells (VSMC) were treated with the specified concentration of extract determined in Figure 1. Values are expressed as mean ± S.D. ** $P < 0.01$ vs control.

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3.3. Silencing of LRP6 increases the expression of MMP2 and MMP13

MMPs are known to play important roles in cellular behaviors such as cell migration, proliferation, differentiation, and angiogenesis. Therefore, when LRP6 was silencing, the expression levels of MMP2, 9, 13, which are indicators of cell migration, were confirmed by Western blotting and q-PCR. Overexpression of LRP6 did not affect MMP expression levels (Figure 4A). When LRP6 was silencing concentration-dependently, significant increases in MMP2 and MMP13 were observed at the protein and mRNA levels. On the other hand, there was no change in MMP9 (Figure 4A and 4B). This study confirmed that silencing of LRP6 induces migration specific to MMP2 and 13.

Next, we confirmed the expression levels of MMP2 and 13, which showed a significant increase in the previous study, through zymography. Since MMP is an enzyme that is released to the outside of the cell and degrades the extracellular matrix, we tried to check its activity in media other than inside the cell. We confirmed that the MMP2 and MMP13 levels increased significantly when LRP6 was silencing (Figure 5A). Additionally, a luciferase assay was performed to confirm the transcription level. Although not shown in the data, there was no significant change in the MMP13 promoter. In the MMP2 promoter, when LRP6 was overexpressed, there was no change, whereas when LRP6 was silencing, it was significantly increased (Figure 5B). Therefore, it was confirmed that LRP6 silencing affects not only the expression level of MMP2 but also the activity and transcription level.

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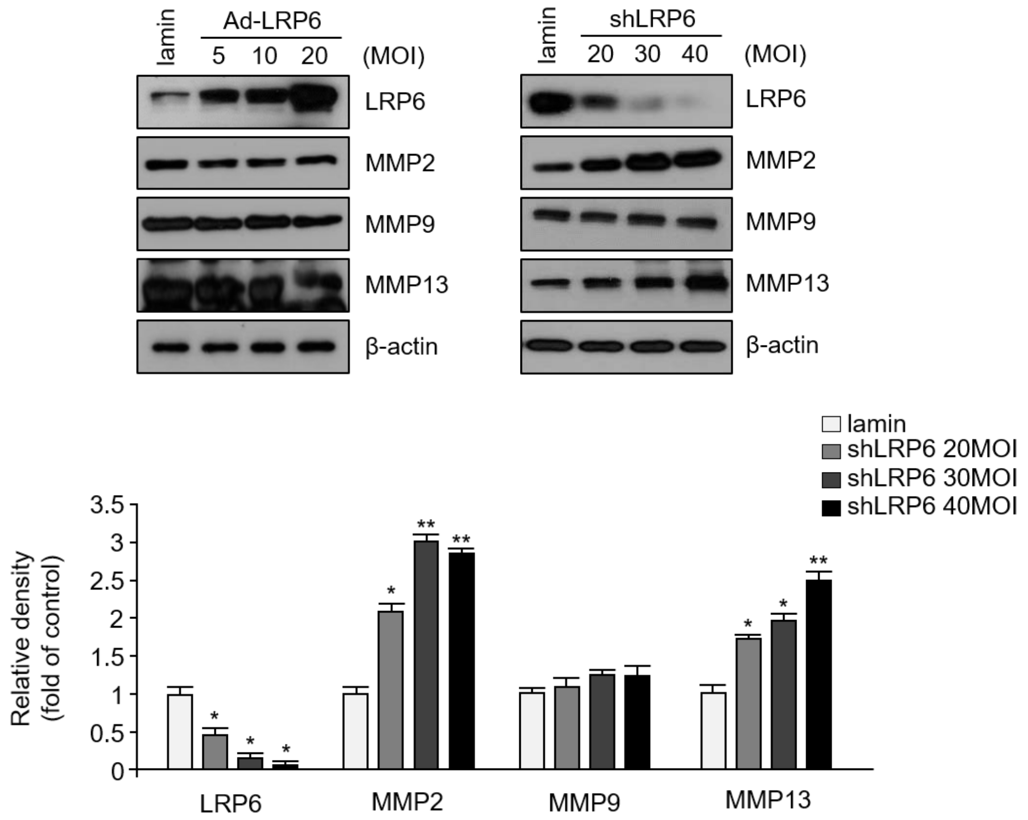

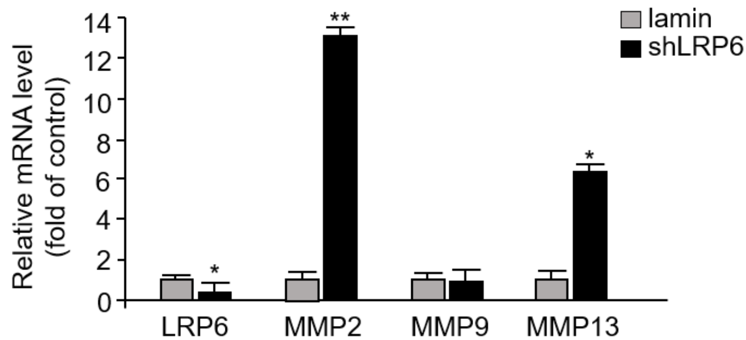

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Figure 4. Effect of shLRP6 on the expression of MMPs

(A) The expression levels of MMP2, 9, 13 confirmed by western blot analysis after infection with Ad-LRP6 and shLRP6. (B) After infection with shLRP6, the expression levels of MMP2, 9, and 13 were confirmed through q-PCR. The experiment was conducted three times. There was no change in MMP9, but a significant increase was shown in MMP2 and 13. Values were represented as mean ± SD. $^*P<0.05$

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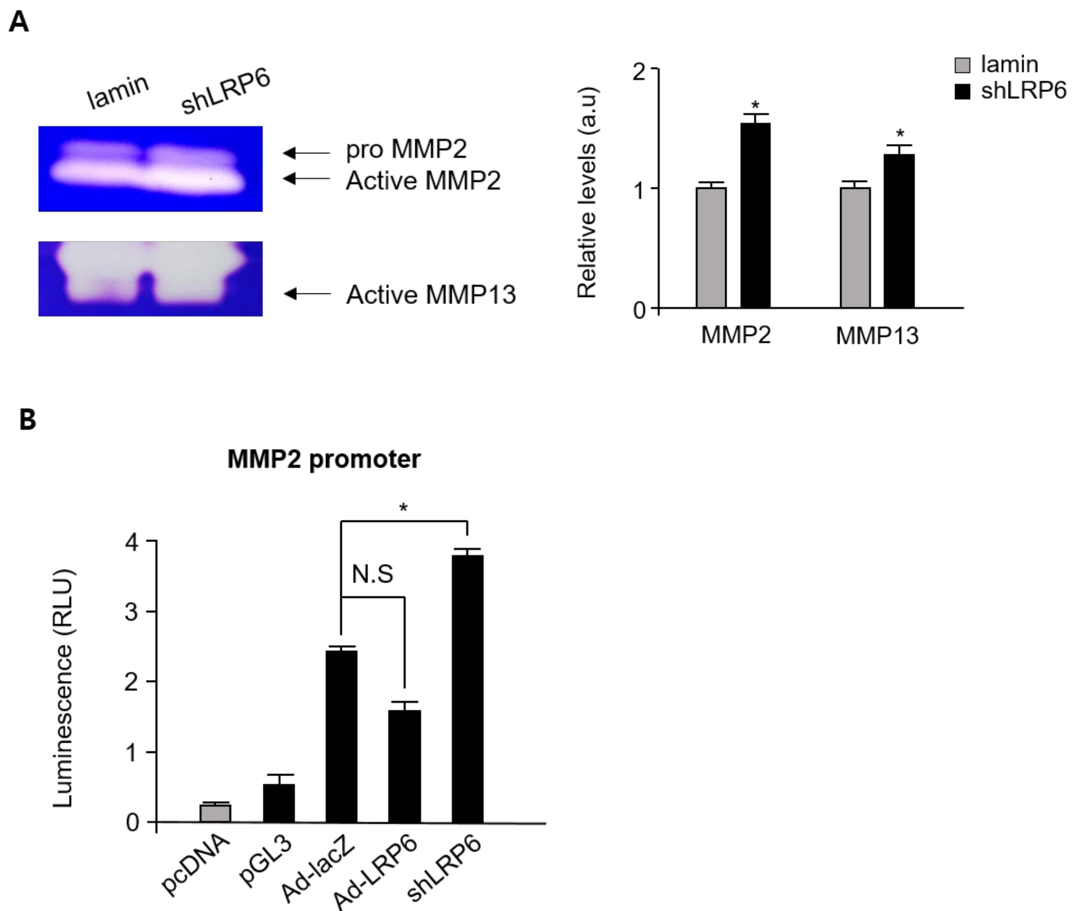

Figure 5. Effects of shLRP6 on MMP activity

(A) Confirmation of the expression level of MMP2, 13 through zymography analysis after shLRP6 infection in VSMC. (B) The activity level of MMP2 was confirmed by luciferase assay after Ad-LRP6 and shLRP6 infection in 293A cells. The experiment was conducted three times. Significant expression was found in MMP2 according to the overexpression and inhibition of LRP6. Values are expressed as mean ± SD. * P <0.05

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3.4. Silencing of LRP6 regulates phosphorylation of FAK and FA-proteins

Based on the above results, we investigated whether the expression of MMP2 and MMP13 by shLRP6 is through the FAK pathway. There have been papers showing that Wnt, which acts as a co-receptor with LRP6, is related to FAK. There were also papers that showed that this FAK regulates VSMC proliferation and neointimal hyperplasia, and also the expression of MMP2 and 9. So, FAK was thought to be related to LRP6 and set it as a target. Western blot analysis was performed to see how the expression levels of Y397 and Y925, the representative phosphorylation sites of FAK, change when LRP6 was silencing in VSMC. As a result, it was confirmed that the protein level of Y397 significantly increased compared to the control, while Y925 decreased compared to the control (Figure 6A). It was an interesting result in contrast. In addition, we wanted to confirm the level of FAK-related proteins. When checking the levels of src, paxillin, talin, and vinculin among numerous proteins, there was no change in src, talin, and vinculin, whereas a significant decrease was observed in paxillin, which is known to be related to Y925 (Fig. 6A).

Therefore, it was confirmed that the silencing of LRP6 affects the phosphorylation of FAK.

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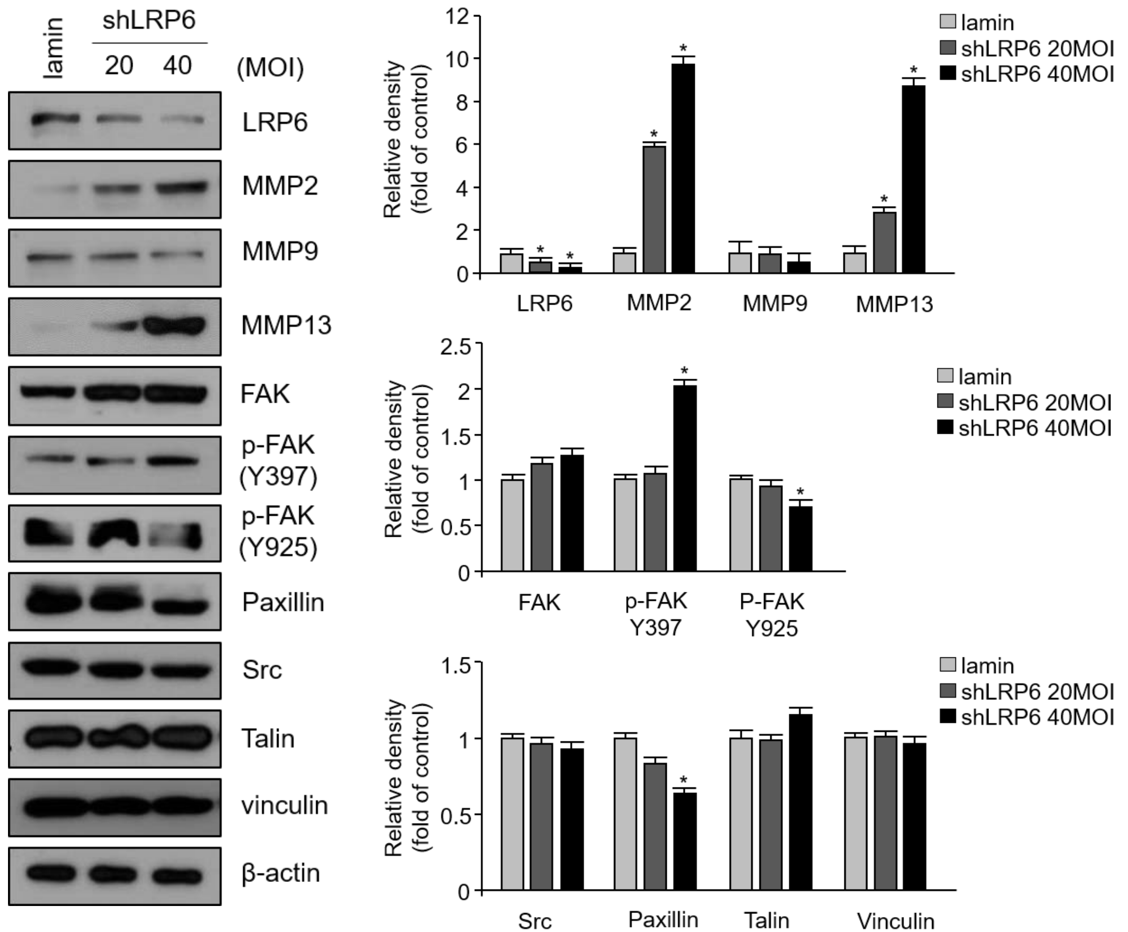

Figure 6. Effect of shLRP6 regulating MMPs on FAK and FA-proteins phosphorylation

(A) After infection with shLRP6 in VSMC, the expression levels of FAK and FA proteins were confirmed through western blot analysis. The experiment was conducted three times. Values are expressed as mean ± SD. * P <0.05

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3.5. Adenovirus expressing FAK mutants or inhibition of FAK

To further study FAK in detail, three mutants, inhibitors, and proteins were used. When FAK is activated, it auto-phosphorylates the Y397 site. So, an adenovirus that mutates this Y397 site was created (Y397F) (Figure 7A). And among the FAK inhibitors, an inhibitor that intervenes at Y397 position and inhibits it was used (Y15) (Figure 7B). FRNK (focal adhesion kinase-related non-kinase), unlike FAK, is a protein that independently expresses and inhibits the phosphorylation of FAK to block its activity (Figure 7C).

These three were treated with VSMC, and the expression was confirmed through Western blot. It was confirmed through HA-tagged on Y397F, Y15 was confirmed through Y397 antibody, a phosphorylation site, and FRNK was confirmed to work well through tagged GFP (Figure 8A-C).

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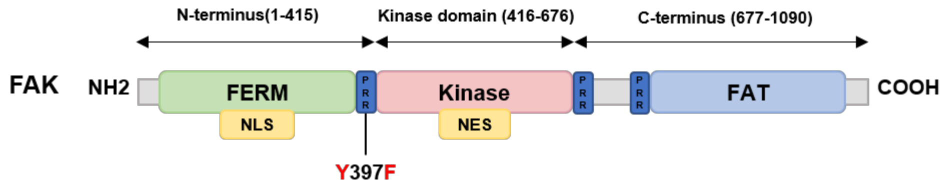

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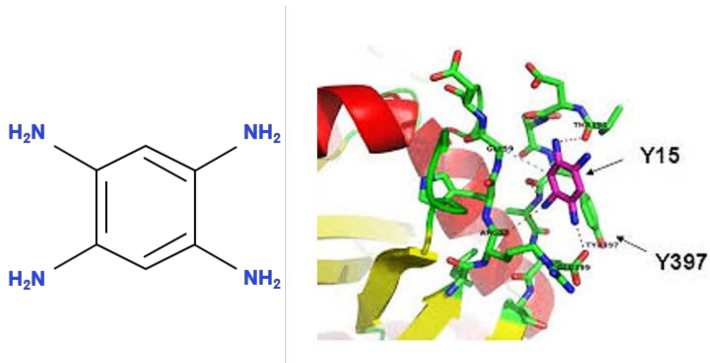

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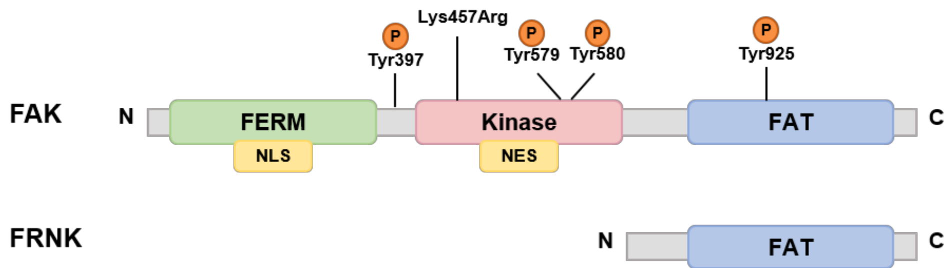

Figure 7. Construction of FAK point mutants adenoviral vector and inhibition of FAK

(A) The structure of the mutation site of Y397 in the FAK domain. (B) FAK inhibitor Y15. (C) The position of FRNK in the FAK domain.

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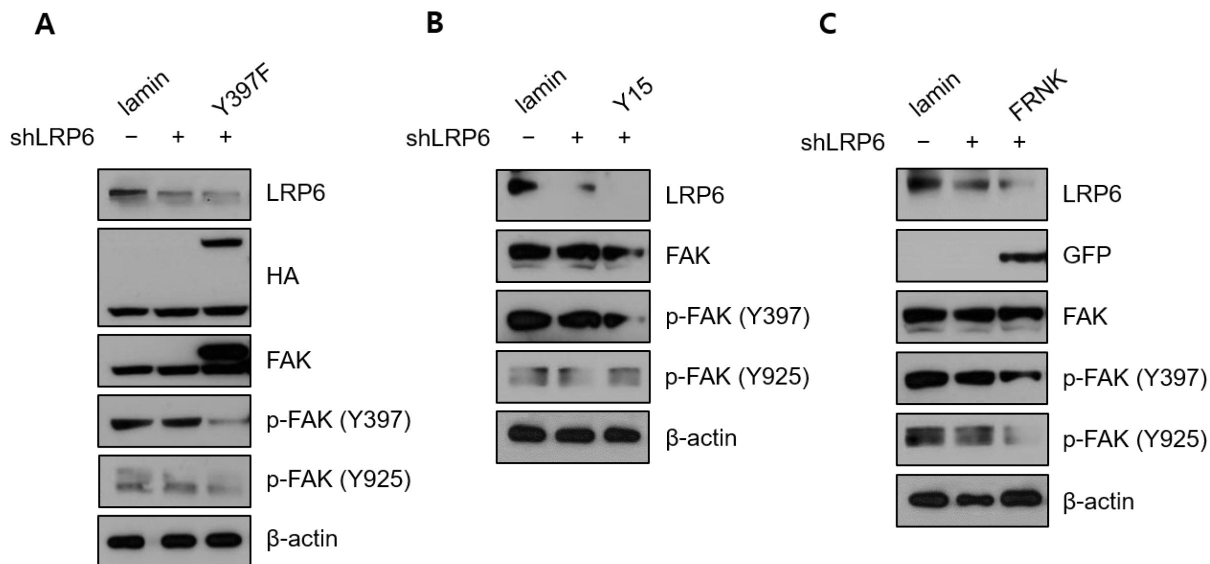

Figure 8. Generation of FAK mutations, FAK inhibitor or inhibition via FRNK transfection

(A) VSMC was treated with FAK mutant Y397F 30 (MOI) to confirm protein expression through Western blot. When producing the FAK mutant virus, HA was tagged. (B) After treatment with 4μg of Y15 chemical, a FAK inhibitor, on VSMC, expression was confirmed through Western blot. (C) After treatment with VSMC, 2μg of FRNK was transfected to confirm protein expression through Western blot. GFP is tagged in the FRNK vector. Values are expressed as mean ± SD. * $P < 0.05$

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3.6. Silencing of LRP6 modulates VSMCs migration by regulating FAK

Two-dimensional wound healing and three-dimensional Boyden chamber cell migration assays were performed to investigate how FAK mutant, Y15, and FRNK affect the increased VSMC migration by shLRP6. As with the previous results, in the 2-D migration assay, the travel distance of shLRP6 was significantly increased in VSMC compared to the control. And when dealing with FAK mutant, Y15 and FRNK, we noticed that the travel distance was reduced in reverse. (Figure 9A). To confirm this result, we performed a three-dimensional movement analysis using a trans well chamber. Cells that migrated through the membrane of VSMC increased in shLRP6 compared to control cells. And it was confirmed that the treatment with FAK mutant, Y15, and FRNK significantly decreased as in 2-D (Figure 9B). As a result, it was confirmed that FAK also affected the migration of VSMC.

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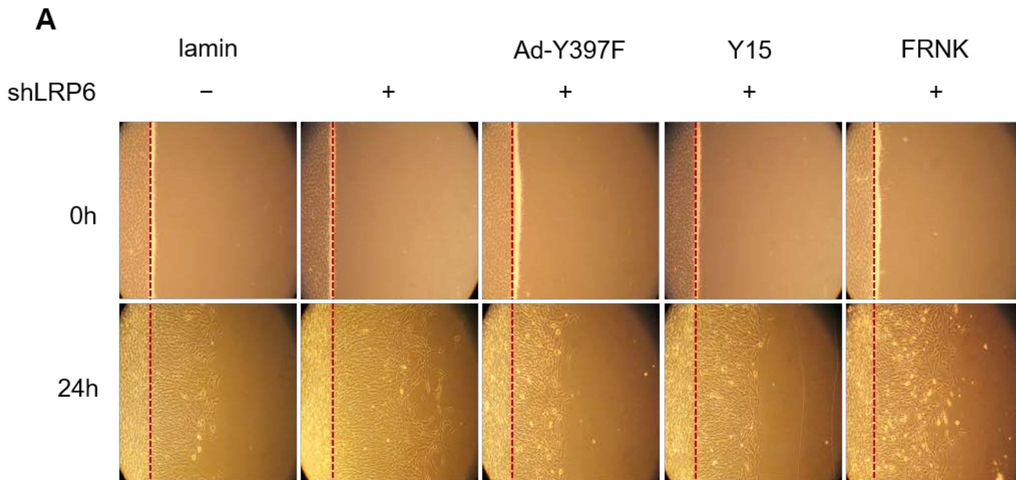

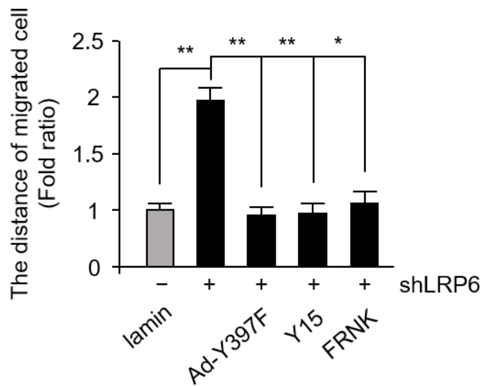

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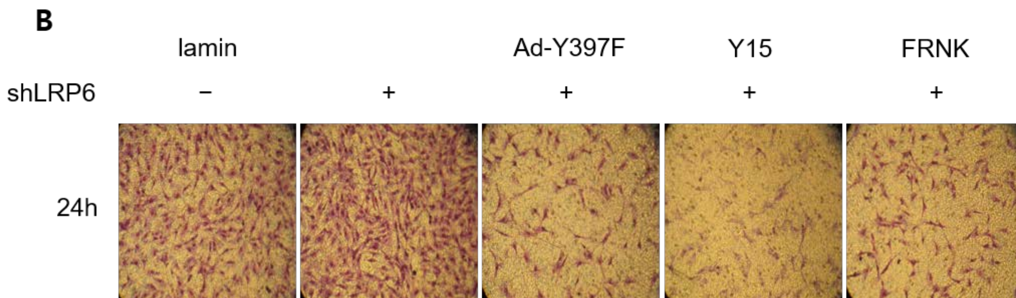

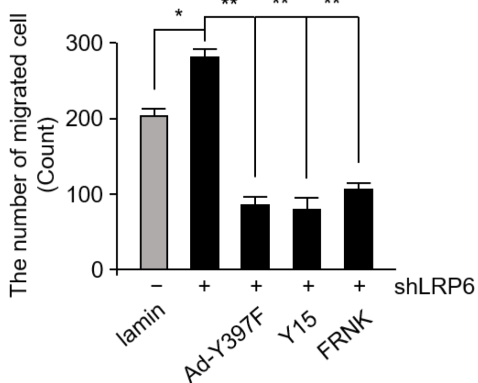

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Figure 9. Involvement of FAK in shLRP6-induced migration of VSMCs

Effect of FAK mutation on the increase of VSMC migration by shLRP6. (A)(B) VSMC was treated with shLRP6, followed by FAK mutant, Y15, and FRNK to perform 2-D wound healing analysis and 3-D Boyden chamber analysis. Values are expressed as mean ± S.D. ** P <0.01 vs control.

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3.7. Inhibition of FAK decreases MMPs in shLRP6-induced migration of VSMCs

The increase in VSMC migration by shLRP6 was reduced by the FAK mutation, and the level of MMP2 was further confirmed by zymography. After 72 hours of treatment with shLRP6 and 48 hours of treatment with FAK mutants, Gelatin SDS-PAGE gels were prepared and confirmed by electrophoresis. Similar to the previous results, it was confirmed that the activity of MMP2, which was increased by LRP6 silencing, was also reduced by FAK mutations (Fig. 10A). Immunocytochemistry has been done to see phenomena in cells. Similarly, shLRP6 was treated for 72 hours, FAK mutants were treated for 48 hours, stained with antibodies, and visualized by laser scanning confocal microscopy. As a result, it was confirmed that the expression of FAK and MMP2 was increased by shLRP6 in cells as well, and there was no change in expression by the FAK mutant (Fig. 10B).

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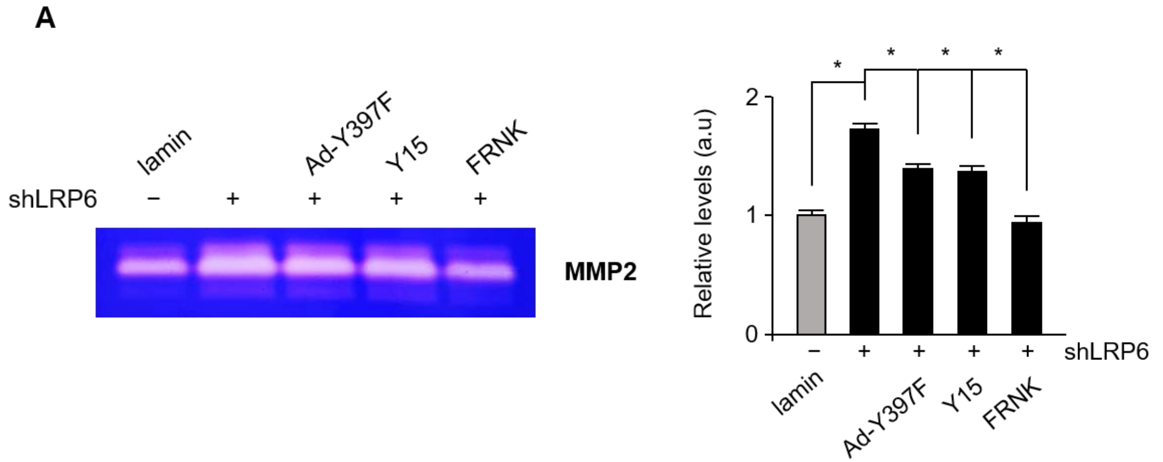

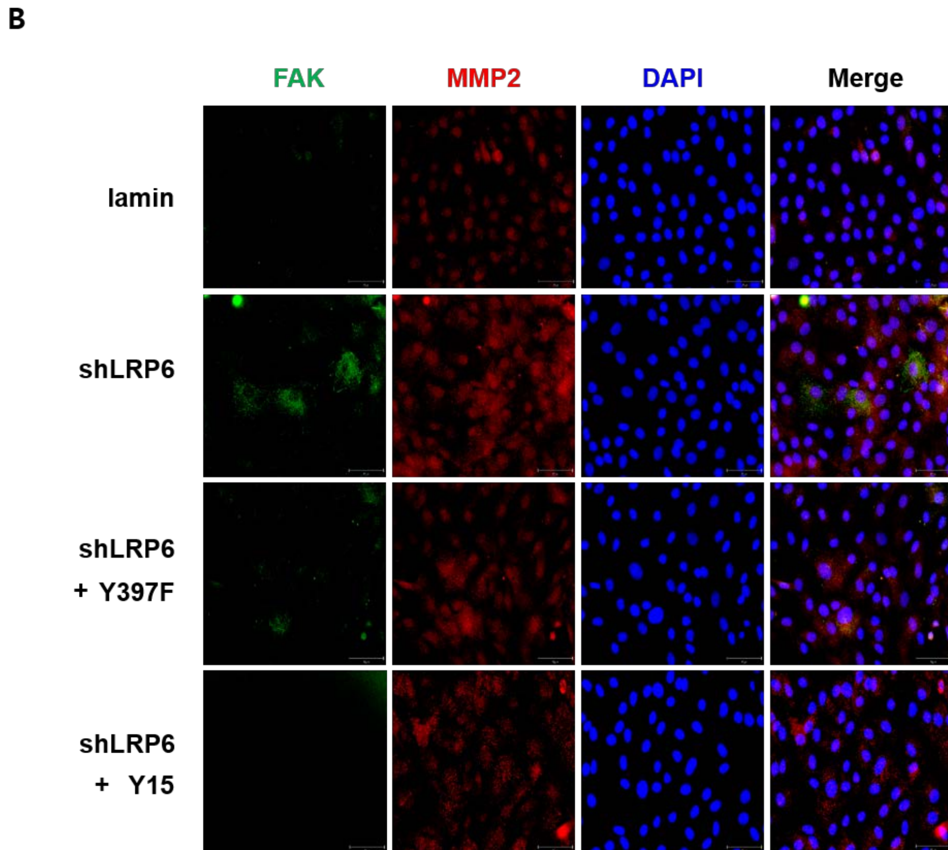

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Figure 10. Involvement of MMPs and FAK in shLRP6-induced migration of VSMCs

Effects of FAK mutants on MMP levels in shLRP6 treated VSMCs. (A) Gelatin zymography confirmed the level of MMP2. The gel was cultured for 24h or 36h to confirm. (B) Immunocytochemistry confirmed the expression levels of FAK and MMP2 in VSMC. Green stained FAK, red-stained MMP2, and blue stained nuclei. Values are expressed as mean ± S.D. ** P <0.01 vs control.

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3.8. Akt is potential mediator of shLRP6 regulation of MMPs and FAK expression

There are many different signaling pathways that regulate the activity of MMP transcription factors. Among these, we tried to confirm the expression levels of proteins related to MAP kinase (Mitogen-activated protein kinase), which is related to FAK. Western blot analysis of Akt, ERK, p38, and c-Jun attempted to identify the signaling pathways involved in the regulation of MMP2 by shLRP6. After treatment with shLRP6 concentration-dependent, expression was confirmed through the antibodies of Akt, ERK, p38, and c-Jun. We found an increase in Akt with the degree of shRLP6 while the other signals remained unchanged (Figure 11A). These results indicate the possibility that Akt may be involved in the regulation of MMP2 by LRP6 in VSMC cell migration.

Based on the previous results, the experiment was conducted by treating each Akt and ERK inhibitor after shLRP6 treatment to see if the Akt signal is really involved. Unlike ERK, it was confirmed that Akt was clearly increased by shLRP6, and showed that the function of the inhibitor was certain. As a result, it was confirmed that the MMP2 and MMP13 increased by shLRP6 was decreased again by the Akt inhibitor (Figure 12A). Based on these results, we determined that the change in the mobility of MMP2 by the regulation of LRP6 could be made by the involvement of the FAk signal and the Akt signal.

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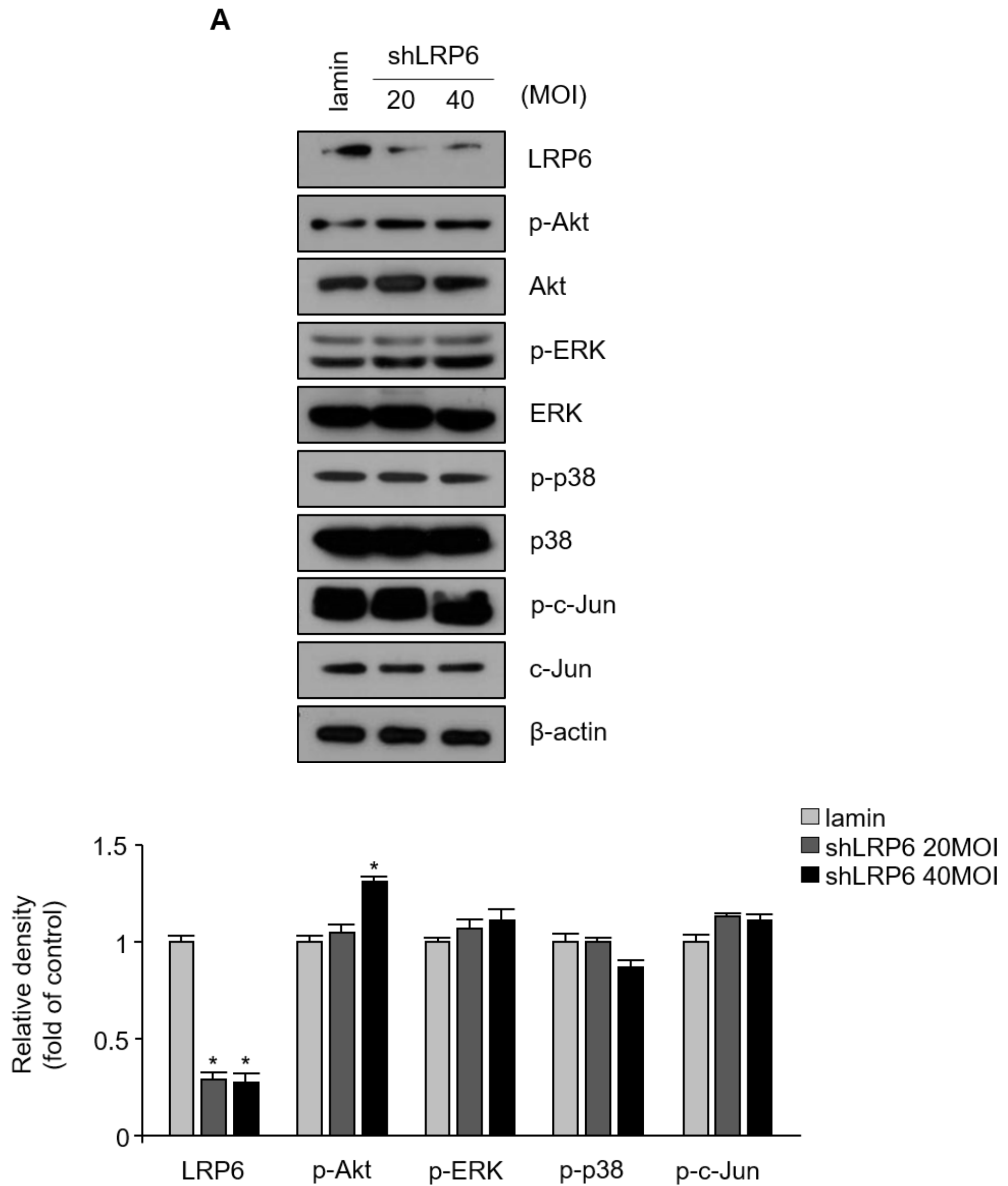

Figure 11. Effect of shLRP6 on phosphorylated Akt

(A) Western blot analysis confirmed the protein level of the MAP kinase-related protein when shLRP6 was treated. Values are expressed as mean ± S.D. ** P <0.01 vs control.

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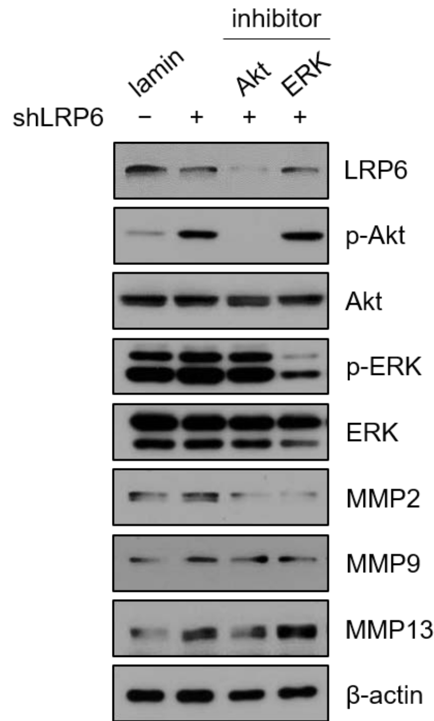

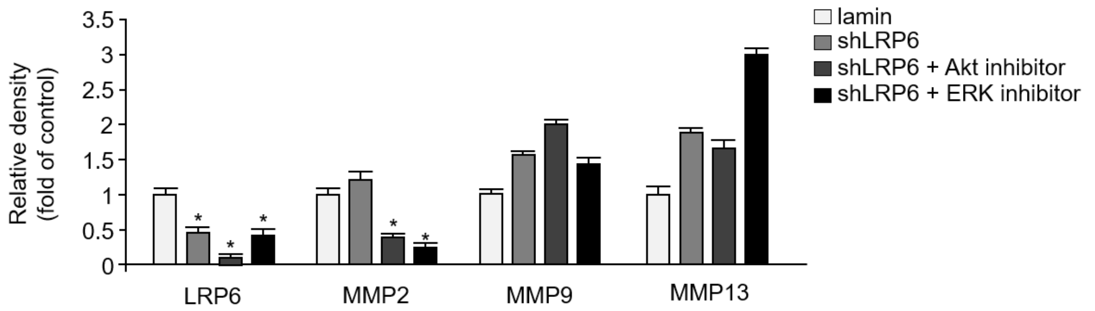

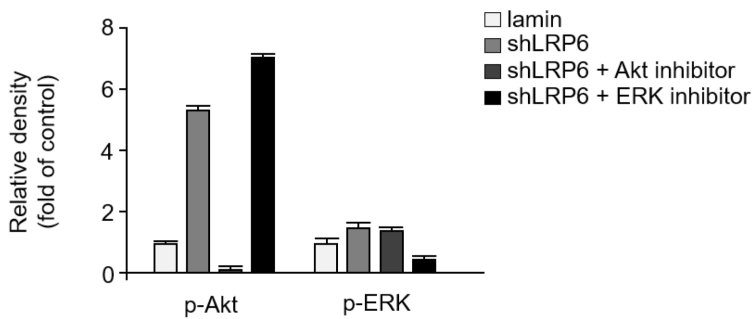

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Figure 12. Effect of shLRP6 on Akt phosphorylation by the inhibitor

(A) Western blot analysis was performed after a total of 72 h after treatment with shLRP6 in VSMC and treatment with each Akt (10μm) and ERK (10μm) inhibitor. Each signal and protein level of MMP were checked. Values are expressed as mean ± S.D. ** $P < 0.01$ vs control.

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3.9. Schematic diagram of LRP6 silencing induced VSMCs migration

A simple schematic was drawn based on the results of the research so far. In vascular smooth muscle cells, when LRP6 located in the membrane is inhibited, the movement of vascular smooth muscle cells increases. With that mechanism, it was confirmed that Y397, the phosphorylation site of FAK, increased, and Y925 decreased. Then, it was confirmed that Akt, a MAP kinase protein related to FAK, was also increased, and as a result, the expression and activity of MMP2 and 13 increased, and finally, it was concluded that migration increased.

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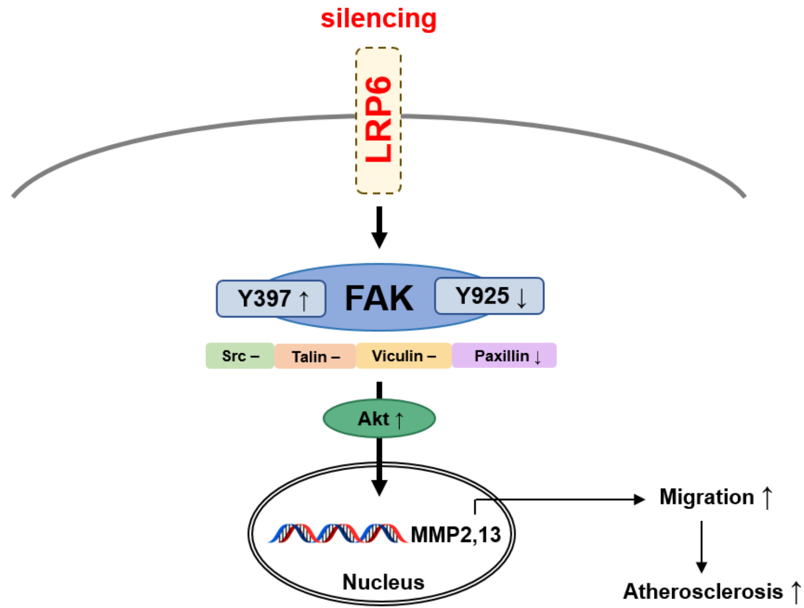

Figure 13. Schematic representation of suggested hypothesis

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4. Discussion

Atherosclerosis is one of the leading causes of high mortality worldwide, and it belongs to representative cardiovascular diseases such as cardiac hypertrophy, heart failure, and myocardial infarction. It is well-known that the initial progression of atherosclerosis, a primary cause of cardiovascular disease, begins with the migration of vascular smooth muscle cells. LRP6 (Low-density lipoprotein receptor-associated protein 6) belongs to the LDLR family and is known as a co-receptor that binds to the Wnt ligand and activates Wnt/β-catenin signaling [10] [39]. LRP6 is associated with the risk of atherosclerosis due to high blood pressure[40] and coronary artery disease (CAD) due to LDL-cholesterol [41]. Also, the LRP6 mutant is an important factor contributing to coronary artery disease (CAD) by impairing Wnt signaling and reducing endothelial cell function and proliferation [21] [42] [43]. Vascular smooth muscle LRP6 promotes atherosclerosis calcification by inhibiting noncanonical Wnt signals in diabetic LDLR knockout mice [44]. In vascular smooth muscle cells, overexpression of LRP6 increases the activity of T cell factor 4 (Tcf-4) and induces the proliferation of vascular smooth muscle cells [20]. Conversely, even when the activity of LRP6 is decreased in the LRP6 (R611C) mouse model, the proliferation of vascular smooth muscle cells is promoted by the platelet-derived growth factor (PDGF) [22]. As such, although the role of LRP6 is not yet clear and many studies are in progress, studies on the mechanism of inhibition of vascular smooth muscle cell migration by LRP6 regulation are not known. Therefore, this study investigated the effect of LRP6 silencing on the mobility of vascular smooth muscle cells causing atherosclerosis.

First, vascular smooth muscle cells were obtained from the thoracic aorta of rats, and LRP6 overexpression and inhibitory adenoviruses were produced to determine the role of LRP6, and it was confirmed that they were well expressed in a concentration-dependent manner through western blot, and then the determined concentration was used in the experiment. In 2D and 3D mobility analysis, there was no significant change in vascular smooth muscle cell mobility upon overexpression of

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LRP6, but it was confirmed that LRP6 inhibition significantly promoted the mobility of vascular smooth muscle cells. However, there was no significant change in proliferative properties through the BrdU assay. Based on this, the expression changes of MMP2, 9, and 13, which are indicators of cell migration, were confirmed through western blot and q-PCR analysis in the experimental group that inhibited the expression of LRP6. As a result, a significant increase was confirmed in MMP2 and 13. By additionally showing a significant increase in MMP2 through zymography and luciferase assay analysis, it was confirmed that the effect of increasing vascular smooth muscle cell migration by LRP6 inhibition was specific to MMP2. In addition, in this study, we tried to find out what role Focal Adhesion Kinase (FAK) plays in the movement of vascular smooth muscle cells increased by LRP6 inhibition. Focal Adhesion Kinase (FAK) is formed in a variety of biological processes such as cell migration, growth, differentiation, survival, and inflammatory reactions. It is well known that it plays a structural role in connecting cells to the extracellular matrix and as a starting point for signal transduction. As a result of confirming Y397 and Y925, which are the phosphorylation of FAK, through western blot, it was confirmed that Y397 significantly increased and Y925 decreased in contrast. Furthermore, additional studies were conducted using adenoviruses that inhibit the functions of Y397 and using the FAK inhibitor Y15 and focal adhesion kinase-related non-kinase (FRNK). In vascular smooth muscle cells that inhibited LRP6, mutants Y397 and Y15, FRNK were treated, and in 2D and 3D mobility analysis, it was confirmed that increased vascular smooth muscle cell mobility by inhibition of LRP6 decreased upon inhibition of FAK phosphorylation. there was. Based on this, the results of zymography, and immunofluorescence staining analysis showed that MMP2 activity, an important indicator of cell migration, was significantly reduced. Additionally, it was confirmed through western blot that Akt is the signaling system of MMP2.

In conclusion, this study confirmed the increase in vascular smooth muscle cell mobility by increasing MMP2, 13 through FAK and Akt signaling during LRP6 inhibition and it was confirmed that LRP6 could be a new target protein that can regulate the mobility of vascular smooth muscle cells.

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However, further studies are needed on FAK-related proteins and MAP kinase proteins by LRP6 silencing in VSMC. In addition, we will conduct an in vivo study to confirm the role of LRP6 in a rat model of atherosclerosis-induced balloon injury.

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