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Role of LRP6 in hypoxia-induced cardiomyocytes death

조선대학교 대학원

의 과 학 과

위 관 환

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저산소 유도된 심근세포 사멸에서 LRP6의 역할

2020년 2월 25일

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LRP6 (Low-density lipoprotein Receptor-related Protein 6)는 LDLR family 에 속하는 막수용체로써 Wnt 신호를 활성화 시키기 위한 co-receptor 로 잘 알려져 있다. Recombinant frizzled1 과 같이 Wnt 신호를 활성화 시키면 심근경색 후 심근손상이 줄어든다는 보고도 있지만, 오히려 심근세포 특이적 β-catenin 저해와 frizzled antagonist 에 의해 Wnt 신호가 억제되었을 경우 심근경색 후 심근비대 와 심장기능이 개선된다는 보고도 있다. 따라서, 심근경색에서 Wnt 신호의 기능은 아직 명확하게 규명되지 않아 논란이 되고 있는 부분이다. 더욱이 Wnt 의 Co-receptor 로 알려진 LRP6 에 대한 심근경색에서의 연구는 전혀 이루어져있지 않다. 그래서 본 연구에서는 저산소 조건에서 LRP6 가 어떤 역할을 하는지 알아보고자 하였다. 먼저 심근세포는 신생백서의 심장에서 획득하였고, LRP6 의 발현이 저산소 유도된 심근세포 사멸에 어떠한 영향을 미치는지 알아 보기 위해 LRP6 과발현 및 저해 아데노바이러스를 제작하였다. LRP6 과발현 및 저해 시킨 심근세포에 저산소를 유도시킨 후 MTT assay 를 통해 세포생존율을 확인한 결과 저산소를 유도 했을 때 대조군 세포는 20%정도 사멸이 일어났는데 LRP6 가 저해 됐을 경우

세포사멸이 40%까지 증가 되는 것을 확인하였다. 세포사멸 신호는 이른 세포사멸을 볼 수 있는 Annexin V 검출하여 확인 했을 때 shLRP6 는 대조군 인 lamin 과 비교하였을 때 apoptotic cell 이 더 많이 검출 되는 것을 확인 하였다. LPR6 과발현은 대조군 인 Ad-lacZ 와 비교하였을 때 유의한 차이는 나타나지 않았다. 늦은 세포사멸을 확인 할 수 있는 caspase-3 activity 는 LRP6 가 저해 되었을 때 증가 되었고 반면에 LRP6 과발현이 되었을 때 감소되었다. 또한, western blot 을 통해서 절단된 caspase-3 발현을 확인했을 때도 앞선 결과와 동일한 패턴을 보였다. 그리고 세포사멸 활성 단백질인 Bax 와 세포사멸 억제 단백질인 Bcl-2 발현 량을 확인하였을 때도 LRP6 의 조절이 저산소에서 세포사멸을 조절한다는 것을 확인 할 수 있었다. 또한 세포사멸과 세포분열 조절에 중요한 역할을 하는 세포사멸 단백질 억제제로 알려진 survivin 또한 LRP6 과발현에선 증가 되었고 LRP6 저해 에서 감소하는 것을 확인했다. 또한 LRP6 의 어떤 부분이 저산소 유도된 심근세포사멸을 조절하는지 알아보기 위해 세 개의 돌연변이를 제작하였다. 돌연변이는 막 수용체 기준으로 N-terminal 이 삭제된 ΔN, C-terminal 이 삭제된 ΔC, 마지막으로 PPPSP motif 의 인산화 site 를 돌연변이 시킨 construct 를 각각 제작하였고, 만들어진 돌연변이들이 제대로 만들어졌는지 전기영동과 염기서열 분석을 통해 확인하였고 아데노바이러스를 제작해서 이 돌연변이들이 잘 발현하는지 확인 하기 위해 농도의존적 처리하여 western blot 을 통해 확인하였다. 본 연구의 결과에서 LRP6 가 저산소 유도된 심근세포의 사멸을 조절할 수 있는 표적 단백질이 될 수 있음을 확인하였다.

1. Introduction

Myocardial infarction (MI), which is caused by the occlusion of a coronary artery leading to irreversible myocardial cell death [1]. A common cause of a sudden blockage of coronary arteries is the formation of blood clots (thrombosis). A thrombus typically forms in the coronary artery that has already narrowed due to atherosclerosis, a condition in which fat deposits (plaques) have accumulated along the inner wall of the blood vessel [2].

Lipoprotein receptor-related protein 6 (LRP6) is a transmembrane co-receptor essential for standard Wnt signaling (Figure 1), binds to Wnt ligands, and cooperates with Frizzled receptors to signal through β-catenin stabilization. Stabilized β-catenin moves to the nucleus and activates Wnt target genes to regulate multiple functions including embryonic development [3], body fat and [4], and bone metabolism [5]. Moreover, the Wnt/β-catenin pathway has been determined to play a role in osteocyte sensing and transducing mechanical stimuli to other bone cells [6]. Also LRP6, LRP5 and Frizzled protein together form a ternary complex that acts as a receptor for the Wnt/β-catenin pathway.

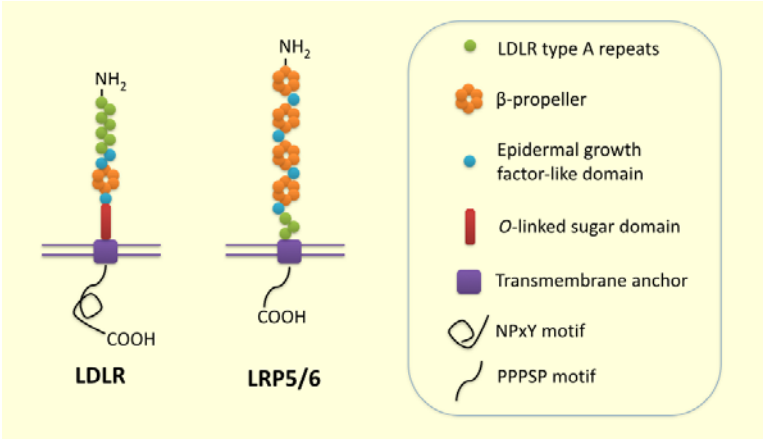


Figure 1. The structure of LRP6 [7].

In the absence of Wnt ligands, cytoplasmic β-catenin protein is constantly degraded by destruction complex, which is composed of APC, Axin, CK-1α, and GSK-3β. While in the presence of Wnt ligands, Wnt/Fzd/LRP-5/6 complex forms to inhibit the degradation of β-catenin. As a consequence, β-catenin accumulates and translocates to the nucleus to trigger the Wnt target genes via TCF and LEF [8]. Wnts protein, a family of 19 secreted lipid-modified cysteine-rich proteins, behaves as locally acting short-range ligands to activate receptor-mediated signaling pathway, whereas β-catenin, a membrane bounded protein, serves as a central mediator of transcriptional coactivator in canonical Wnt/β-catenin signaling [9]. Wnt/β-catenin signaling exhibits a pivotal role in the development of various organs systems, such as spinal cord, heart, mammary glands, skin, gut, lungs, brain, bones, eyes, cartilage, pancreas, liver, teeth, kidney, hematopoietic, and reproductive system [10], [11], regeneration of skin and hair, and repair of lung and liver after injury [12], [13], [14], [15]. Furthermore, Wnt/β-catenin signaling also has a significant role in proliferation, cell fate, migration, polarity, morphogenesis, apoptosis, genetic stability and instability [16], [17], [18]. While aberrant Wnt/β-catenin signaling leads to metabolic disorders, inflammatory ailments and pathological defects such as cancer, hypertension, and fibrosis [19], [20].

Wnt receptors and co-receptors, including Frizzled (seven-pass transmembrane GPCRs; so far, 10 human Fzd genes have been discovered) [21], and low-density lipoprotein receptor-related proteins-5 and -6 [22], receptor-like tyrosine kinase (RYK) [23], receptor tyrosine kinase-like orphan receptor (ROR) [24], and protein tyrosine kinase-7 (PTK-7) [25] are significantly concerned with Wnt/β-catenin signaling. also the Dkk-1 and Dkk-3 are the most studied members of this family; these can inhibit the Wnt signaling by binding to LRP-5/6 and then degrade the coreceptor; thereby considered as potential targets in diseases with an aberrant Wnt signaling activity [26], [27].

The canonical or β-catenin/TCF-dependent Wnt signaling is strongly dependent on β-catenin for activation. Wnt ligands and other signaling cascades, such as hepatocyte growth factors (HGF), prostaglandin and E-cadherin, act as

extracellular regulators of β-catenin [28], [29]. Briefly, under steady state condition, the cytoplasmic β-catenin pool is firmly regulated via phosphorylation by destruction complex, and destruction complex is composed of tumor suppressor adenomatous polyposis coli (APC), axis inhibition protein (Axin; scaffold protein), casein kinase-1α (CK-1α) and glycogen synthase kinase-3β (GSK-3β) [30] in the absence of Wnt ligands, β-catenin undergoes phosphorylation for the ubiquitylation and proteasomal degradation, firmly controlled by GSK-3β, thus maintaining the cytoplasmic β-catenin pool [31]. Rescuing β-catenin from phosphorylated destruction complex by Wnt ligands constitutes an essential step in β-catenin stabilization and nuclear translocation. in the presence of Wnt ligands, formed Wnt/Fzd/LRP-5/6 complex induces the membrane translocation of Axin that binds to the cytoplasmic tail of LRP-5/6 via phosphorylation either by GSK-3β or by CK-1α, and then regulates the downstream signaling via (Dvl) protein activation through phosphorylation [32]. And non-canonical Wnt signaling, gene expression is entirely independent of β-catenin, thereby also known as β-catenin-independent signaling; these signaling pathways include planar cell polarity and Ca+2-dependent pathways [33].

Furthermore LRP6, a receptor of Wnt pathway, is over expressed and promotes the β-catenin-dependent Wnt signaling in cancer [34] and TNBC cells [35]. The suppression of LRP6 expression inhibition Wnt/β-catenin signaling and decrease the levels of survivin in breast and prostate cancer cells [36]. LRP6 has been reported to play a role in bone absorption and formation, but LRP5 appears to affect bone formation but not bone absorption [37]. In cardiovascular systems, LRP6 dysfunction contributes to the development of coronary artery disease due to defective Wnt signal activation [38] LRP6 has been reported to play a role as a scaffold protein to regulate cardiac gap junction assembly [39]. However, the role of LPR6 in cardiomyocytes is not known. The study was conducted to find the role of LRP6 in cardiomyocytes. The experiment also explored the role of LRP6 knockdown in cardiomyocytes.

2. Materials and Methods

2.1. Preparation of adenovirus

The LRP6 overexpression adenovirus was generated using a ViraPower™ adenovirus expression system (Invitrogen) and named as LRP6Ad. LRP6 is a VSVG-tagged LRP6 in the pcDNA construct was kindly provided by cell signaling which was cloned in the pENTR1A dual selection vector. An adenoviral vector expressing lacZ-β-galactosidase was used as the control and was named LacZ Ad. The constructs were then used in a LR recombination reaction with the pAd/CMV/V5/DEST gateway vector to generate an adenoviral expression clone. Then, the constructed adenoviral vector was transfected into 293A cells using Lipofectamine 3000 reagent (Invitrogen life Technologies) after digesting with the Pac I restriction enzyme. Adenoviral particles were titered at a multiplicity of infection (MOI) using the Adeno-X™ qPCR titration kit (Takara, Clontech). Cardiomyocytes were infected with adenoviruses at MOI of 5 and 10 for 48 h.

The LRP6 silencing adenovirus (shLRP6) was generated using a BLOCK-iT™ adenoviral RNAi expression system (Invitrogen) expressing a rat LRP6-targeting shRNA. The target nucleotide sequence of the oligo duplexes was as follows: 5'-GGU UGU UCC CAU UUG UGU U-3'. Double-stranded oligonucleotides cloned in the BLOCK-iT™ U6 entry vector were used in a LR recombination reaction with pAd/BLOCK-iT™-DEST vector to generate an adenoviral expression clone. The pAd-GW/U6-lamin^{shRNA} adenoviral vector (lamin) was used as a control. Cardiomyocytes were infected with adenoviruses at MOI of 10, 20, and 30 for 72 h.

2.2. Cell viability

For cell viability assay, experimental cells were evaluated by both the MTT and Trypan blue assay. MTT assay was measured by CellTiter 96 Aqueous One Solution Cell Proliferation Assay (Promega) according to the manufacturer's instructions. The absorbance was measured at 490 nm using an ELISA reader

(TECAN, infinite M200 PRO). For Trypan blue assay, cells were detached by trypsinization and stained with 0.4% Trypan blue dye. The number of viable cells was counted under microscope using a hemacytometer counter. The viability of the control was regarded as 100%.

2.3. Apoptosis assay

Hypoxia-induced cardiomyocytes apoptosis rate was determined by using the Annexin V-Cy3 apoptosis detection kit (Sigma-Aldrich) or colorimetric Caspase 3 Assay kit, (Sigma-Aldrich) according to the manufacturer's instructions, respectively. Annexin V-Cy3 labeled cells were then visualized by a laser scanning confocal microscope (Fluoview FV1000, Olympus). For the detection of Caspase-3 activity, cell lysate was combined with an equal amount of substrate reaction buffer with a caspase-3 colorimetric substrate (Ac-DEVD-pNA), and then absorbance was measured at 405 nm using an ELISA reader (TECAN, infinite M200 PRO).

2.4. Western blotting

Cardiomyocytes were lysed in lysis buffer (phosphate-buffered saline containing 1% Triton X-100, protease inhibitor cocktail, and 1 mM phenylmethylsulfonyl fluoride (PMSF)). Protein concentrations were determined using a Bradford protein assay kit. Equal quantities of protein were separated on 10% sodium dodecyl sulfate-polyacrylamide gel via electrophoresis and transferred to a polyvinylidene difluoride (PVDF, Bio-Rad) membrane. The membranes were incubated with anti-LRP6 primary antibodies then, the membranes were incubated with a 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.

2.5 Primary culture of rat neonatal cardiomyocytes

Neonatal rat ventricular cardiomyocytes (NRVCMs) were isolated from the hearts of 2-4 day-old Sprague-Dawley rats. Briefly, isolated hearts were washed with Dulbecco's phosphate-buffered saline solution (pH 7.4, WELGENE) and minced to approximately 0.5 mm^3 sized pieces. The heart tissues were then treated with 5 mL collagenase II (1.4 mg/mL, 270 units/mg, Gibco BRL) solution for 5 min, the supernatant was removed, and then washed with alpha modification minimum essential medium (α-MEM) containing 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin solution (Capricorn Scientific GmbH). The pellet was resuspended in collagenase II solution and incubated in a 37°C humidified chamber containing 5% CO_2 for 7 min. This procedure was repeated until the tissue was completely digested. The resulting cell pellet was resuspended in α-MEM and the cells were allowed to attach to a 100-mm culture dish for 30 min at 37°C. Non-adherent NRVCMs were cultured for experiments with α-MEM containing 10% FBS, 1% penicillin/streptomycin solution, and 0.1 mM 5-bromo-20-deoxyuridine (BrdU, Sigma-Aldrich) to eliminate fibroblast expansion. After 24 h of incubation, the cell culture medium was changed to α-MEM (containing 10% FBS, 1% penicillin/streptomycin solution)

2.6 Hypoxia treatment

To induce hypoxia, cardiomyocytes were carefully washed 2 times with hypoxic media (serum free and replacing with N_2 gas for 30min) and subjected to hypoxia to mimic the *in vivo* condition of myocardial ischemia. The cells were placed in an incubator at 37˚C. N_2 (95%) and CO_2 (5%) were flushed into the incubator to bring the oxygen content down to 1% monitored by an oxygen probe (Vision Scientific). Normoxic cardiomyocytes were maintained at 37°C in a separate incubator with a normoxic atmosphere.

2.7 Statistical analysis

All quantified data from at least triplicate samples were analyzed with SPSS 13.0 software. Data are expressed as mean ± SD. Statistical comparisons

between two groups were performed using Student's t-test. Statistical comparisons among multiple groups were performed using analysis of variance (ANOVA). A two-tailed $P < 0.05$ was considered statistically significant

3. Results

3.1. Hypoxia decreases LRP6 expression in cardiomyocytes

This study investigated the expression of LRP6 in hypoxia-induced cardiomyocytes. Isolated neonatal rat cardiomyocytes were exposed to hypoxic (O_2 3%) or normoxic condition for 8h (O_2 20%). As shown in Figure 2A and B, the expression of LRP6 was downregulated by hypoxia induction.

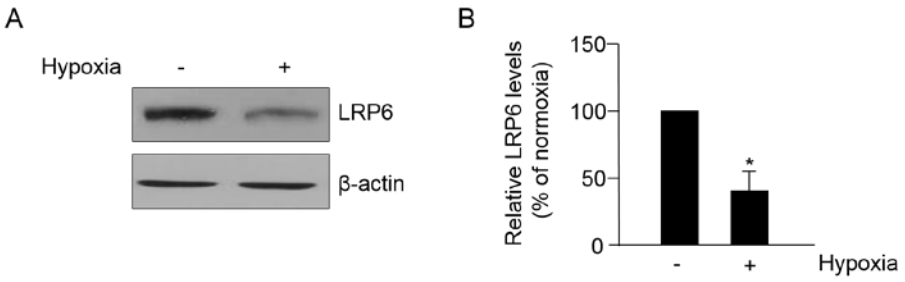


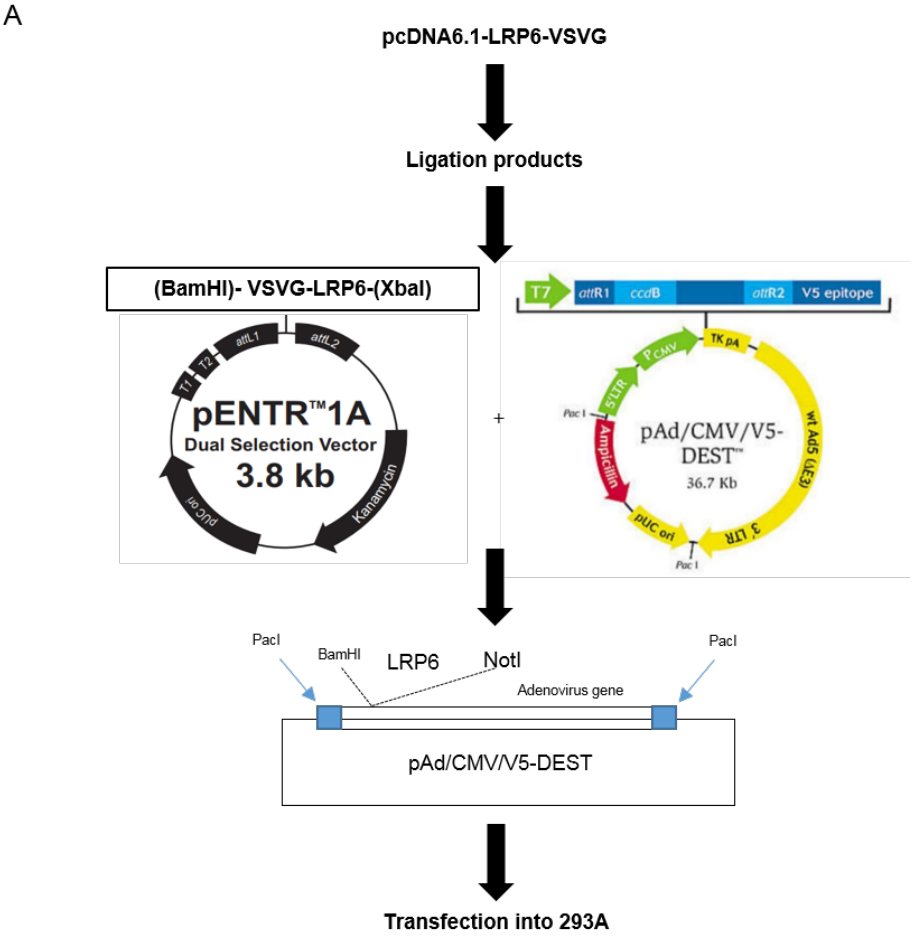
Figure 2. Alteration of LRP6 expression in hypoxic cardiomyocytes

(A) Representative expression of LRP6 exposed to hypoxia for 8h (n=4). Cardiomyocytes were exposed to normoxic or hypoxic condition, and then cells were harvested for western blotting. (B).Relative LRP6 expression level was normalized to β-actin. Values were represented as mean ± SD. *P<0.05

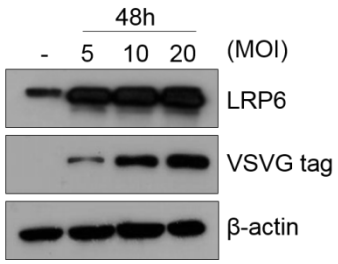
3.2. Adenovirus expressing LRP6 or shLRP6

To determine whether altered expression of LRP6 affected the hypoxic response in cardiomyocyte, this study constructed adenoviral vector to overexpress the VSVG tagged-LRP6 (Ad-LRP6) cDNA or to express an LRP6 targeting shRNA (shLRP6) for silencing endogenous expression (Figure 3A). Next experimentally infected Ad-LRP6 or shLRP6, respectively in cardiomyocytes for 48h or 72h. These results indicated that both overexpression of LRP6 and silencing of LRP6 were successfully constructed in concentration-dependent manner (Figure 3B and C).

A



B



C

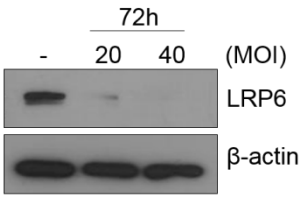


Figure 3. Construction of LRP6 or shLRP6 adenoviral vector

(A) A shematic illustration of generating adenoviruses. (B)(C) Primary neonatal rat cardiomyocytes were infected with adenoviruses expressing full-length LRP6 or shRNA targeting LRP6 at the indicated MOI values. (B) Effect of VSVG-tagged LRP6 overexpression. (C) Effect of LRP6 silencing in cardiomyocytes was verified by western blotting. The results represent the findings from three independent experiments. Relative LRP6 expression level was normalized to β-actin. Values were represented as mean ± SD.

3.3. Silencing of LRP6 promotes cardiomyocytes death in hypoxia

To investigate the effect of LRP6 in hypoxic cardiomyocytes, we measured cell viability using a MTT assay. Isolated neonatal rat cardiomyocytes were infected with Ad-lacZ, Ad-LRP6, lamin or shLRP6, respectively then exposed to hypoxia for 4h or 8h. LacZ-β-galactosidase (Ad-lacZ) and lamin were used as an infection control.

The MTT assay is a colorimetric assay that measures mitochondrial succinate dehydrogenase reduction. MTT enters the cell, enters the mitochondria, where it is reduced to an insoluble dark purple formazan product. The cells are then solubilized with an organic solvent and the released solubilized formazan reagent is measured spectrophotometrically. The level of activity is an indicator of cell viability because MTT reduction occurs only in metabolically active cells [44].

As shown in Figure 4A and B, these results confirmed that the hypoxic induction condition of this experiment was successfully established by confirming the decreased cell viability with the induction of hypoxia in time-dependent manner in cardiomyocytes. Together, the results showed that silencing of LRP6 promotes further decrease of cell viability by approximately 40% compared to lamin control cells under hypoxic condition (Figure 4B). However, overexpression of LRP6 had no significant difference on cell viability compared with Ad-lacZ control cells (Figure 4A).

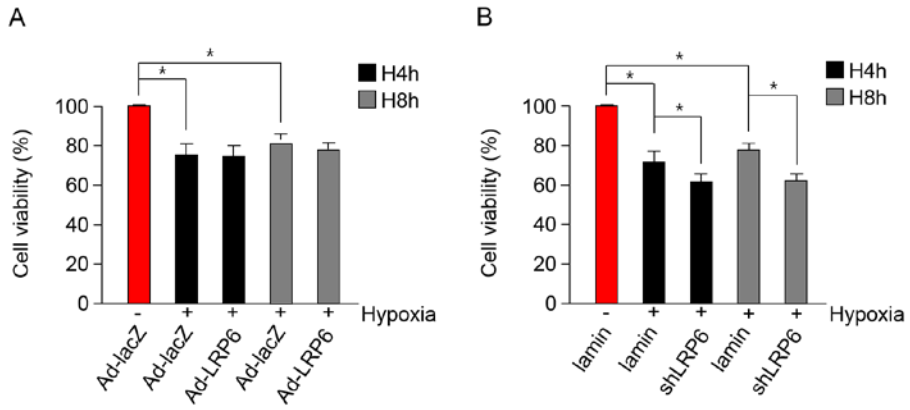


Figure 4. Effect of LRP6 on cell viability in hypoxia

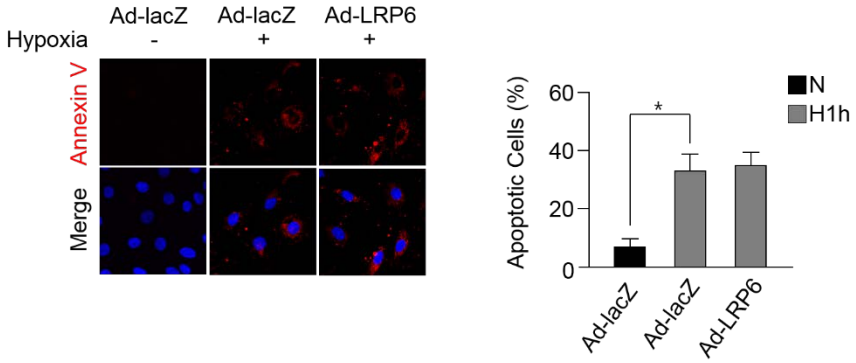
(A) Cardiomyocytes were infected with Ad-lacZ or Ad-LRP6, respectively, then exposed to normoxia (indicated by "N") or hypoxia (indicated by "H") (O_2 1.2%) for the indicated times. The cell viability was determined by MTT assay (n=4), Cell viability was normalized with normoxic Ad-lacZ control. (B) Cardiomyocytes were infected with lamin or shLRP6, respectively, then exposed to normoxia or hypoxia for the indicated times. Cell viability was normalized with normoxic lamin control. Values were represented as mean ± SD. *P<0.05

3.4. Silencing of LRP6 activates caspase-3

Based on above results, next study investigated whether cardiomyocytes death by LRP6 regulation is via apoptosis pathway. Annexin V was performed to investigate whether cardiomyocytes induce cell death through apoptoic signal when hypoxia-induced cardiac cells were infected with shLRP6. As a result, the cardiomyocytes were infected with LRP6, induced hypoxia, and Annexin V showed a slight early apoptotic death compared to the control group. However, when infected with cardiomyocytes, shLRP6 increased apoptiotic death more than the control group (Figure 5A and B).

In the next study, hypoxia-caspase to investigate what pathway induces cell death via LAP6 overexpressed or silenced in induced cardiomyocytes -3 activities were performed. As a result, when LRP6 was silenced in cardiomyocytes, caspase-3 activity in which hypoxia was induced increased and reverse LRP6 was overexpressed, capase-3 activity decreased (Figure 5C and D). Therefore, it was confirmed that apoptosis was induced through caspase-3 signaling. Moreover, cardiomyocytes were infected with LRP6 overexpression and silencing to induce hypoxia, and Western blotting was performed to confirm capsase-3 expression. As a result, the same pattern as in the above experiment was obtained (Figure 5E).

A



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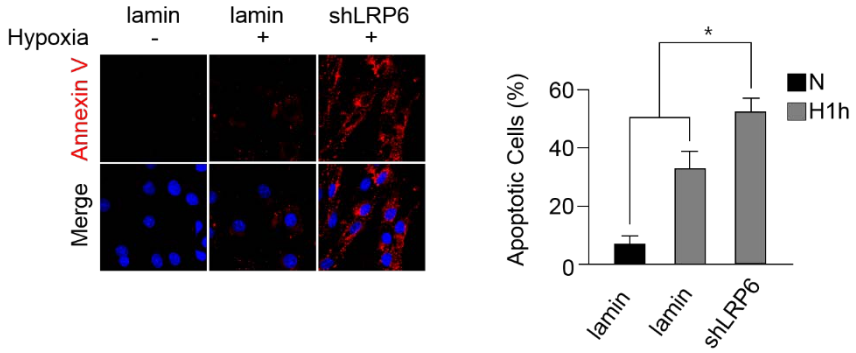


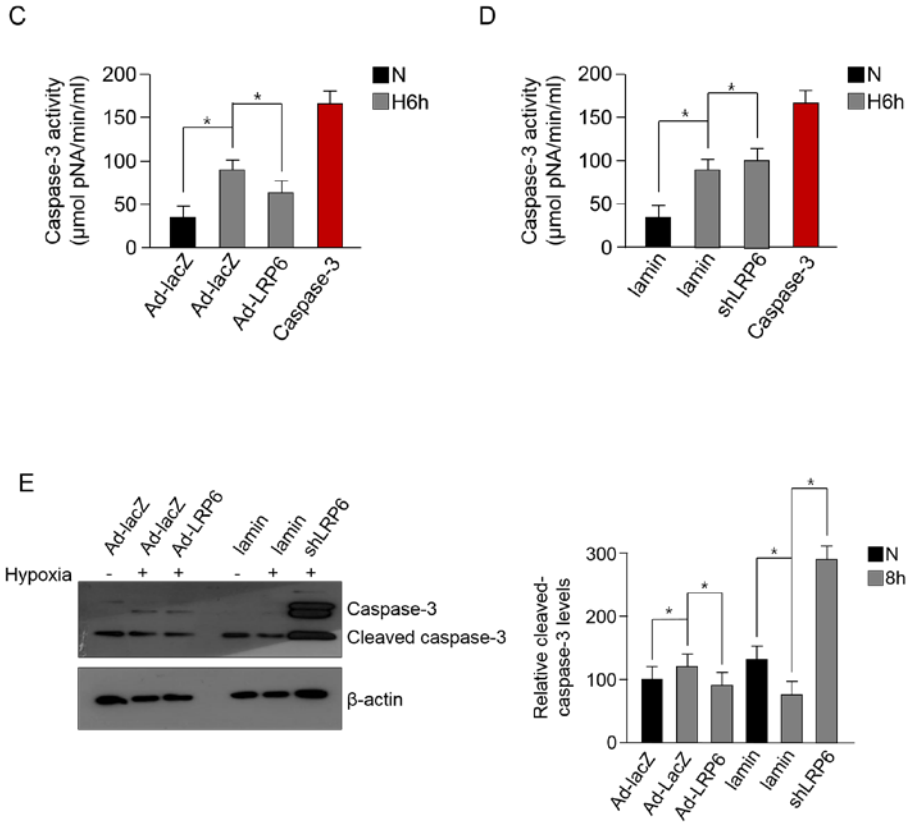


Figure 5. Effect of LRP6 on caspase-3 in hypoxic cardiomyocytes

Cardiomyocytes were infected with Ad-lacZ, Ad-LRP6, lamin and shLRP6, respectively, then exposed to normoxia or hypoxia for the indicated times. (A)(B) Representative fluorescence image of apoptosis staining with Annexin V-Cy3.18 (red) and DAPI (blue). The apoptosis rate was quantified by SIBIA software. (C)(D) Measurement of caspase-3 activation (n=4). (E) Expression of caspase-3 and cleaved caspase-3 were verified by western blotting. Relative LRP6 expression level was normalized to β-actin. Values were represented as mean ± SD. *P<0.05

3.5. Silencing of LRP6 regulates Bcl-2 and survivin-regulated apoptotic pathway

Cell death is regulated by apoptosis mechanisms, which are largely divided into extracellular and intracellular signals. The mechanism of apoptosis by intracellular signaling is relatively well known by p53. p53, a representative tumor suppressor, is located on chromosome 17 and when DNA is damaged, it promotes the transcription of $p21^{WAF1}$, blocks cell cycle progression and DNA synthesis, reduces the expression of Bcl-2, and increases the expression of Bax. To induce apoptosis. Bcl-2 and Bax can bind to each other as homodimers or heterodimers. The homopolymers of Bcl-2 act in the direction of preventing apoptosis, while the homopolymers of Bax act in the direction of inducing apoptosis, respectively. Therefore, infected Ad-LRP6 and Ad-shLRP6 to cardiomyocytes and induced hypoxia, followed by western blotting to confirm the expression levels of Bcl-2 and Bax. Likewise, when Ad-shLRP6 was infected, the expression of Bax increased and Bcl2 decreased in reverse. Thus, the study confirmed that apoptosis is a mechanism of apoptosis when Ad-shLRP6 is infected (Figure 6A and B).

In the next study, a western blot was performed to determine the expression level of survivin, known as an inhibitor of apoptosis protein (IAP), which plays an important role in the regulation of apoptosis and cell division [45], [46]. As a result, the expression level of survivin was increased when infecting cardiomyocytes with Ad-LRP6 and inducing hypoxia. However, when shLRP6 was infected, the expression level decreased sharply (Figure 7A and B).

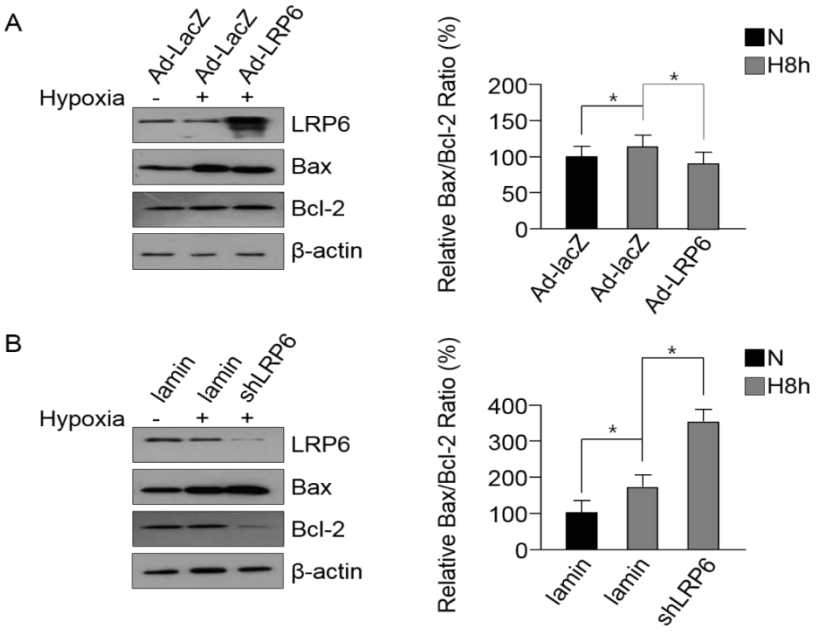


Figure 6. Effect of LRP6 on Bax/Bcl-2 ratio in hypoxic cardiomyocytes

(A)(B) Expression of Bax and Bcl-2 in Ad-LRP6 and silencing LRP6 infected cardiomyocytes as determined by western blot analysis. The experiments were performed in triplicate. Bax expression increased while Bcl-2 expression decreased after hypoxia-induced cardiomyocytes. Values were represented as mean ± SD. *P<0.05

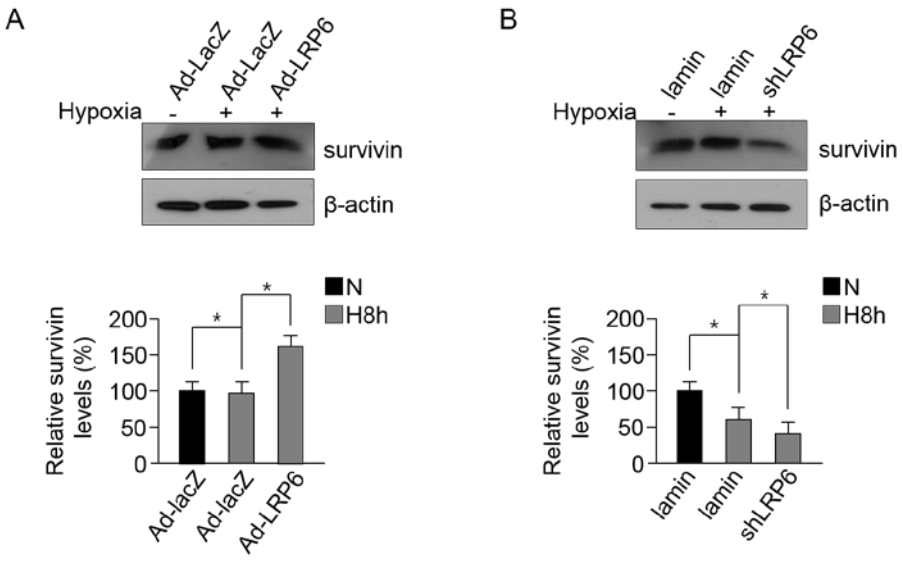


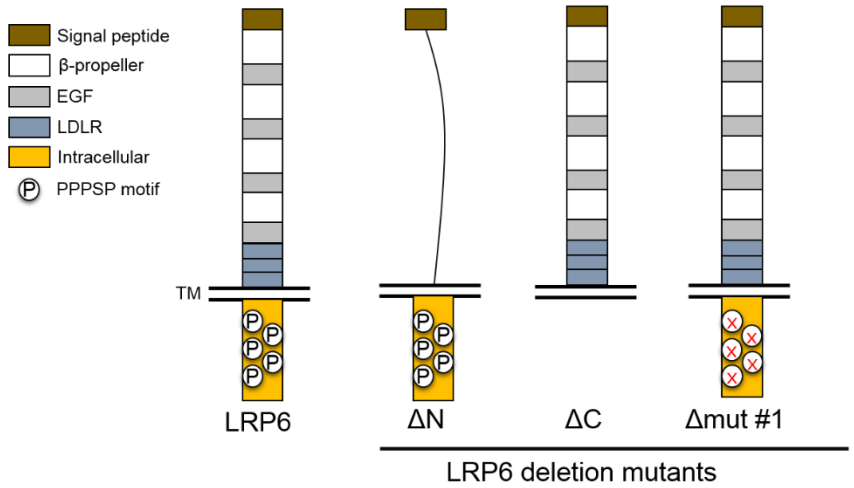
Figure 7. Effect of LRP6 on survivin expression in hypoxic cardiomyocytes

(A) Expression of survivin in Ad-LRP6 and silencing LRP6 infected cardiomyocytes as determined by western blot analysis (n=3). Values were represented as mean ± SD. *P<0.05

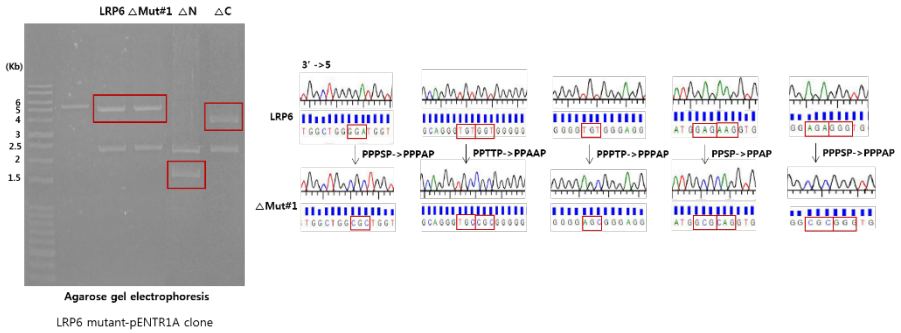
3.6. Construction of LRP6 mutants

In this study, three mutants were constructed to determine which domains of LRP6 regulate hypoxia-induced cardiomyocyte death. Mutant produced ΔN, ΔC, and Δmut # 1 as shown in (Figure 8A). ΔN deletes the upper N-terminal of the transmembrane and ΔC deletes the C-terminal, the lower part of the transmembrane. Finally, ΔMut # 1 mutated the PPPSP phosphorylation site. After that, (Figure 8B) each mutant was properly prepared by electrophoresis and sequencing, and then adenovirus was prepared and Western blotting was carried out by infecting the mutants in a concentration-dependent manner (Figure 8C, D and E). As a result, it was confirmed that each mutant was well expressed.

A



B



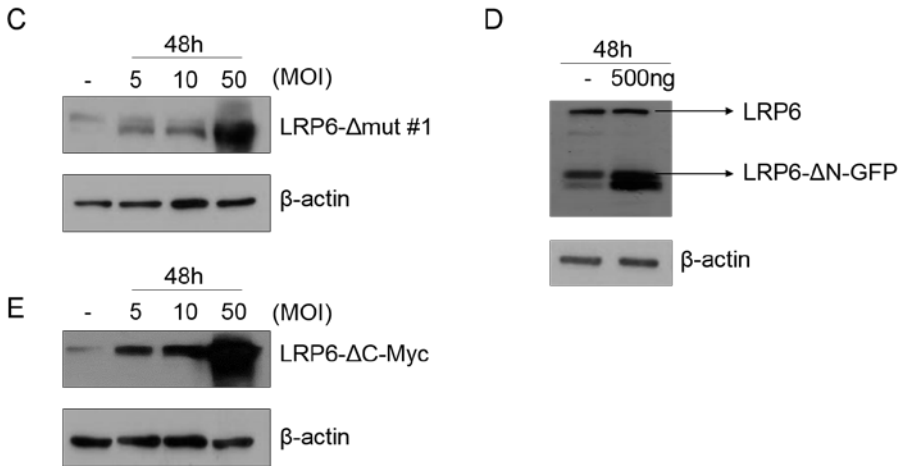


Figure 8. Generation of adenovirus expressing LRP6 mutants

(A) Schematic diagram of LRP6 mutations. (B) Electrophoretic patterns of LRP6 mutants and their sequences. (C-E) Western blot for LRP6 mutants in cardiomyocytes.

4. Discussion

LRP6, a member of the LDL receptor-related family, is a co-receptor of the canonical Wnt signaling pathway and is involved in various developmental processes in various systems by stabilizing β-catenin. [3] Genetic variation in the LRP6 gene is associated with the risk of hyperlipidemia [48], metabolic syndrome [38], and atherosclerosis [49] in the general population. LRP6 polymorphism has been reported to be associated with an increased risk of myocardial infarction [50] or non-small cell lung cancer [51]. Impaired LRP6 function in the cardiovascular system alters the vascular smooth muscle cell phenotype [52], disrupts endothelial cell function [53], and leads to the development of obstructive coronary artery disease (CAD) [54]. Vascular smooth muscle LRP6 promotes calcification of atherosclerosis in diabetic LDLR knockout mice [55]. However, the function of cardiac LRP6 has not been well studied. Several studies have shown that cardiomyocytes die when LRP6 is knocked down in the absence of glucose. Alternatively, this experiment will investigate the role of LRP6 in cardiomyocyte death in hypoxia.

The results showed that LRP6 expression decreased in hypoxia-induced cardiomyocytes. It was also confirmed by MTT analysis that hypoxia decreased cell viability induced from muscle cells of LRP-silent heart. However, the cell viability of cardiomyocytes overexpressing LRP6 did not change significantly. Therefore, when mitochondrial activity was measured by MTT analysis, the cells were not completely dead and the enzyme activity was maintained, so I thought there was no big difference in the number. It was confirmed that cells overexpressing LRP6 were protected when the cell apoptosis signal was confirmed.

So the next experiment confirmed the cell apoptosis signal. First, when hypoxia was induced in LRP6-overexpressing cardiomyocytes by annexin V staining that could confirm early apoptosis, it was not significantly significant

compared to lacZ. However, when LRP6 was silenced, more apoptotic cells could be seen than lamin.

When caspase-3 activity was confirmed, silencing of LRP6 showed high activity, and overexpression of LRP6 decreased. It was confirmed by western blotting. We confirmed by Western blotting that Bax and Bcl-2, which can validate or suppress these apoptosis, showed similar effects, and survivn expression also appeared. Therefore, in this study, in order to confirm that any domain of LRP6 regulates cell death, each deletion mutants can be produced and used for experiments. After that, it was finally confirmed that the expression increased in a concentration-dependent manner through western blotting.

This study confirmed the role of LRP6 in hypoxia-induced cardiomyocyte death. Future studies will clarify and investigate the signaling pathways regulated by LRP6, identify the resulting mutant and myocardial cell death regulatory domains, and use the in vivo model of myocardial infarction in vivo to examine the effects of regulating LRP6. Will experiment in *in vivo*.

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