

RESEARCH ARTICLE

BMPR2 Editing in Fibroblast NIH3T3 using CRISPR/Cas9 Affecting BMPR2 mRNA Expression and ProliferationDwi Aris Agung Nugrahaningsih^{1,*}, Eko Purnomo², Widya Wasityastuti³,
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Abstract

BACKGROUND: Bone Morphogenetic Protein Receptor II (BMP2) deficiency is associated with the pathologic development of pulmonary vascular changes in Pulmonary Arterial Hypertension (PAH). Fibroblast is the most abundant cell in vascular. However, there is only a little information regarding the effect of BMP2 deficiency in fibroblast. This study aims to understand the effect of BMP2 deficiency in fibroblasts.

METHODS: This study applied the CRISPR/Cas9 technique to edit BMP2 in NIH-3T3 cells. The transfection of CRISPR/Cas9 for BMP2 editing into NIH-3T3 cells was done by using chitosan nanoparticles. The evaluation of BMP2 and Transforming Growth Factor

(TGF)- β mRNA expression was done using Quantitative real-time polymerase chain reaction. The assessment of edited NIH-3T3 cells proliferation was done using a scratch test assay.

RESULTS: The BMP2 mRNA expression of CRISPR/Cas9-edited group was lower than the untreated group. The proliferation of the CRISPR/Cas9-edited group was higher than the untreated group. The TGF- β mRNA expression of CRISPR/Cas9-edited and untreated groups was similar.

CONCLUSION: BMP2 deficiency in fibroblast increase the fibroblast ability to proliferate.

KEYWORDS: BMP2, PAH, fibroblast NIH-3T3, CRISPR/Cas9, proliferation

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Introduction

Pulmonary Arterial Hypertension (PAH), characterized by dyspnea, inability to tolerate exercise, and pulmonary vascular resistance, often leads to right ventricular failure and death.(1) Based on The World Health Organization (WHO) classification, PAH belongs to group 1 of Pulmonary

Hypertension. This group includes several subgroups, namely Heritable Pulmonary Arterial Hypertension (HPAH), Idiopathic Pulmonary Arterial Hypertension (IPAH), and Associated Pulmonary Arterial Hypertension (APAH) with several conditions, *i.e.*, connective tissue disease, congenital heart disease, or xenobiotics exposure. (2) The pathological changes in PAH include an increase in pulmonary vascular contractility, vascular endothelial

dysfunction, and a progressive proliferation of vascular pulmonary vascular endothelial and smooth muscle cells leading to pulmonary vascular occlusion.(3)

Genetic variation is one of the crucial factors associated with PAH. The primary gene suggested to be involved in PAH is Bone Morphogenetic Protein Receptor II (BMPR2). More than 80% of HPAH patient and 6-40% of IPAH patients has a mutation of BMPR2.(4-7) PAH development correlates with BMPR2 functional changes or BMPR2 reduced expression.(8) Patient with both primary (idiopathic) and familial PAH shows a low expression of BMPR2 protein. Patients with secondary PAH also show a low expression of BMPR2 in their vascular.(9) Several mutations in BMPR2 associated with PAH have been identified that includes nonsense, missense, frameshift mutation, and significant gene rearrangements.(10)

BMPR2 belongs to the Transforming Growth Factor Beta (TGF- β) cell-signaling superfamily. Besides TGF- β , there are several other ligands for BMPR2, such as Bone Morphogenic Proteins (BMPs), and several growth differentiation factors.(8) Previous conducted review shows that BMPR2 deficiency is related to the increase of the thickness of vascular intimal.(11) BMPR2 knocked out in Pulmonary Artery Endothelial Cell (PAEC) increases PAEC apoptosis. Silencing mRNA BMPR2 in PAEC using siRNA also increases PAEC proliferation and migration.(12-13) In Pulmonary Smooth Muscle Cell (PASMC), BMPR2 is an essential factor for the development of PAH, which includes two main things: increased proliferation and decreased apoptosis. BMPR2 signal dysregulation is associated with a characteristic of Smooth Muscle Cell (SMC) in PAH. (14) However, there are limited data regarding the effect of BMPR2 deficiency in fibroblast, which is one of the main components in the vascular wall structure.

Methods

The study utilized mouse embryonic fibroblast, NIH-3T3 (ATCC® CRL-1658, ATCC, Manassas, VA, USA). The study was done after receiving ethical clearance from the ethical committee Faculty of Medicine, Public Health, and Nursing Universitas Gadjah Mada with reference number: KE/FK/0868/EC/2019.

Cell Culture and Treatment

The NIH 3T3 cell was cultured in DMEM (Gibco, Waltham, MA, USA) completed media containing 10% FBS (Gibco,

New York, USA) and 2% penicillin-streptomycin (Gibco) and incubated in a 5% CO₂ incubator. The experiment was done two times, and each experiment was done in triplicate.

Target Site Selection

The gRNA was predesigned from integrated DNA Technology (design ID: Mm.Cas9.BMPR2.1.AB). The gRNA sequence was GAGCACGTGTTATGGTCTGT, and the Protospacer Adjacent Motif (PAM) was GGG.

Ribonucleoprotein Complex Formation and Transfection

To examine the effect BMPR2 deficiency in fibroblasts, we edited the BMPR2 gene in NIH 3T3 using CRISPR-Cas9 techniques. Genome editing was performed based on the CRISPR CAS-9 kit manual (Alt-R® CRISPR-Cas9 crRNA; Alt-R® CRISPR-Cas9 tracrRNA; Alt-R® S.p. Cas9 Nuclease V3, Integrated DNA Technologies INC, Coralville, IA, USA). Briefly, Ribonucleoprotein (RNP) Complex was prepared by mixing 1 μ L 100 μ M cr-RNA and 1 μ L 100 μ M Tracr-RNA. Twelve μ L 1 μ M Cas-9 was then added to RNP complex mixture, and the mixture was incubated for 5 minutes. The CRISPR-Cas9 RNP complex was incorporated into the cell using chitosan nanoparticles. The RNP complex mixture were mixed with 0.2% chitosan in DMEM, and 66.6 μ L 0.05% tripolyphosphate (TPP). The mix of RNP, chitosan, and TPP was used to replace the cell medium during transfection. The cells were incubated in 5% CO₂ incubator for 6 hours for transfection.

Genome Editing Evaluation

Genome editing evaluation was done by sequencing. The chromatogram was examined using free online software, Inference of CRISPR edits (ICE) (Synthego, Redwood City, CA, USA). The primers used for sequencing were GAATCAAGAACGGCTGTGTGC (forward) and GATCCTGCCGTGTGCATCATA (reverse).

mRNA Expression Evaluation

The mRNA isolation was done using the FavorPrep™ Tissue Total RNA Mini Kit FATRK 001-2 (Favorgen, PingTung, Taiwan). The cDNA was synthesized with the [RP1400]ExcelRT™ Reverse Transcription Kit II (SMOBIO Reverse Transcription Kit II, Hsin Chu City, Taiwan). The RT-PCR (GoTaq® qPCR Master Mix A6001, Promega Cooperation, Madison, WI, USA) was performed using 40 cycles of the following thermal conditions: pre-denaturation at 95°C for 1 min, denaturation at 95°C for 30 Sec, and annealing at 61°C for 30 Sec.

Cell Proliferation Evaluation

The CRISPR/Cas9-edited and untreated NIH-3T3 cells were seeded in the microplate, incubate overnight and then a scratch was made using the tip of a 10 μ L tip with a longitudinal line at the base of the culture disk. The cells were observed and photographed using Optilab (PT MICONOS, Sleman, Indonesia) and incubated for 24 hours. The cells were observed and photographed again after 24 hours. The change of the scratch width was measured by comparing the distance before and after 24 h at the same point.

Statistical Analysis

The data were presented as mean \pm SEM. Statistical analysis of mRNA expression and cell proliferation means was performed by an unpaired T-test using GraphPad Prism 9.0.0 (GraphPad Software, Inc., San Diego, CA).

Results

The BMPR2 gene was successfully edited in NIH 3T3. Figure 1A showed sanger sequence results of untreated and CRIPRS-Cas9-edited samples from ICE software and indel percentages of the editing. The sequence analysis status using ICE analysis was succeeded. Indel percentage, which

presents the difference between CRISPR/Cas9-edited and untreated samples sequences, was 85%. The discordance plot (Figure 1B) showed the level of alignment per base between the untreated (represented in an orange curve) and the CRISPR/Cas9-edited sample (represented in a green curve). The orange and green lines should close together before the utting site (horizontal black dot line) and far apart after the cutting site, representing the sequence discordance between the untreated and CRISPR/Cas9-edited sample. The discordance plot portrays detected indel as blue bar on the x axis and the percentage of the indel on the y axis.

Figure 2 showed the BMPR2 mRNA expression in untreated and BMPR2 mRNA expression in CRISPR/Cas9-edited samples. The BMPR2 mRNA expression in the CRISPR/Cas9-edited group was lower compare to those on the untreated group (0.4323 \pm 0.05 vs. 1.326 \pm 0.43, respectively).

The closure of scratch area in scratch wound assay represented proliferation of the cells. The representative picture and analysis were shown in Figure 3 and 4. The picture and analysis demonstrating that cell proliferation in the cell-free area after 24 hours incubation was significantly accelerated in the CRISPR/Cas9-edited group when compared to the untreated group. The percent proliferation of NIH 3T3 cell in the CRISPR/Cas9-edited vs. the untreated group is 71.69 \pm 1.61 vs. 55.01 \pm 3.71, respectively.



Figure 1. The result of ICE analysis. A: Comparison of Sanger sequencing of untreated versus CRIPSR/Cas9-edited samples. Horizontal black underlines region is the guide sequence. The horizontal red underline is PAM site. The vertical black dotted line represents the actual cut site. B: Indels and discordance plots (the orange color is untreated, the green color is edited sample and the horizontal black dot line is the cutting site).

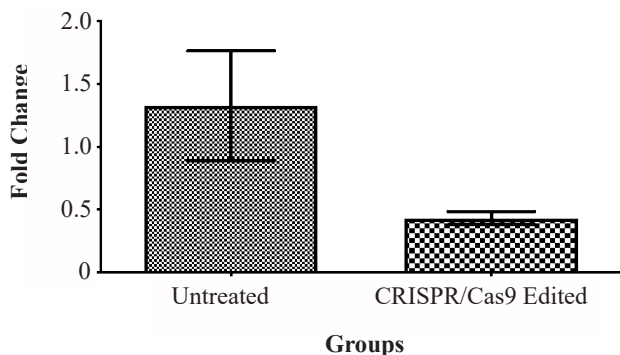


Figure 2. Fold change in BMPR2 mRNA expression of the NIH 3T3. Tested with unpaired T-test, $p < 0.05$.

The TGF- β mRNA expression in the CRISPR/Cas9-edited sample showed higher expression compared to those on the untreated sample. However, the difference was not significant statistically. Figure 5 showed the TGF- β mRNA expression in untreated and CRISPR/Cas9-edited group. The TGF- β mRNA expression in CRISPR/Cas9-edited group was similar to those on untreated group (2.450 ± 0.98 vs. 1.043 ± 0.29 , respectively).

Discussion

BMPR2 editing in NIH 3T3 fibroblast was successfully achieved using the CRISPR/Cas9 technique with indel

percentages of 85% that means 85% of the detected sequences are different from the untreated. We had done five experiments to edit the BMPR2 gene using CRISPR/Cas9 before we got the editing result presented in this manuscript. However, each experiment has different nanoparticle concentration conditions, the CRISPR/Cas9 reagent concentration, or the length of incubation with RNP.

The deletion of BMPR2 in NIH 3T3 results in the decrease of BMPR2 mRNA expression. The BMPR2 edited NIH 3T3 cell also showed an increase in cell proliferation when measured using scratch wound assay. After 24 hours of incubation, the scratch area in BMPR2 CRISPR/Cas9-edited NIH 3T3 cell was closed faster than those in the untreated group. The area closure in scratch wound assay is known to be the result of cell proliferation and migration. However, since the system is not under starvation conditions in our setting, proliferation is supposed to be the predominant way causing the closure of scratch wound assay area.(15)

The development of PAH is associated with vascular inflammation, fibroproliferative development, and remodelling. Remodelling the pulmonary vascular in PAH results from an imbalance between proliferation and death of pulmonary vascular cells.(16) Patient with PAH shows a low expression of BMPR2 protein in their vascular.(9) In our study, BMPR2 deletion in NIH3T3 fibroblast showed low BMPR2 mRNA expression but high NIH3T3 proliferation.

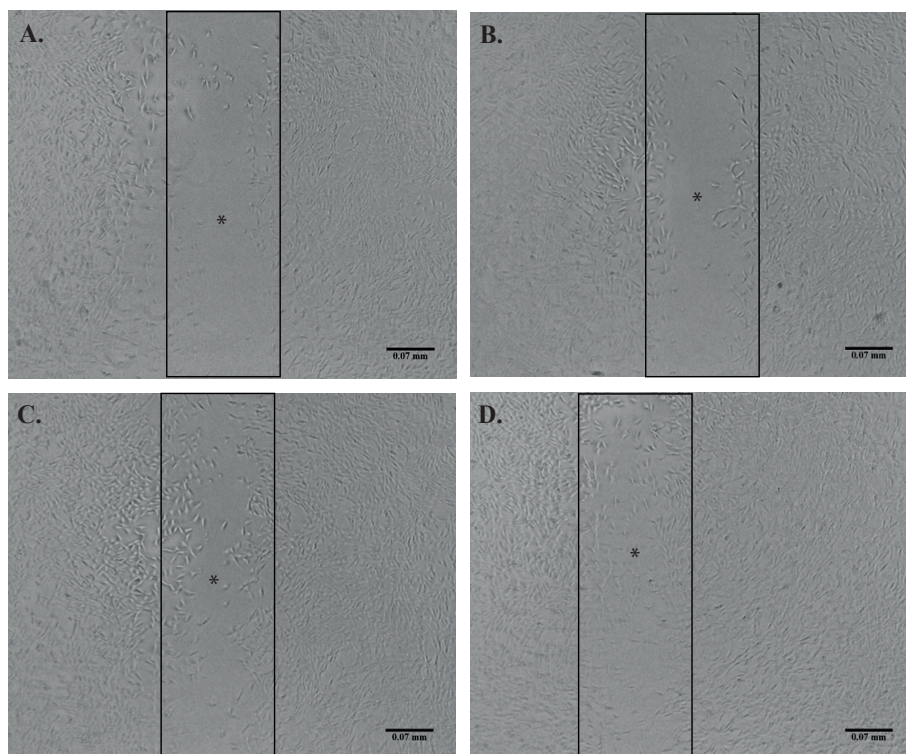


Figure 3. Representative pictures of scratch wound healing assays compare to cell proliferation in the cell-free region (*). A: Untreated group at 0 hour; B: CRISPR/Cas9-edited group at 0 hour; C: Untreated group at 24 hour; D: CRISPR/Cas9-edited group at 24 hour. Size bar=0.07 mm.

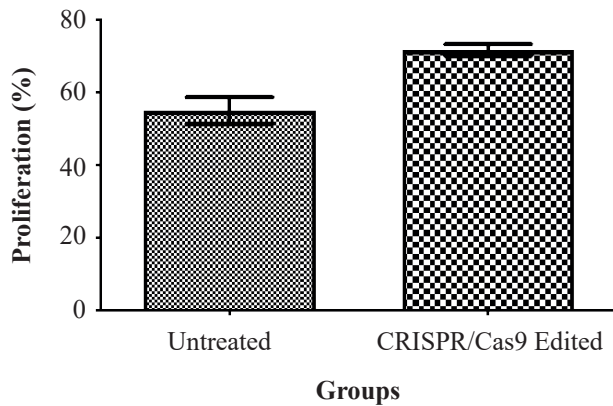


Figure 4. Percent proliferation of NIH 3T3 cell. Tested with unpaired T-test, $p < 0.05$.

Our results suggest that low BMPR2 expression might contribute to fibroblast proliferation.

More than 300 different genetic variations in BMPR2 have been found in PAH patients.(17) The variations include missense mutations, in-frame exon deletions, nonsense, and frameshift were identified in the BMPR2 coding region that is predicted to result in BMPR2 truncation and or loss of BMPR2 mRNA expression.(18) The previous study has demonstrated that some of the BMPR2 nonsense mutation cause nonsense mediated decay and finally result in haploinsufficiency.(19) There are 13 exons in BMPR2 that encode the primary functional domain of BMPR2. Exon 2-3 encodes the extracellular ligand-binding domain, exon 4 encodes the transmembrane domain, exon 5-11 encodes the serine/threonine kinase domain and exon 12-13 encodes the long cytoplasmic tail of BMPR2 protein. Variations have been found in all of those domains which most of the variations associated with PAH.(20)

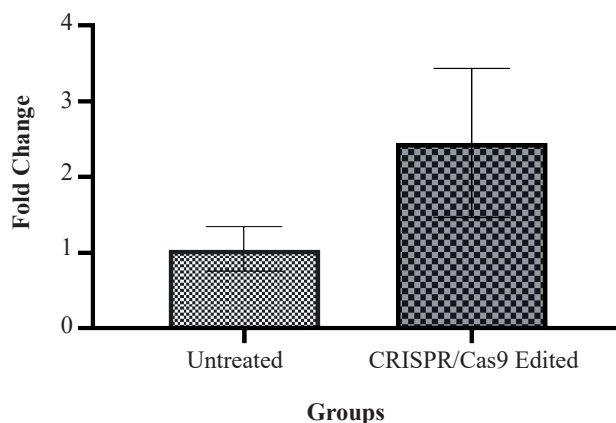


Figure 5. Fold change in TGF beta mRNA expression of the NIH 3T3. Tested with unpaired T-test, $p < 0.05$.

In this study, the guide RNA is targeted to edit exon 2 of the BMPR2 of the NIH 3T3. The editing of BMPR2 exon 2 was successfully done showed as high indel percentages. This study demonstrated that the edited BMPR2 in NIH 3T3 shows lower BMPR2 mRNA expression. We suggest that disruption in exon 2 of BMPR2 caused transcription disruption of BMPR2 mRNA expression. Interestingly, the BMPR2 edited NIH 3T3 cell also shows an increase of proliferation that was shown using scratch wound assay. The previous study in Human Pulmonary Artery Smooth Muscle Cells (HPASMCs) showed that suppression of BMPR2 signal increases HPASMCs proliferation and migration.(21) An *in vivo* study in rats with a deletion on exon 1 of BMPR2 showed that the rats showed low expression of BMPR2 and developed PAH with a proliferative phenotype of pulmonary microvascular.(22)

BMPR2 is the type 2 serine-threonine kinase receptor of TGF- β 1.(8) In the presence of ligand, BMPR2 will phosphorylate the type 1 receptor, which then activates both canonical and non-canonical signal-transducing targets. In the canonical pathway, R-Smads 1, 2, 3, and 8 will be activated. The activated R-smad will interact with smad-4 and be translocated to the nucleus. This translocation will subsequently regulate the transcription of several genes, including cell migration, proliferation, and apoptosis.(23) In the non-canonical pathway, several signals transducing target *i.e.*, Extracellular Signal-Regulated Kinase (ERK), proto-oncogene tyrosine-protein kinase Src (*c-Src*), and p38 Mitogen-Activated Protein Kinase (MAPK) will also be activated. These pathways are related to the regulation of cytoskeletal dynamics and cell survival.(24) Therefore, disruption of BMPR2 such as in BMPR2 deficiency, cell proliferation is increase.

Our study also shows that BMPR2 knocked out fibroblast cells, and the TGF- β mRNA expression was similar to those on untreated. The TGF- β signaling is involved in the vascular remodelling.(25) There are controversies regarding BMPR2 and TGF- β relation in pulmonary hypertension. Some studies showed that increased activity of TGF- β is related to reduced activity of BMPR2 in pulmonary hypertension.(26,27) However, another study showed that both BMPR2 and TGF- β involve the development of elastic fibre structure independently. Our study reinforces the hypothesis that BMPR2 expression is not influencing TGF- β expression.(28) Therefore, strategies to restore a balance between TG- β and BMPR2 signals are needed to develop pulmonary hypertension treatment.

Conclusion

Although TGF β mRNA expression was similar between CRIPSR/Cas9 edited and untreated NIH 3T3 fibroblast, the disruption of BMPR2 in NIH 3T3 fibroblast resulted in low expression of BMPR2 mRNA, and high cell proliferation. Taken together, BMPR2 deficiency in fibroblast increase the fibroblast ability to proliferate.

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Authors Contribution

DAAN, EP, RM, NA, TH were planning the idea of the study; EP and WW were drafting the manuscript; DAAN and WW were analyzing the data; RM and NA were interpreting the study result; meanwhile DAAN, RM, NA, and TH were supervising the study.

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