Gene Therapy of Radiation Fibrosis Using Adenovirus Expressing Decoy Wnt Receptor (sLRP6E1E2)

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Gene Therapy of Radiation Fibrosis Using Adenovirus Expressing Decoy Wnt Receptor (sLRP6E1E2)

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Special thanks to my wife, inspiring me in all aspect and my beloved parents who devoted their life for our family. And I really want to share the delight with my lovely daughter Chae Won and adorable son Chae Joon. I also would like to thank my parents-in-law for unwavering support.

I would like to dedicate my dissertation to all the people in the above. I expect this small achievement will be the base of great advances.

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ABSTRACT

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(Directed by Professor Dae Hyun Lew)

Progressive fibrosis of the dermal tissues is a challenging complication of radiotherapy, since mechanism of action is not fully understood and there are only few available treatments. The canonical wnt/β-catenin signaling pathway plays an important role in fibrotic reaction as well as epithelial-to-mesenchymal transition. We verified whether that inhibition of wnt/β-catenin signaling pathway with sLRP6E1E2 that is binding to extracellular wnt ligands would ameliorate radiation-induced fibrosis both *in vitro* and *in vivo*. Radiation with a single dose of 2Gy not only facilitated fibrotic reaction in cultured human dermal fibroblasts via activated wnt/β-catenin pathway, but is also responsible for epithelial to mesenchymal transition of cultured keratinocytes, developing collagen-producing mesenchymal cells. sLRP6E1E2-expressing adenovirus treatment exerted anti-fibrotic actions in irradiated cultured dermal fibroblasts and keratinocytes. In a mouse model, a single fraction

of 15Gy was delivered to the dorsal skin of each mouse. Thirty mice were randomized into three groups: PBS, control adenovirus (dE1-k35) and decoy wnt receptor-expressing adenovirus (dE1-k35/sLRP6E1E2). The mice were observed for 16 weeks, and there was no significantly different gross change observed in comparison with controls. In semi-quantitative analysis with Masson's trichrome staining, excessive collagen deposition was suppressed by sLRP6E1E2-expressing adenovirus treatment. These results support that modulation of wnt/β-catenin pathway has the potential to decrease the severity of radiation-induced dermal fibrosis.

Key words: radiation, fibrosis, fibroblast, adenovirus, gene therapy

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I. INTRODUCTION

Radiotherapy is certainly an effective treatment to achieve control of solid tumors such as head and neck cancer, breast cancer and soft tissue sarcoma. But collateral injury to the surrounding healthy tissues may result in toxicity and frequently limits the therapeutic dose of radiation that can be delivered.[1] Since skin is usually the first site of entry of radiation beam, variable degrees of skin reactions can occur. In the acute phase of radiation injury of the skin, erythema may be evident within hours of irradiation, and desquamation and ulceration appear at higher doses of radiation. These acute events can be addressed symptomatically and generally resolve without exacerbation.[2] However, progressive fibrosis of the dermal tissues that is a late adverse effect of radiation is not always resolved. Pain, limited range of motion, and poor cosmesis are well-described adverse events attributed to radiation fibrosis of

the skin.[3] Unfortunately, there are few anti-fibrotic therapies available to mitigate the fibrotic process.

Tissue fibrosis is the excessive accumulation of collagen and other extracellular matrix components following breakdown in the normal balance of extracellular matrix synthesis and degradation.[4] A lot of mediators are involved in this fibrotic reactions and act on fibroblasts to cause tissue fibrosis. The TGF-β signaling pathway is not only a key mediator of fibroblast activation, but also the wnt/β-catenin signaling pathway plays an important role in fibrotic reactions.[5] In the setting of radiation, β-catenin mediates the effects of radiation in fibroblasts, and its modulation has the potential to decrease the severity of radiation-induced complications.[4]

In this study, we tested the hypothesis that inhibition of wnt/β-catenin signaling pathway would ameliorate radiation fibrosis. Inhibition of the pathway was accomplished by using adenovirus gene therapy that expressed wnt antagonists that bind wnt ligands to block interaction with receptors.[6] Low-density lipoprotein receptor–related protein 6 (LRP6), a wnt ligand, is required for activation of the canonical wnt/β-catenin pathway.[7] A novel soluble wnt receptor, sLRP6E1E2, which is composed of the LRP6 E1 and E2 regions was used to inhibit the pathway. Therefore, we explored the biological effects of sLRP6E1E2 binding to extracellular wnt ligands after irradiation *in vitro* and *in vivo* study.

II. MATERIALS AND METHODS

1. Cell Cultures and *in Vitro* Irradiation

Human dermal fibroblasts (HDF) and HaCaT keratinocytes (American Type Culture Collection, Manassas, VA, USA) were seeded at passage 4 in 6 well plates and grown in a complete cell culture medium consisting of Dulbecco's Modified Eagle Medium (DMEM) with 100 U/ml penicillin and 100 lg/mL streptomycin sulphate, and 10 % fetal bovine serum (FBS) at 37°C in a humidified atmosphere containing 5 % CO2 until forming a confluent monolayer. An X-RAD320 irradiator (Precision X-Ray, North Branford, Connecticut, USA) was used to irradiate cell cultures with a single dose of 2Gy radiation.

2. Treatment with Adenovirus Expressing Decoy Wnt Receptor

To examine the biochemical function of soluble LRP6 receptor (sLRP6E1E2), constructs of the E1 and E2 extracellular domains (wnt-binding sites) of LRP6 were generated, and FLAG-tagged sLRP6E1E2 was subcloned into a pCA14 shuttle vector.[8,9] This pCA14/sLRP6E1E2 vector was co-transformed with a replication-incompetent adenovirus 5/35 chimeric vector (dE1-k35), generating pdE1-k35/sLRP6E1E2.[10] These recombinant plasmids were transfected into HEK293 cells to generate dE1-k35/sLRP6E1E2 (**Fig. 1**). The replication-incompetent dE1-k35 was

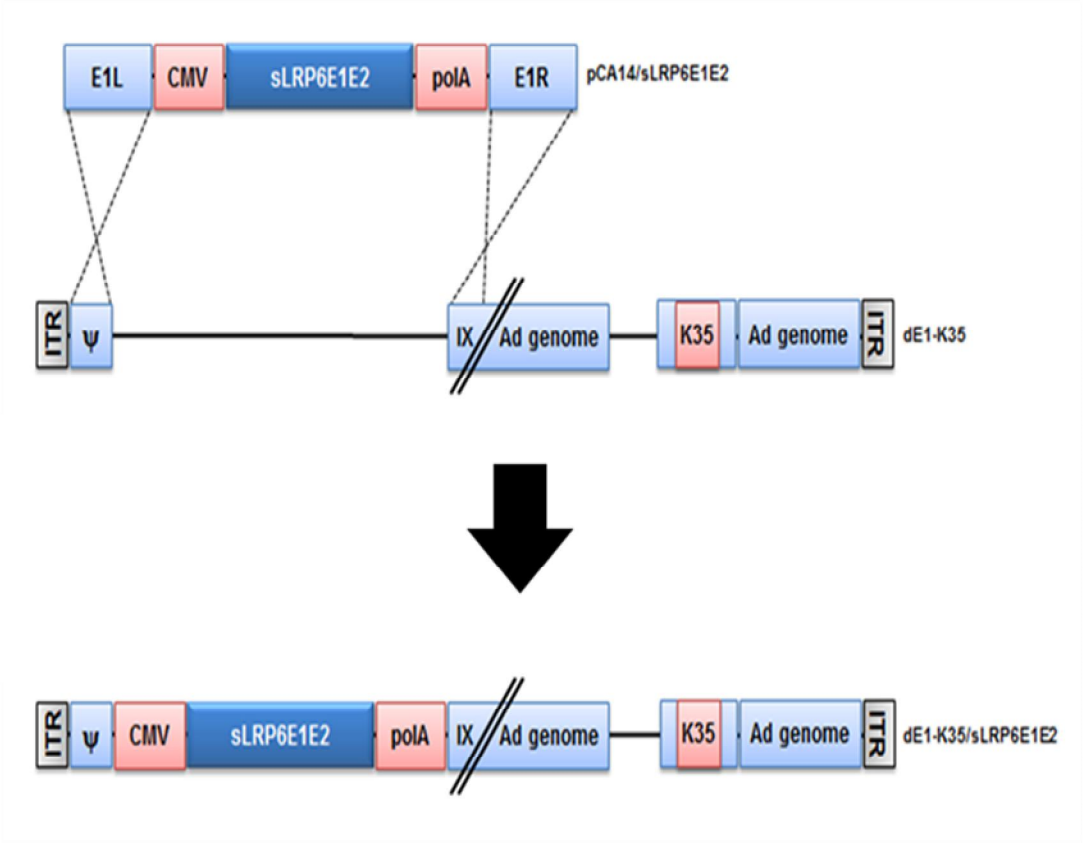


Figure 1. Schematic representation of the genomic structure. Constructs of the E1 and E2 extracellular domains that is wnt-binding sites was transfected into HEK293 cells using pCA14 shuttle vector to generate adenovirus expressing soluble LRP6 receptor (sLRP6E1E2).

used as a negative control.[11] The titers of generated adenoviruses were determined with optical density of the viral genome. One hundred MOI of each dE1-k35/sLRP6E1E2 (sLRP6E1E2 expressing virus) and dE1-k35 (control virus), was transfected into cultured dermal fibroblasts and HaCaT cells. Irradiation to cell cultures was followed immediately by viral transfection.

3. RNA and Protein Analysis

To evaluate the RNA levels of collagen type I/III, TGF-β, smad-2, 3 in cultured HDF and E-cadherin, wist, vimentin, slug in cultured HaCaT cells, quantitative real-time PCR (qRT-PCR) was performed 48 hours after irradiation. Total RNA was prepared with RNeasy Mini Kit (Qiagen, Hilden, Germany), and complementary DNA was prepared from 0.5 μg of total RNA by random priming using a first-strand cDNA synthesis kit (AccuPower™ RT PreMix, Bioneer, Daejeon, Korea), under the following conditions: 95°C for 5 minutes, 37°C for 2 hours, and 75°C for 15 minutes. Applied Biosystems TaqMan primer/ probe kits were used to analyze mRNA expression levels by use of an ABI Prism 7500 HT Sequence Detection System (Applied Biosystems, Foster City, CA, USA). Target mRNA levels were measured relative to an internal glyceraldehyde-3-phosphate dehydrogenase (GAPDH) control. For cDNA amplification, AmpliTaq Gold DNA polymerase was activated by 10 minutes' incubation at 95°C; this was followed by 40 cycles

with 15 seconds at 95°C and 1 minute at 60°C for each cycle. To measure cDNA levels, the threshold cycle at which fluorescence is first detected above baseline is used, and a standard curve is drawn between starting nucleic acid concentrations and the threshold cycle. The mRNA expression levels were normalized to the levels of GAPDH housekeeping genes, and then relative quantization was expressed as fold-induction compared with control conditions. The following primers were used: Hs00164004_m1 (collagen type I), Hs00164103_m1 (collagen type III), Hs00998133_m1 (TGF-β), Hs00183425_m1 (smad 2), Hs00969210_m1 (smad 3), Hs 01023894_m1 (E-cadherin), Hs 00185584_m1 (vimentin), Hs 00950344_m1 (slug), Hs 00361186_m1 (Twist), and Hs99999905_m1 (GAPDH, reference).

Western blot analysis was performed to examine the proteins associated with wnt/β-catenin signaling pathway in cultured cells and animal tissues. Cultured cells or tissues were lysed in 50 mM Tris-HCl (pH 7.6), 1% Nonidet P-40, 150 mM sodium chloride, and 0.1 mM zinc acetate in the presence of protease inhibitors. Protein concentration was determined by the Lowry method (Bio-Rad, Hercules, CA, USA), and 20 μg of each sample was separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis. The gels were then transferred electrophoretically onto a polyvinylidene difluoride membrane (Millipore, Billerica, MA, USA). The membrane was blocked with blocking buffer for 1 hour and then incubated overnight at 4°C with primary antibodies against β-catenin (Cell Signaling Technology, Beverly, MA, USA),

wnt (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) and actin (mouse monoclonal, Sigma-Aldrich, St Louis, MO, USA). After a 2-hour incubation at room temperature with the secondary antibodies horseradish peroxidase-conjugated rabbit antibody (Santa Cruz Biotechnology) horseradish peroxidase-conjugated mouse antibody (Santa Cruz Biotechnology), the protein bands were visualized using ECL detection kit (Thermo, Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions. Protein expression was analyzed using ImageJ software (National Institutes of Health, Bethesda, MD, USA).

4. Immunocytochemistry for Collagen Type I

After 48 hours post-radiation, cultured cells were washed twice with PBS, fixed in 4% paraformaldehyde for 15 minutes at room temperature, and then permeabilized by incubation for 15 min with 0.01% tween 20 in PBS. The samples were blocked with 5% bovine serum albumin followed by incubation with anti-collagen 1 (1:100, Abcam, Cambridge, MA, USA) overnight at 4°C. The next day, cells were washed with PBS and incubated with bovine anti-rabbit IgG-FITC (1:200, Santa Cruz Biotechnology) secondary antibody for 2 hours at room temperature. Cells were mounted on slides by using mounting solution containing DAPI (Vector Laboratories, Burlingame, CA, USA). Slides were mounted with and cells were viewed under a confocal microscope system (LSM700, Olympus, Center Valley, PA, USA).

5. Mouse Model and Experimental Protocols

Animals were handled according to national and international guidelines, in an animal facility accredited by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC). The number of animals used was minimized, and all necessary precautions were fulfilled to mitigate pain and suffering. Protocols were approved by the Institutional Animal Care and Use Committee at Yonsei University health system.

Thirty six four- to five-week-old mice were randomized into three groups according to the injection materials: PBS, control adenovirus (dE1-k35) and decoy wnt receptor-expressing adenovirus (dE1-k35/sLRP6E1E2). A single fraction of 15Gy was delivered to the dorsal skin (dimension: 1.5×2cm) of each mouse using an X-RAD320 irradiator, ensuring that >90% of the prescribed dose would be limited to skin depth. On 1st, 3rd, 5th, 7th, 9th, 11th weeks after irradiation, PBS or virus were directly injected into each irradiated dorsal skin. In each mouse, 5×10^{10} VP/ml of viruses were administrated. The mice were housed and observed for 16 weeks to allow toxic effects of radiation to develop. On 2nd, 4th, 6th, 8th, 10th, 12th weeks after irradiation, digital photograph and laser Doppler flowmetry (Periflux system 5000, Perimed AB, Jarfalla, Sweden) were taken. On 4th, 8th, 12th, 16th weeks, mice were euthanized (n=3, each period), and tissues were harvested for histologic analysis.

6. Clinical Evaluation of Mouse Model

Gross changes of the skin after a single fraction irradiation of 15Gy observed and complications related to the irradiation such as erythema, dry desquamation, moist desquamation, ulceration, hair loss and necrosis were recorded. Irradiated areas were designed on dorsum of mice before radiation, and the marked areas were followed up by a digital photograph. The Scion image (NIH-Scion Corp., Frederick, MD, USA) program, by which the affected area could be digitally measured, was performed by two blinded observers. Using the program, the length of the image was converted to the actual length and the surface area was calculated. The sequential changes of irradiated area were analyzed.

To assess changes in blood flow within the irradiated area, the Periflux system 5000 was used. Skin vascularity was taken from the center of the irradiated area, using laser Doppler flowmetry. Data were measured three times at 1 min intervals and the mean value was obtained. Data were presented as perfusion unit (PU).

7. Quantification of Collagen Production

To analyze the collagen fibers, Masson's trichrome staining was performed. The staining solution was prepared with Bouin's solution (picric acid solution, 75 ml; 37 % formalin, 25 ml; glacial acetic acid, 5 ml), Weigert's iron hematoxylin solution (hematoxylin, 1 g; 95 % ethanol, 100 ml; ferric chloride,

2 g; concentrated HCl, 1 ml; distilled water, 95 ml), Biebrich scarlet-acid fuchsin solution (1 % Biebrich scarlet, 90 ml; 1 % acid fuchsin, 10 ml; Glacial acetic acid, 1 ml), phosphomolybdic–phosphotungstic acid (phosphomolybdic acid, 2.5 g; phosphotungstic acid, 2.5 g) and an aniline blue solution (aniline blue, 2.5 g; distilled water, 100 ml; glacial acetic acid, 2 ml).

A semi-quantitative analysis of the synthesis of collagen fibers was executed using MetaMorph® image analysis software (Universal Image Corporation, Buckinghamshire, UK). Results are expressed as the average optical density (OD) for five different digital images. OD quantifies the opacity of objects when exposed to transmitted light, and it can be thought of as analogous to the inverse of the gray scale values, which pertain to the amount of spectral, or reflected, light.

8. Immunofluorescence Assay

Mouse skin tissue sections were deparaffinized, rehydrated, blocked with 5% goat serum and incubated with anti E-cadherin anti-body (Acris antibody, INC., San Diago, CA, USA) and vimentin (Santa Cruz Biotechnology) overnight at 4°C. The next day, tissues were then washed with PBS and incubated with bovine anti-rabbit IgG-FITC and Texas Red-conjugated bovine anti-mouse IgG (Santa Cruz Biotechnology) secondary anti-body for 2 h at room temperature. Tissues were mounted on slides by using mounting solution containing DAPI (Vector Laboratories). Slides were mounted with

and cells were viewed under a confocal microscope system (LSM700).

9. Statistical Analysis

Each experiment was performed in triplicate. Results are expressed as mean ± standard error of the mean (SEM). Group results were compared by one-way analysis of variance, followed by post hoc Student's *t*-test for unpaired observations or Bonferroni's correction for multiple comparisons. $P<0.05$ was considered significant.

III. RESULTS

1. Influences of Irradiation in Fibroblasts and Keratinocytes

The representative cells that composed skin, keratinocytes and dermal fibroblasts, were selected to determine the effect of irradiation in skin tissues. A single dose of 2Gy irradiation led to activation of dermal fibroblast after 48 hours. RNA analysis showed an increase of mRNA in collagen type I, III, TGF-β and smad-2, 3 (**Fig. 2A**). There was a substantial increase of collagen fibers as well as an activation of TGF-β/smad pathway. The results of immunocytochemistry for collagen type I in dermal fibroblasts also revealed that radiation facilitated production of collagen type I (**Fig. 2B**), suggesting that irradiation causes fibrotic reaction.

In irradiated keratinocytes, epithelial-to-mesenchymal transition (EMT) was verified by mRNA expression of EMT-related markers. We hypothesized that irradiated keratinocytes would be converted into collagen-producing mesenchymal cells by the mechanism of EMT, and converted mesenchymal cells ultimately produce collagen fibers. **Fig. 3A** showed that irradiated keratinocytes exhibited up-regulation of an epithelial marker E-cadherin by qRT-PCR. Conversely, mesenchymal markers (i.e., twist, vimentin and slug) were markedly down-regulated in irradiated keratinocytes, suggesting that EMT occurred (**Fig. 3A)**. Furthermore, the results of immunocytochemistry for collagen type I proved that irradiated keratinocytes eventually produced

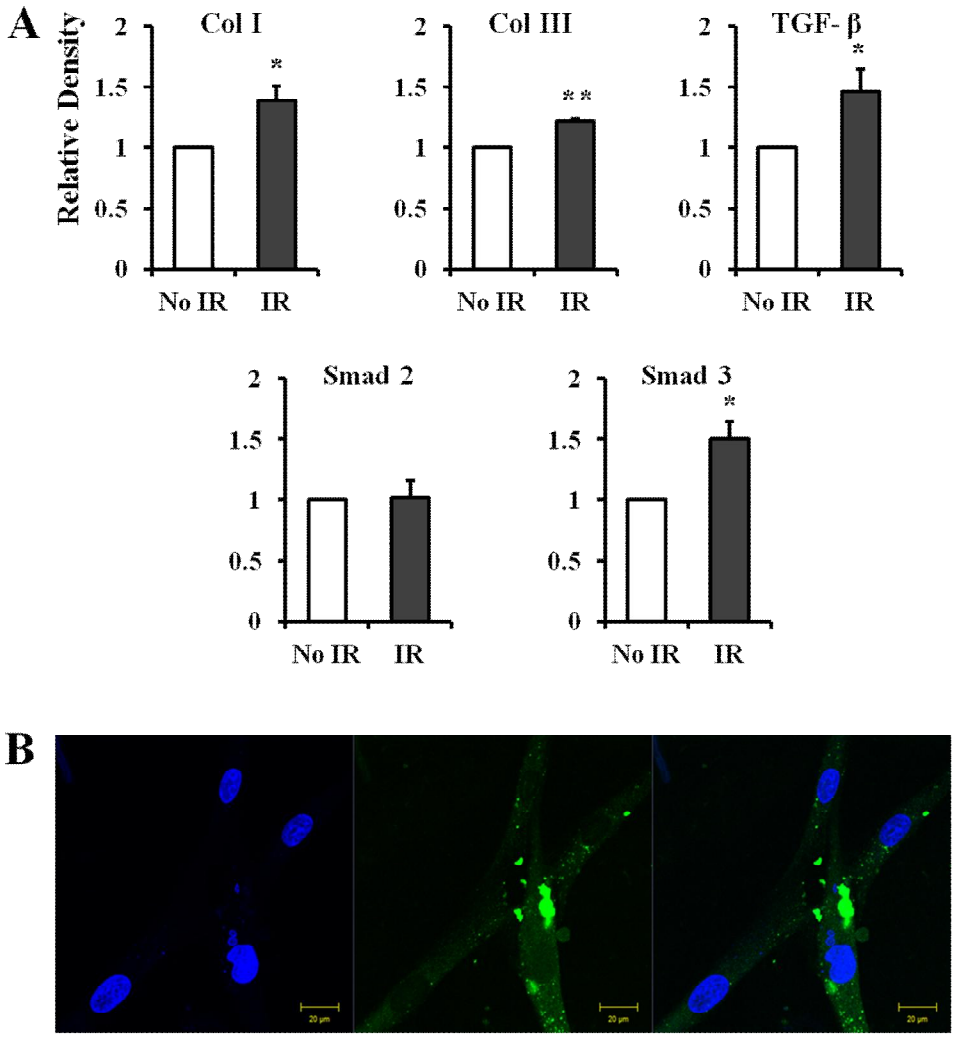
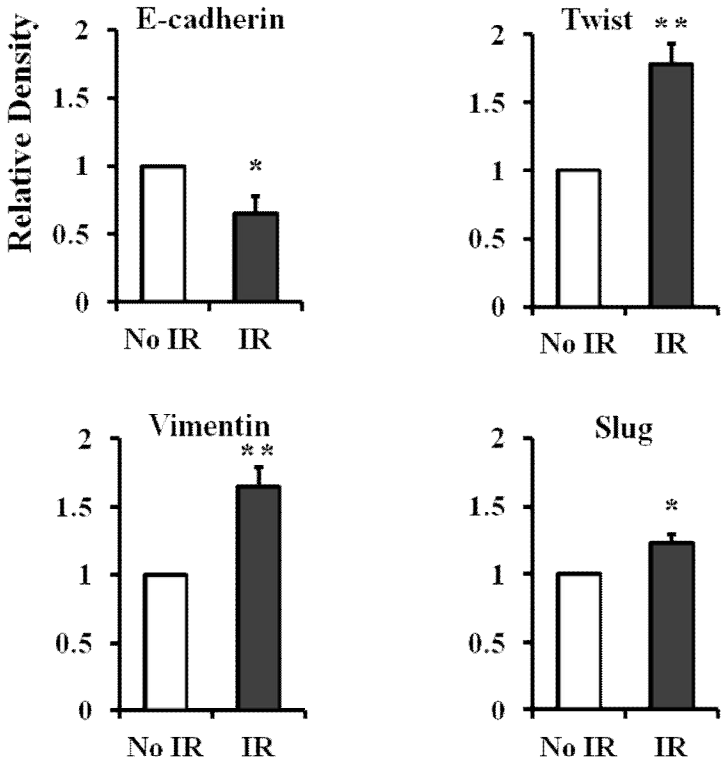


Figure 2. Radiation facilitates fibrotic reaction in cultured dermal fibroblasts. **A.** The expressions of mRNA were increased on collagen type I, III, TGF-β and smad-2, 3. Col: collagen; No IR: not irradiated; IR: irradiated; * $p<0.05$; ** $p<0.01$. **B.** The results of immunocytochemistry showed that irradiated fibroblasts involve collagen type I. Blue: DAPI stain; green: collagen type I stain.

A



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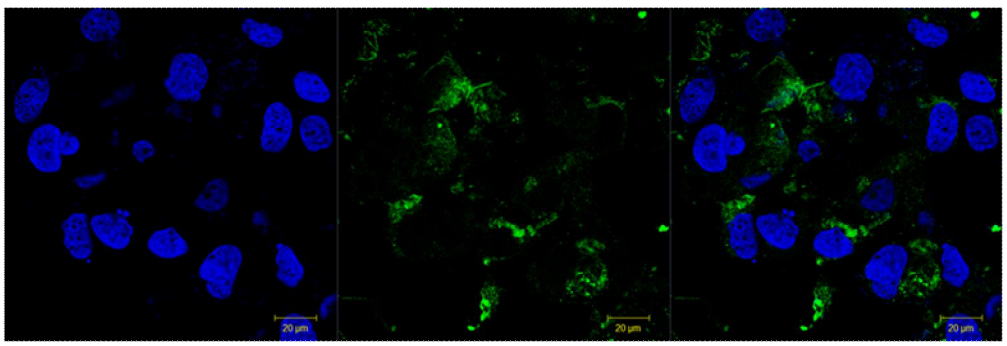

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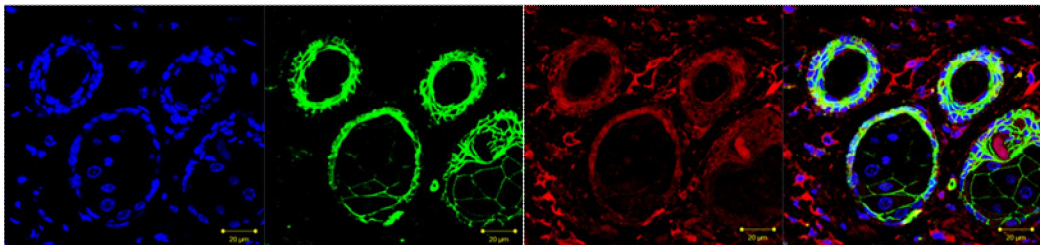


Figure 3. Radiation is responsible for epithelial-to-mesenchymal transition (EMT) of cultured keratinocytes. **A.** The level of mRNA was increased on E-cadherin (epithelial marker), while the levels was decreased on twist, vimentin and slug (mesenchymal markers). No IR: not irradiated; IR: irradiated; * $p<0.05$; ** $p<0.01$. **B.** The immunocytochemistry demonstrates that irradiated keratinocytes ultimately produce collagen type I. Blue: DAPI stain; green: collagen type I stain. **C.** *In vivo* study shows EMT is facilitated. Dermal tissues of a mouse were irradiated with a single dose of 15Gy and above finding was observed after 8 weeks post-radiation. Blue: DAPI stain; green: E-cadherin; red: vimentin.

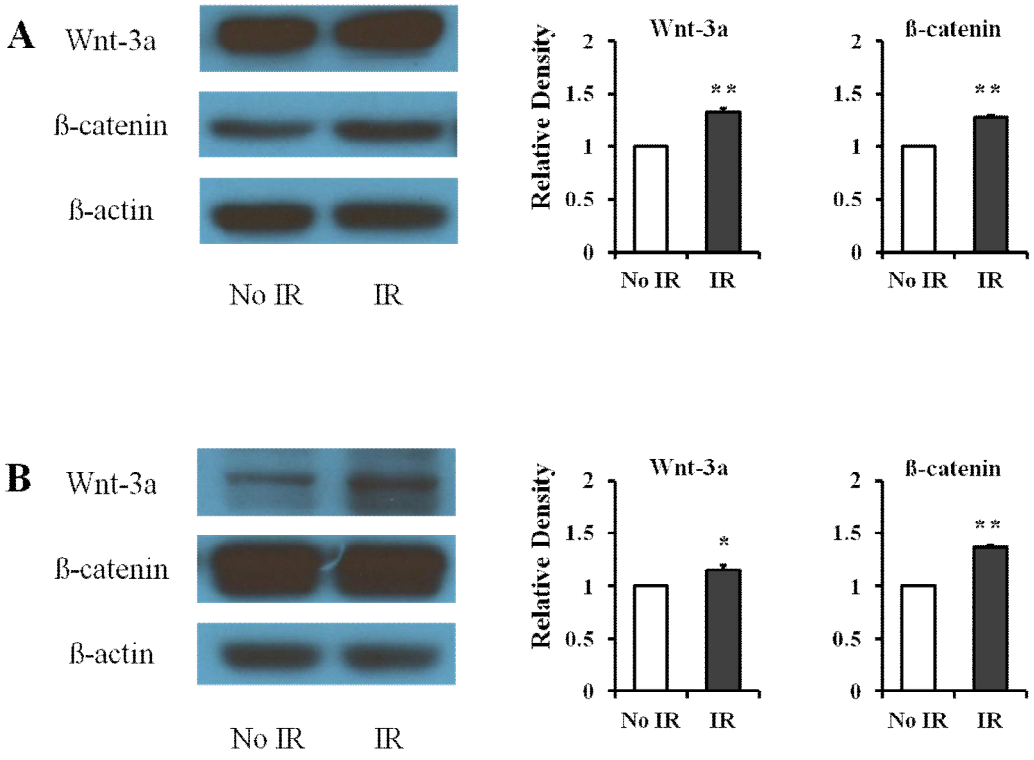


Figure 4. Radiation activates wnt/β-catenin pathway. **A.** The proteins, wnt-3a and β-catenin which are key factors of wnt/β-catenin signaling pathway, were highly expressed in irradiated fibroblasts. **B.** Those proteins were also increased in irradiated keratinocytes. No IR: not irradiated; IR: irradiated; * $p<0.05$; ** $p<0.01$.

the type I collagen fibers (**Fig. 3B)**. Similar results were observed in a mouse model. Immunofluorescence assay revealed that double staining of E-cadherin and vimentin was positive in irradiated dermal tissues of mice (**Fig. 3C)**.

We have started with the premise that wnt/β-catenin signaling pathway would be activated in the setting of radiation, thus the decoy wnt receptor-expressing adenovirus was used to inhibit wnt/β-catenin pathway. To determine whether the radiation was attributable to activation of wnt/β-catenin pathway, we examined wnt-3a and β-catenin at the protein level. Cultured cells were irradiated, and protein was analyzed by Western analysis. Both proteins of wnt-3a and β-catenin in fibroblasts and keratinocytes were increased after radiation (**Fig. 4**). Therefore, it is suggested that radiation is responsible for activation of wnt/β-catenin pathway in cultured fibroblasts and keratinocytes.

2. *In Vitro* Anti-fibrotic Effect of Decoy Wnt Receptor

Before transfection into the cultured cells, the generated adenovirus, dE1-k35/sLRP6E1E2 (sLRP6E1E2 expressing virus) and dE1-k35 (control virus), were assessed for identifying the sLRP6E1E2 gene inserted. The gene was amplified by polymerase chain reaction, and sLRP6E1E2 gene that is 1.5kb was verified only in dE1-k35/sLRP6E1E2, not in control virus (**Fig. 5**). The viruses (100 MOI) were transfected into cell cultures immediately after 2Gy radiation.

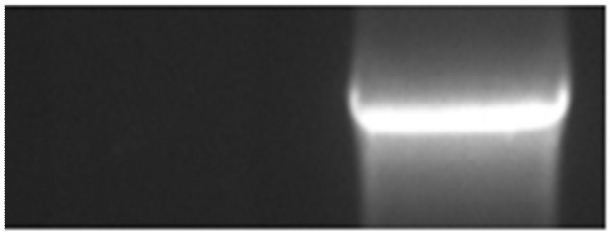
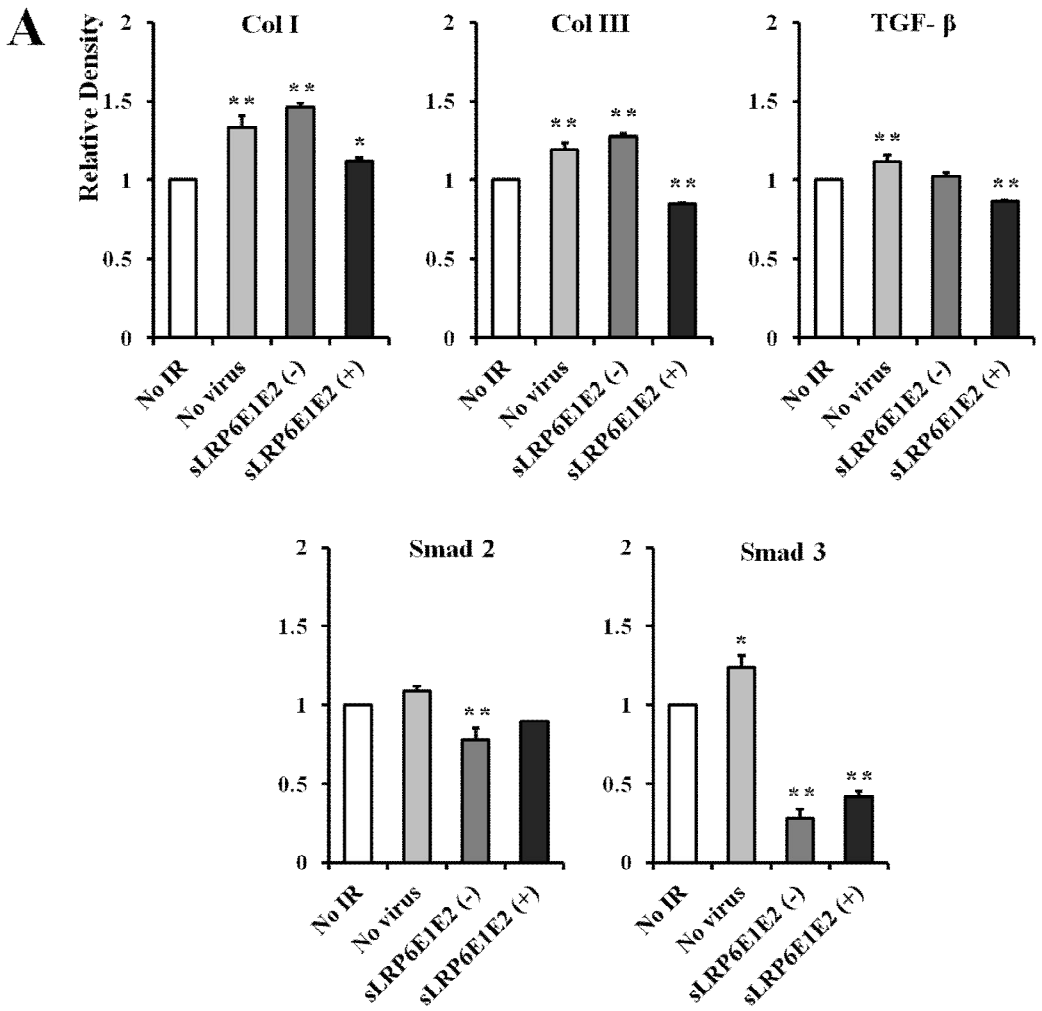


Figure 5. Identification of sLRP6E1E2 gene. The sLRP6E1E2 gene that is amplified by polymerase chain reaction was identified in dE1-k35/sLRP6E1E2, not in control virus (dE1-k35).

The wnt/β-catenin pathway regulates a wide range of cellular functions including fibrosis.[6,12] To test the effects of sLRP6E1E2 on *in vitro* fibrosis, cells treated with PBS or transfected with dE1-k35 or dE1-k35/sLRP6E1E2. At 48 hr after radiation, collagen type I and III production was reduced by 23% and 34% respectively in dermal fibroblast cultures transfected with dE1-k35/sLRP6E1E2, compared to dE1-k35-transfected controls (**Fig. 6A**). Especially, collagen type III was dramatically reduced rather than the level of non-irradiated state. The TGF-β/smad pathway was activated in PBS or control virus, however it was down-regulated by sLRP6E1E2-expressing virus (**Fig. 6A**). In immunocytochemistry for collagen type I staining in dermal fibroblasts treated with sLRP6E1E2, collagen type I fibers were not observed (**Fig. 6B**). Taken together, these findings suggest that sLRP6E1E2-expressing viruses suppress the radiation-induced fibrosis in cultured fibroblasts.

EMT is known to be an important process in fibrosis,[4] and the wnt/β-catenin pathway may play an important role in this process.[6] As mentioned above, irradiated keratinocytes was proved to produce collagen fibers. Therefore we investigated by qRT-PCR whether sLRP6E1E2-expressing viruses could inhibit EMT process and collagen synthesis in irradiated keratinocytes. We found that mRNA expressions of almost markers, whether epithelial or mesenchymal, were not significantly different from the level of mRNA in non-irradiated cells, contrary to previous results (**Fig. 7A**), indicating that EMT in irradiated keratinocytes was inhibited by treatment with



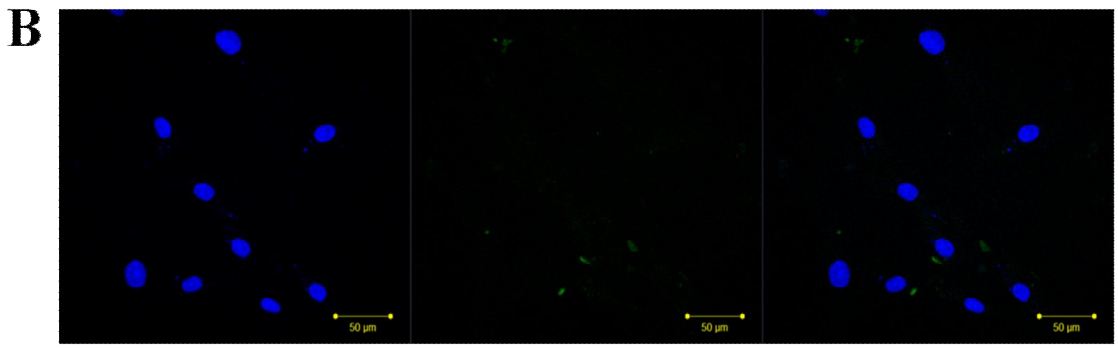
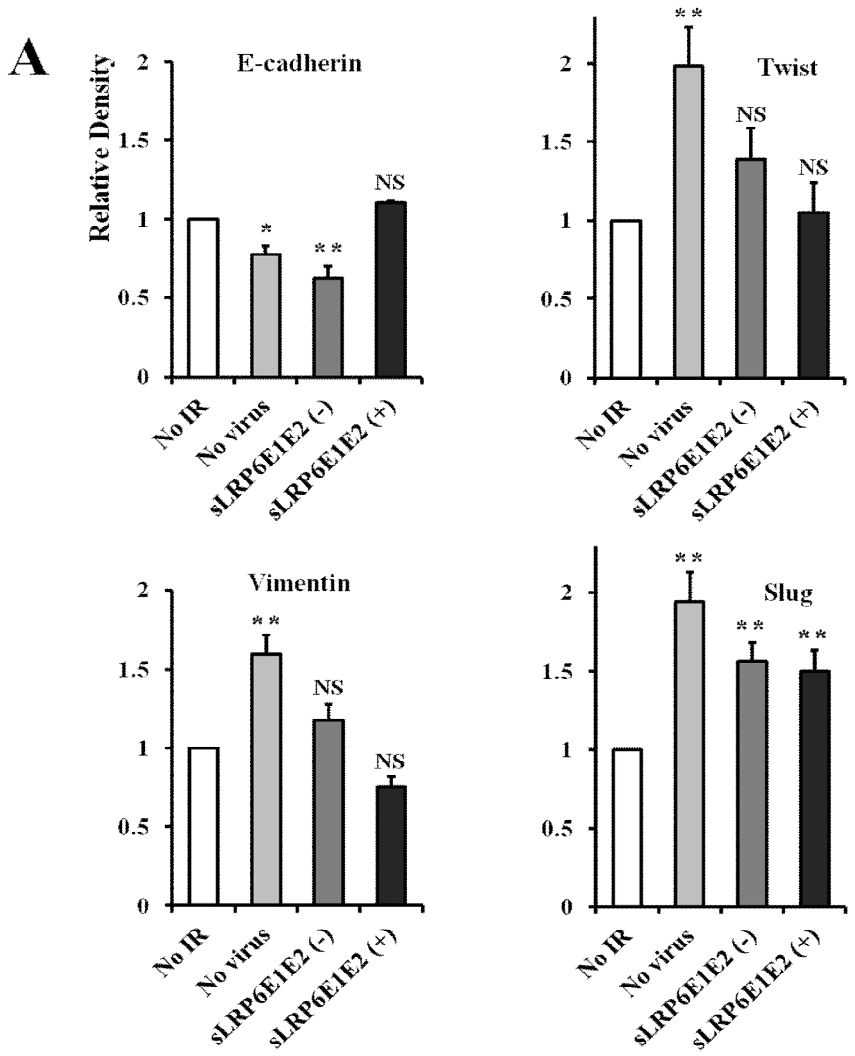


Figure 6. sLRP6E1E2 exerts anti-fibrotic actions in irradiated cultured fibroblasts. **A.** At 48 hr after radiation, collagen type I and III production was reduced in dermal fibroblast cultures transfected with dE1-k35/sLRP6E1E2 compared to dE1-k35-transfected controls. The expression of mRNA related to TGF-β/smad pathway was also reduced sLRP6E1E2-expressing virus. Col: collagen; No IR: not irradiated; No virus: irradiated, but no virus applied; sLRP6E1E2 (-): irradiated and control virus applied; sLRP6E1E2 (+): irradiated and sLRP6E1E2-expressing virus applied; * $p<0.05$; ** $p<0.01$. **B.** In immunocytochemistry for collagen type I staining in dermal fibroblasts treated with sLRP6E1E2, collagen fibers were not observed unlike the results in **Figure 3B**. Blue: DAPI stain (right panel); green: collagen type I stain (middle panel, but not observed); left panel: a merged image.



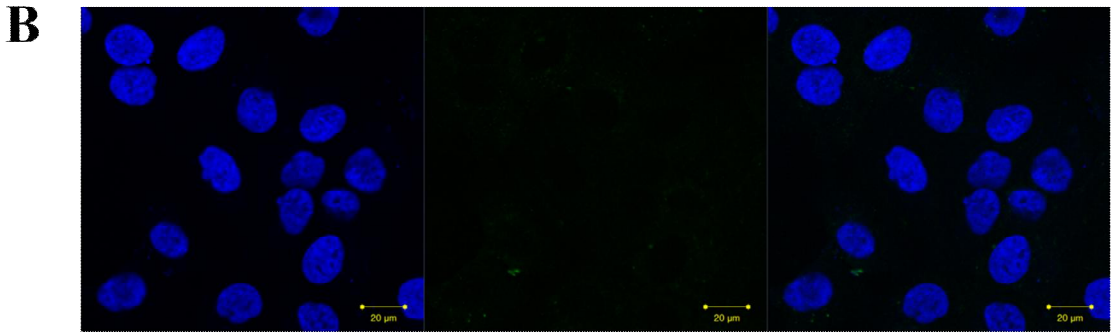
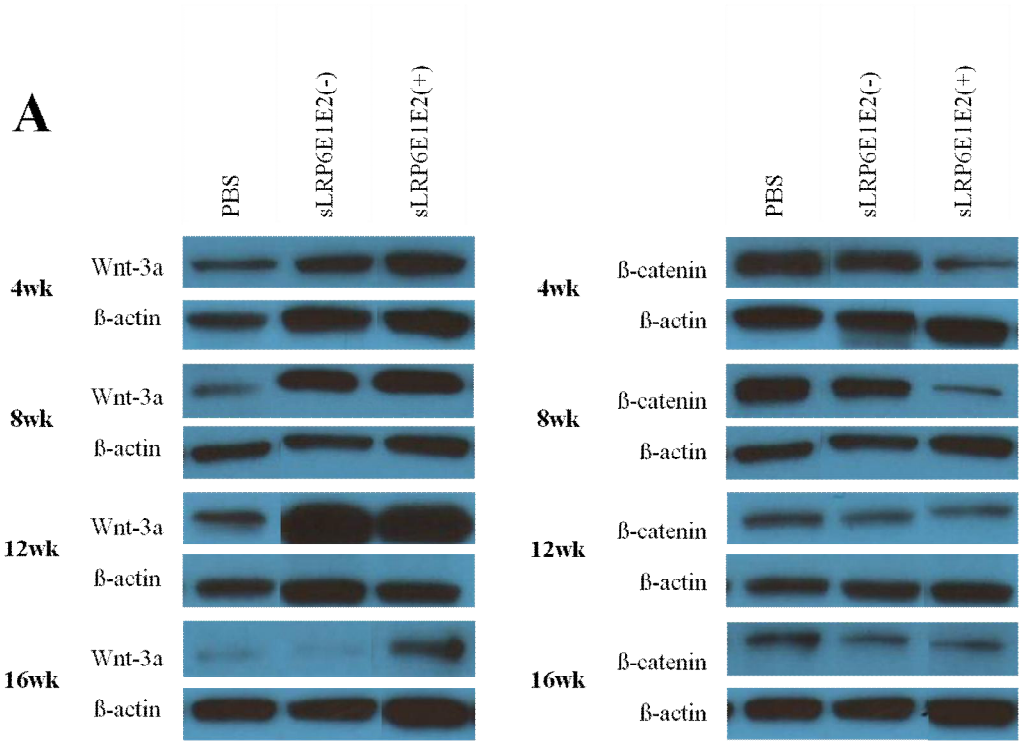


Figure 7. sLRP6E1E2 inhibits EMT in irradiated cultured keratinocytes. **A.** mRNA expressions of E-cadherin, twist, vimentin in irradiated cultured keratinocytes transfected with sLRP6E1E2-expressing virus were not significantly different from the mRAN level in non-irradiated cells. Col: collagen; No IR: not irradiated; No virus: irradiated, but no virus applied; sLRP6E1E2 (-): irradiated and control virus applied; sLRP6E1E2 (+): irradiated and sLRP6E1E2-expressing virus applied; * $p<0.05$; ** $p<0.01$; NS: not significant. **B.** In immunocytochemistry for collagen type I, collagen fibers in irradiated keratinocytes was not observed after treatment with sLRP6E1E2-expressing virus. Blue: DAPI stain (right panel); green: collagen type I stain (middle panel, but not observed); left panel: a merged image.

dE1-k35/sLRP6E1E2 viruses. In immunocytochemistry for collagen type I, collagen fibers in irradiated keratinocytes treated with sLRP6E1E2 was not produced as well (**Fig. 7B**).

3. Alterations of Wnt/β-Catenin Pathway in Irradiated Mice

We next evaluated alterations of wnt/β-catenin pathway in the setting of radiation in a mouse model, according to treatment with PBS or control adenovirus (dE1-k35) or decoy wnt receptor-expressing adenovirus (dE1-k35/sLRP6E1E2), by Western blot analysis of wnt-3a and β-catenin. From the point of view of PBS control, the level of wnt-3a began increasing after irradiation and had a peak at 12 weeks, while β-catenin increased rapidly at 4 weeks, peaked at 8 weeks, and decreased through 12-16 weeks after irradiation (**Fig. 8**). It suggests that radiation activates the wnt/β-catenin pathway during initial 12 weeks and subside until 16 weeks post-radiation. When treated with sLRP6E1E2-expressing virus, the rate of increase and the peak in the protein expression of wnt-3a was markedly pronounced. However, the β-catenin protein was less expressed with a lower peak than PBS control and dE1-k35 control virus. These findings may be explained by the hypothesis that the level of β-catenin in dE1-k35/sLRP6E1E2 group generally decreases due to the action of sLRP6E1E2 which suppresses the wnt/β-catenin pathway, while the level of wnt-3a in the same group elevates for compensation of suppressed wnt/β-catenin pathway as a positive feedback.



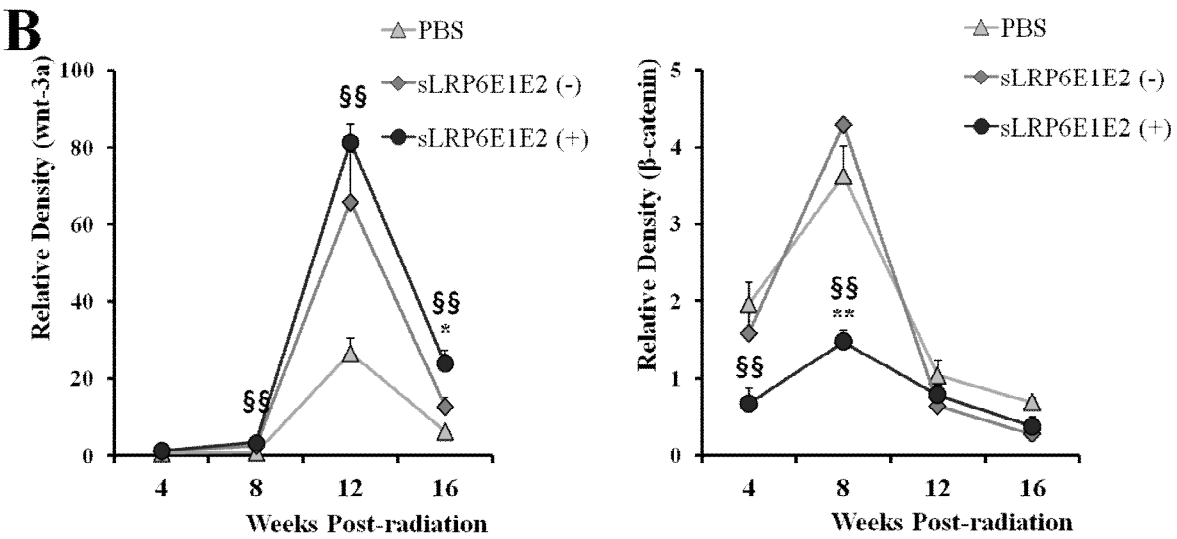


Figure 8. Alterations of wnt/β-catenin pathway in a mouse model. **A.** The wnt-3a and β-catenin proteins were detected by Western blot as time passed. **B**. Relative density of the wnt-3a (left panel) and β-catenin (right panel) compared to control protein as detected using densitometry, depending on the post-radiation time. PBS: PBS applied group without viruses; sLRP6E1E2 (-): control virus applied group; sLRP6E1E2 (+): sLRP6E1E2-expressing virus applied group; § $p<0.05$, §§ $p<0.01$ versus PBS; * $p<0.05$, ** $p<0.01$ versus sLRP6E1E2(-).

4. Clinical Results in a Mouse Model

Through a preliminary study for the dose-dependent response to radiation in a mouse model, the dose of radiation was determined as a single fraction of 15Gy in the current study because higher dose of radiation would induce desquamation or ulceration on the skin that activates wound healing and fibrosis process (data not shown). As we focus on the radiation fibrosis in this study, fibrosis caused by wound healing should be excluded not to affect the results. Furthermore, the situation with 15Gy radiation is similar to a real clinical setting in human.

After a single fraction of 15Gy delivered to the dorsal skin, tiny dry desquamations appeared in approximately one-third of each group within 4 week and fields receiving radiation healed without ulceration. On 8, 12 and 16 weeks post-radiation, hair loss was observed in half of each group. There was no definite difference between groups (**Fig. 9**). Upon examination by planimetric analysis, the previously marked areas were followed up as **Fig. 10**. The areas gradually increased, and almost values between each group showed no significant differences. It means that the radiation dose of 15Gy could not cause tissue contracture within experimental period, although radiation-induced of fibrosis occurred.

It is known that microvascular injury provides stimulus of fibrosis.[4] We examined cutaneous blood flow by laser Doppler flowmetry. However, we could not find any differences between groups, even if the values of

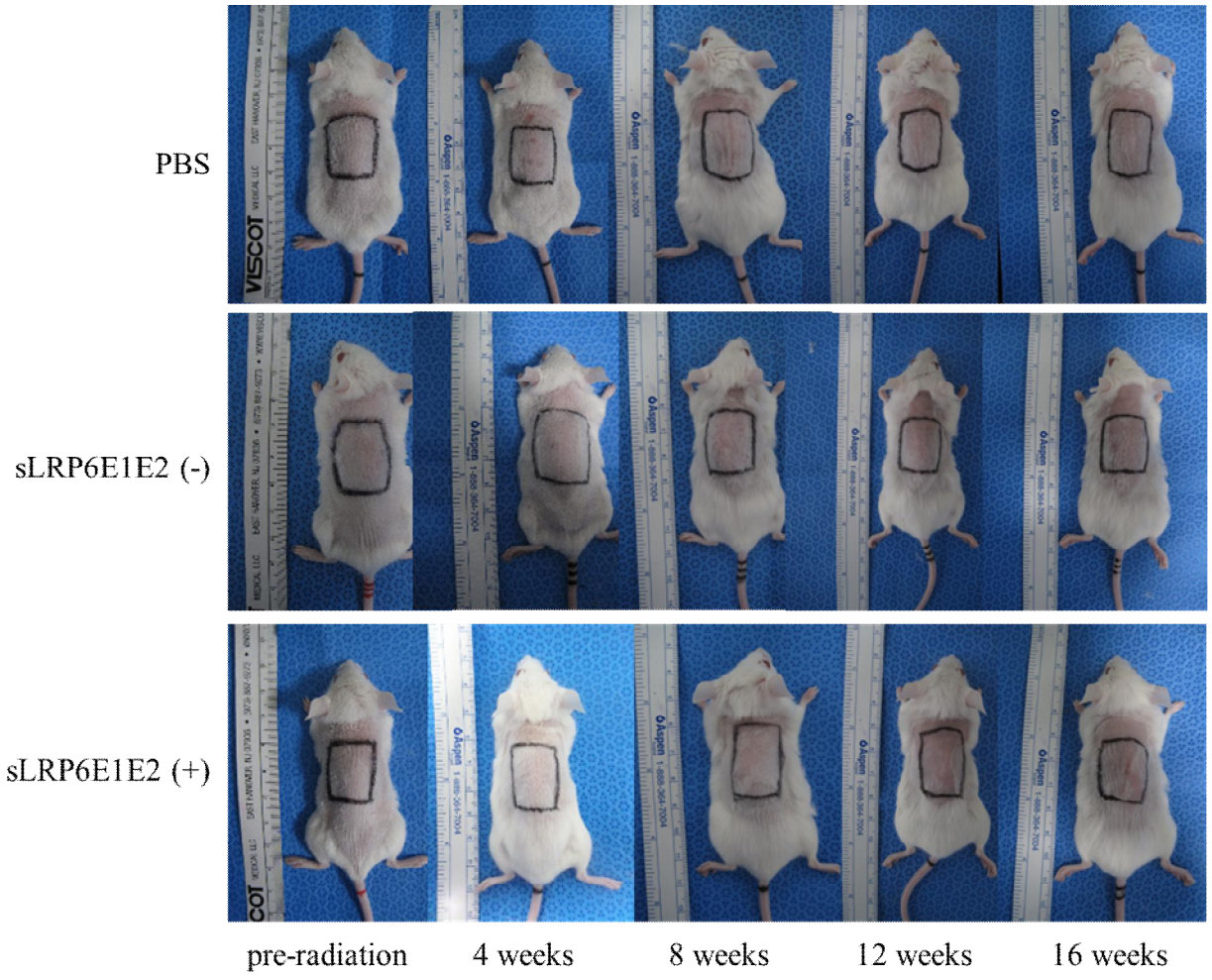


Figure 9. Gross changes of the skin. Tiny dry desquamation and hair loss are observed, but there is no ulceration. No significant difference is noted between groups.

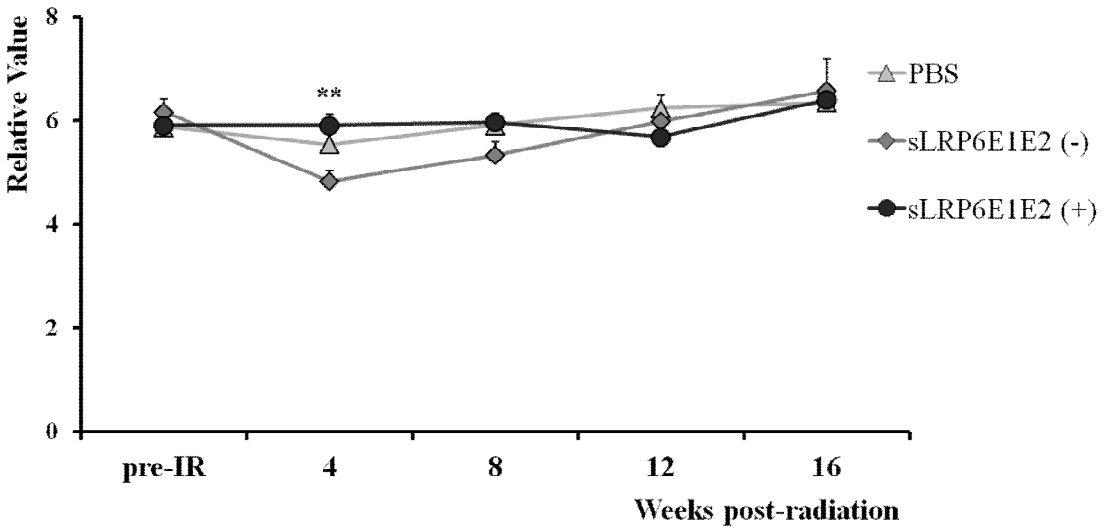


Figure 10. Planimetric analysis of irradiated area. The result shows gradual increase with time, but generally there is no significant difference between groups. PBS: PBS applied group without viruses; sLRP6E1E2 (-): control virus applied group; sLRP6E1E2 (+): sLRP6E1E2-expressing virus applied group; § $p<0.05$, §§ $p<0.01$ versus PBS; * $p<0.05$, ** $p<0.01$ versus sLRP6E1E2(-).

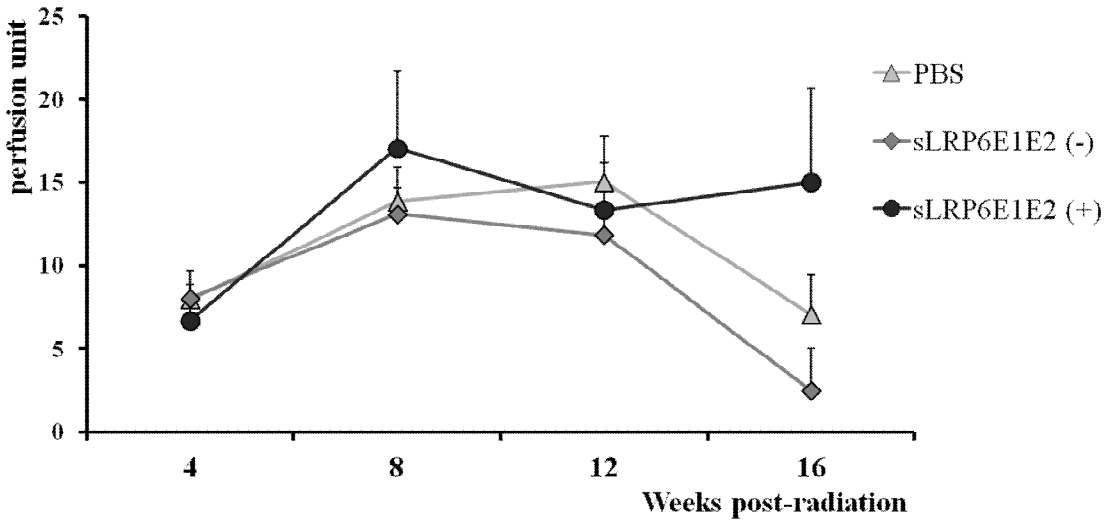
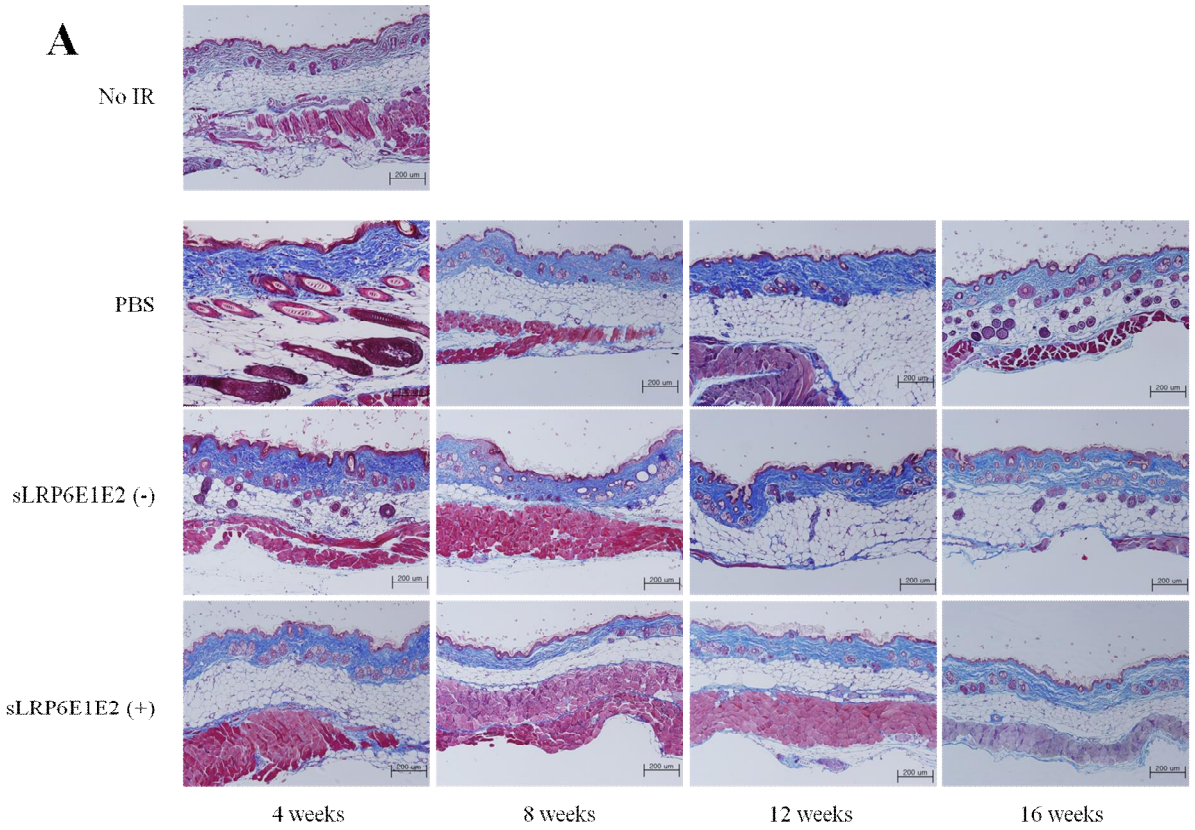


Figure 11. Cutaneous blood flow by laser Doppler flowmetry. The values were taken from center in the irradiated areas. The values of sLRP6E1E2-treated group are higher than other groups at 8 and 16 weeks post-radiation, but there is no significant difference between groups. PBS: PBS applied group without viruses; sLRP6E1E2 (-): control virus applied group; sLRP6E1E2 (+): sLRP6E1E2-expressing virus applied group.

sLRP6E1E2-treated group were higher than other groups at 8 and 16 weeks post-radiation. The result is shown in **Fig. 11**.

5. *In Vivo* Anti-fibrotic Effect of Decoy Wnt Receptor

To measure collagen production in a mouse model, we performed Masson's trichrome staining and semi-quantitative analysis of the synthesis of collagen fibers. Generally, the amount of collagen fibers was increased and dermal tissues were thickened in irradiated groups (PBS, sLRP6E1E2 (-) and sLRP6E1E2 (+) group) compared to normal dermal tissue. Radiation is responsible for increased production of collagen. Among the irradiated groups, there was decrease trend of collagen expression with time in sLRP6E1E2-treated group, while mice in other control group had a higher level of collagen expression (**Fig. 12**). The lack of increase in expression of type 1 collagen in sLRP6E1E2 treated group suggests that wnt/β-catenin pathway play an important role in radiation-induced fibrosis.



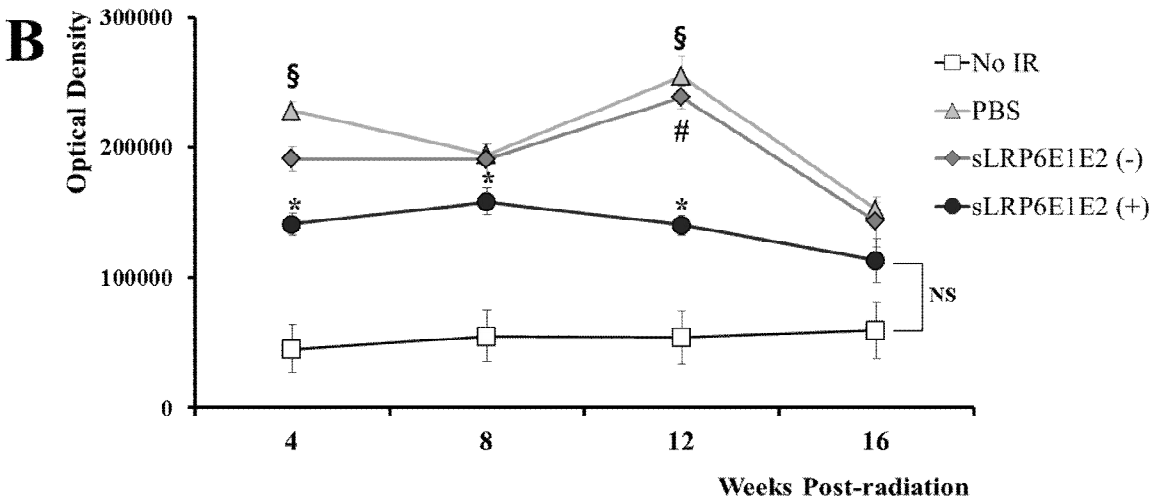


Figure 12. Quantification of collagen production. **A**. Histologic views of un-irradiated normal tissue and irradiated tissues of PBS control group, control virus group and sLRP6E1E2 treated group. **B**. A semi-quantitative analysis of the synthesis of collagen fibers using MetaMorph® image analysis software (Universal Image Corporation, Buckinghamshire, UK). The results shows decrease trend in sLRP6E1E2 treated group compared to other irradiated tissues, while the expression is higher than normal tissue except on 16 weeks post-radiation. No IR: not irradiated normal tissue; PBS: PBS applied group without viruses; sLRP6E1E2 (-): control virus applied group; sLRP6E1E2 (+): sLRP6E1E2-expressing virus applied group; § $p<0.05$, versus PBS; # $p<0.05$, versus sLRP6E1E2(-);* $p<0.05$, versus No IR.

IV. DISCUSSION

We investigated whether inhibition of the canonical wnt/β-catenin pathway reverse radiation fibrosis. sLRP6E1E2 expressing adenovirus was used to inhibit a wnt ligand-dependent process. In an irradiated mouse model, elevation of β-catenin level has been verified, and we found that overproduction of collagen type I that is a representative of extracellular matrix components could be controlled by inhibition of the wnt/β-catenin pathway. It means that inhibition of the β-catenin mediator is effective in ameliorating radiation-induced fibrosis.

The identification of a lot of pathways from initial stimulus to collagen synthesis offers various therapeutic targets. Among them, we selected the wnt/β-catenin pathway as a target to control the radiation-induced fibrosis, although TGF-β is known to be a key factor for the fibrotic reaction.[13] Reactive oxygen species generated by radiation exposure are immediate activators of TGF-β, and then activated TGF-β initiates a process of upregulated collagen synthesis.[14] Recent studies suggest that the effect of TGF-β on wound healing is mediated by β-catenin and a similar process wnt/β-catenin signaling might contributes to radiation fibrosis.[15-17] We hypothesized that inhibit of β-catenin would provide a significant mitigation of radiation-induced fibrosis in dermal tissues.

Before developing a strategy to reduce the progression of radiation fibrosis,

we should closely consider the treatment startpoint and endpoint. Radiation-induced fibrosis is presented in the late phase, even after decades. The perpetuation of fibrotic reaction may not require continued paracrine stimulation by fibrogenic cytokines, relying on positive autocrine.[4] It is postulated that collagen synthesis is subsequently continued by autocrine induction of connective tissue growth factor.[18,19] Whether early activation of TGF-β is relevant to late effect of radiation fibrosis lacks confirmation. However, several studies could ameliorate radiation-induced fibrosis by inhibition of the early TGF-β pathway.[20,21] In the present *in vivo* study, the results of control group revealed that the protein level of wnt-3a and β-catenin began increasing after irradiation with a peak at 12 weeks and 8 weeks respectively, and decreased through 12-16 weeks, suggesting the activation of wnt/β-catenin pathway is confined within 16 weeks post-radiation. Synthesis of collagen type I in a mouse model was increased even in early phase, although radiation-induced fibrosis is a late effect in human. The current result is consistent with previous reports targeting to human with early deposition of collagen fibers.[22,23] In this respect, radiation fibrosis in dermis is clinically manifested as a late effect, however collagen deposition starts immediately after irradiation. Therefore, we applied the treatable virus after irradiation until 11 weeks post-radiation when β-catenin subsides.

In the past, it was known that locally resident mesenchymal cells are the only source of collagen producing cells, but other origins of fibroblasts have

indentified. They are epithelial cell and endothelial cell, capable of undergoing EMT and endothelial-mesenchymal transition (EndoMT).[24] Circulating fibrocytes from bone marrow are another circulating fibroblast precursor.[25] We focused on the EMT as a source of fibroblasts. The wnt/β-catenin pathway plays an important role in the EMT process as well as fibrotic reaction.[6] It is proposed that epithelial cells in dermal tissues may transform into fibroblasts, and we assumed that inhibition of the wnt/β-catenin pathway decreases the EMT process. Accordingly, fibrotic activity is expected to be suppressed by decreasing number of fibroblasts. *In virto* study confirmed the hypothesis as irradiated keratinocytes produced collagen fibers and the collagen synthesis was blocked by the treatable virus. In summary, the inhibition of wnt/β-catenin pathway could ameliorate radiation-induced fibrosis by the two mechanisms: one is direct suppression of fibrogenic activity; the other is indirect suppression of fibrogenic activity by lessening the number of fibroblasts.

IV. CONCLUSION

We examined whether inhibition of wnt/β-catenin pathway with soluble LRP6 receptor (sLRP6E1E2) expressing adenovirus would ameliorate radiation-induced fibrosis. Radiation facilitates fibrotic reaction in cultured keratinocytes as well as cultured human dermal fibroblasts, while sLRP6E1E2 exerts anti-fibrotic actions in irradiated cultured cells. In a mouse model, although there were no significant differences between groups with regard to gross change, change of irradiated area and cutaneous blood flow, excessive collagen deposition was controlled in sLRP6E1E2-treated group. Taken together, these results support that modulation of wnt/β-catenin pathway has the potential to decrease the severity of radiation-induced dermal fibrosis.

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ABSTRACT (IN KOREAN)

Wnt를 억제하는 sLRP6E1E2 발현 아데노바이러스를 이용한 방사선 섬유화에 대한 유전자 치료

<지도교수 유대현>

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이 동 원

방사선 치료 후 발생하는 피부조직의 섬유화는 치료하기 어려운 합병증 가운데 하나이다. 방사선 섬유화는 아직까지 그 기전이 명확히 규명되지 않았으며, 효과적인 치료법도 없는 실정이다. 고전적인 wnt/β-catenin 신호전달경로는 섬유화 과정에서 결정적인 역할을 하며, 상피-중간엽 이행 현상에서도 중요한 역할을 하는 것으로 알려져 있다. 본 연구에서는 sLRP6E1E2를 발현하는 아데노바이러스를 이용한 유전자 치료를 통하여 wnt/β-catenin 신호전달경로를 억제하여, 방사선 섬유화를 개선시킬 수 있는지 생체 외 실험과 생체 내 실험에서 알아보고자 하였다. 생체 외 실험에서는 2Gy의 방사선을 배양된 인간 진피섬유모세포와 표피세포에 조사하였다. 방사선 조사에 의하여 wnt/β-catenin 신호전달경로는 활성화 되었으며, 조사된 진피섬유모세포에서는 콜라겐 합성이 증가되었다. 조사된 표피세포에서는 상피-중간엽 이행이 발생하였으며, 결국에는 콜라겐이 합성된 것을 확인할 수 있었다. sLRP6E1E2 발현 아데노바이러스 처리를 하게 되면, 섬유화 과정과 상피-중간엽 이행이 억제되면서 배양된 세포에서의 콜라겐 합성이 감소하였다. 한편, 생체 내 실험에서는 생쥐를 대상으로 15Gy의

방사선을 등의 피부에 조사하였다. 30 마리의 생쥐를 PBS 대조군과 control virus를 주사한 대조군, sLRP6E1E2 발현하는 바이러스를 주사한 실험군으로 분류하였다. 방사선 조사 후 16주 간 관찰하였으며, 그룹 간에 육안적인 변화에는 차이가 없었다. Masson's trichrome 염색을 이용한 반정량적 분석에 의하여 대조군에서는 지나친 콜라겐 섬유의 침착이 관찰되었으나, sLRP6E1E2 발현 실험군에서는 억제되는 효과를 확인할 수 있었다. 이러한 결과들을 종합해 볼 때, wnt/β-catenin 신호전달경로의 조절을 통하여 피부조직의 방사선 섬유화를 최소화할 수 있는 계기를 기대해 볼 수 있다.

핵심되는 말: radiation, fibrosis, fibroblast, adenovirus, gene therapy