SUPPORTING INFORMATION

Activation of β2-adrenergic receptor signals suppresses mesenchymal phenotypes of oral squamous cell carcinoma cells

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S1 SUPPORTING MATERIALS AND METHODS

S1.1 Cell culture and reagents

Human oral squamous carcinoma cell lines, SAS and HSC-4 were obtained from the RIKEN BioResource Center Cell Bank (Tsukuba, Japan). HEK-Blue TGF-β reporter cells (HEK293 cell-derived TGF-β responsive reporter cells) were purchased from InvivoGen (San Diego, CA, USA). The cells were maintained in DMEM (Nacalai Tesque, Kyoto, Japan) supplemented with 10% fetal bovine serum (FBS; Sigma, St. Louis, MO, USA), 100 units/mL penicillin, and 100 μg/mL streptomycin (Nacalai Tesque).

S1.2 Establishment of oral cancer cell line-based reporter system to screen for low molecular weight compounds inducing mesenchymal epithelial transition (MET)

The experimental procedures were approved by the Genetically Modified Organisms Safety Committee of Tokyo Medical and Dental University (registration number: G2019-026C). The MET-reporter cell-based system was established using a reporter construct PEcadZsG containing green fluorescent protein, ZsGreen-1, under control of CDH1/E-cadherin promoter.[1] The SAS cells (2.5×10^5 cells) were plated into 6-well plate and transfected with PEcadZsG using Lipofectamine 2000 Transfection Reagent (Thermo Fisher Scientific, Waltham, MA, USA), followed by isolation of G418-resistant stable transfectants. A stable clone SAS-A3 showing the best response to SB431542 or TGF-β1 was used for chemical library screening.

S1.3 Chemical library screening

The 1,600 low molecular weight well-characterized compounds (10 mM stock solutions) owned by Chemical Biology Screening Center of Tokyo Medical and Dental University (TMDU) were used in screening. The MET reporter clone SAS-A3 was seeded into 24-well plates (1.0×10^5 cells/well) and incubated for 16 h at 37ºC in 5% CO_2. The medium was replaced with DMEM, 10% FBS containing chemical compounds at the concentration 10 μM and the cells were incubated for 72 h. The inhibitor of TGF-β signals, SB431542, and TGF-β1 were used as positive and negative controls, respectively. After 72 h-treatment, cells were washed with PBS (-) and observed under All-in-One fluorescent microscope, BZ-X710 (Keyence, Osaka, Japan). Compounds increasing fluorescent intensity and inducing tight interaction between cells were considered as positive and used for further evaluation.

S1.4 Immunocytochemistry

The SAS or *ADRB2* KO SAS cells were seeded on cover glasses placed into 12-well tissue culture plates and incubated for 16 h at 37ºC in 5% CO_2 followed by culture on the absence (control/-) or the presence of 1 ng/ml TGF-β1, 10 μM SB431542, or 10 μM isoxsuprine for 72 h. The cells were then fixed with an ice-cold mixture of methanol-acetone (1:1) for 20 s and blocked with 1% BSA, followed by incubation with anti-E-cadherin (1: 200 dilution, 3195; Cell Signaling Technology, Danvers, MA, USA), anti-SM22α (1: 2000 dilution, ab14106; Abcam, Cambridge, UK) or anti-vimentin (1: 1000 dilution, ab92547; Abcam) antibodies. The proteins were visualized with anti-mouse IgG (H+L) Alexa Fluor 488-conjugated (A-11001; Thermo Fisher Scientific) and anti-rabbit IgG (H+L) Alexa Fluor 594-conjugated (A-21207; Thermo Fisher Scientific) secondary antibodies depending on the experiment. Hoechst33342 (Cell Signaling Technologies) was used for nuclear staining. Images were obtained All-in-One fluorescent microscope, BZ-X710 (Keyence). ImageJ software (National Institutes of Health, Bethesda, MA, USA) was used for the quantification of vimentin-positive area in each field of view (n=5) and the number of Hoechst-positive cells. The values represented in each observed field in relation to the number of cells.

S1.5 Smad2/3/4-responsive reporter assay (HEK-Blue TGF-β reporter assay)

TGF-β/Smad signal activation was determined using HEK-Blue TGF-β reporter cells. HEK-Blue cells were seeded into 96-well plates (1.0×10^5 cells/well) and incubated for 16 h at 37ºC in 5% CO_2. The medium was replaced with 100 μL of serum-free DMEM and the cells were incubated for 2 h. The SB431542 or isoxsuprine were then added to the cells (final concentration 10 μM) and the cells were incubated for additional 1 h, followed by addition of 1ng/ml TGF-β1 and further incubation for 24 h. A mixture of SB431542 and TGF-β1 in serum-free DMEM was used as a positive control for inhibition of TGF-β signals. The activation of TGF-β/Smad signals was detected with the QUANTI-Blue substrate (InvivoGen). Fraction of medium was mixed with substrate and incubated for 30 min at 37ºC. The colorimetric changes of the substrate by the released SEAP were quantified at 640 nm using a spectrophotometer.

S2 SUPPORTING REFERENCES

1. Harazono Y, Muramatsu T, Endo H, *et al.* miR-655 is an EMT-suppressive microRNA targeting ZEB1 and TGFBR2. *PLoS One* 2013; **8**: e62757.

TABLE S1. Primers for quantitative RT-PCR

Transcript	Primer	Sequence (5' to 3')
ACTB (β-actin)	5'	TCACCCACACTGTGCCCATCTACGA
	3'	CAGCGGAACCGCTCATTGCCAATGG
CDH2 (N-cadherin)	5'	TTCCTTGCTTCTGACAATGG
	3'	ATGTCATAATCAAGTGCTG
FN1 (Fibronectin)	5'	AAACCAATTCTTGGAGCAGG
	3'	CCATAAAGGGCAACCAAGAG
IL6 (IL-6)	5'	CTTCGGTCCAGTTGCCTTCT
	3'	TGGAATCTTCTCCTGGGGGT
PMEPA1 (TMEPAI)	5'	TTTGTGGGAATGGCTTTGCG
	3'	GAGGACACTGGGCTTCAACA
TAGLN (SM22α)	5'	TCAAGCAGATGGAGCAGGTG
	3'	GCTGCCATGTCTTTGCCTTC
VIM (Vimentin)	5'	GCAAAGATTCCACTTTGCGT
	3'	GAAATTGCAGGAGGAGATGC

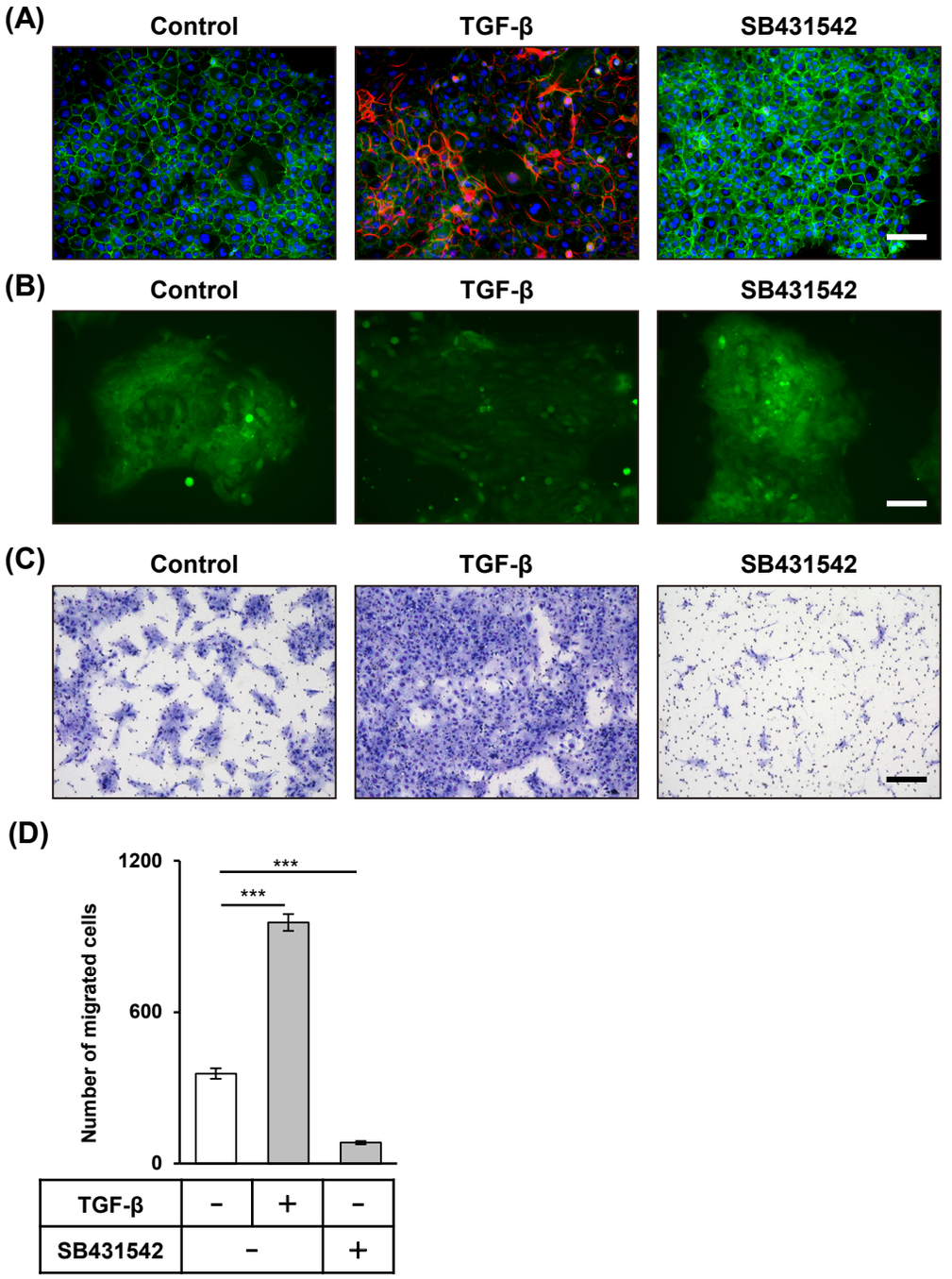


FIGURE S1 Effect of TGF-β signals on the mesenchymal characteristics of SAS and SAS-A3 oral cancer cells stably expressing green fluorescent protein under the control of E-cadherin promoter. SAS (A, C) and SAS-A3 (B) cells were cultured in the absence (-/control) or presence of 1 ng/mL TGF-β1 or 10 μM SB431542 for 72 h, followed by fluorescence immunostaining (A) for E-cadherin (green), SM22α (red) and nuclei (blue) and observation under fluorescent microscope (B). (C, D) The cells migrated for 48 h were stained (C) and counted (D). Scale bar: 100 μm (A, B), 200 μm (C). Data are represented as mean ± S.D. ***P<0.001.

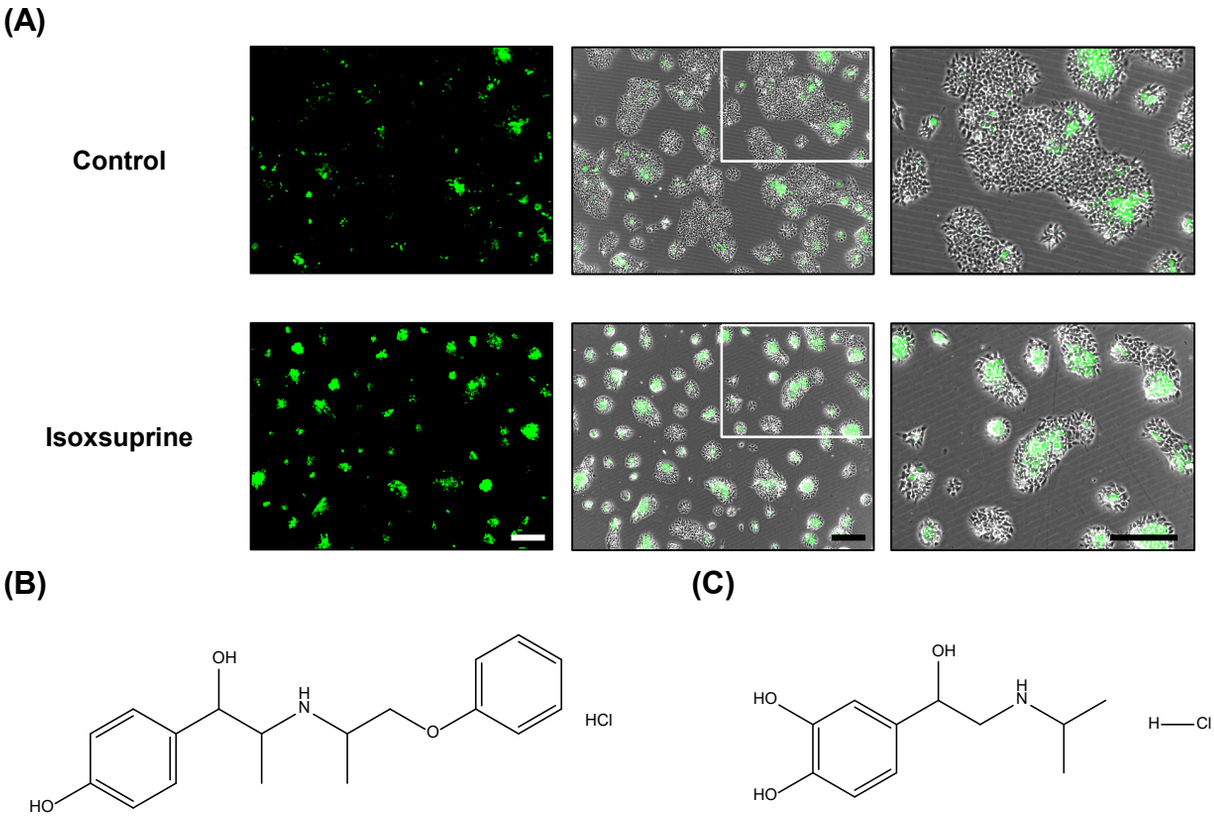


FIGURE S2 Identification of isoxsuprine as a low molecular weight compound that induces MET of SAS-A3 cells. (A) SAS-A3 cells were cultured in the absence (control) or presence of 10 μM isoxsuprine for 72 h followed by observation under fluorescence microscope and brightfield microscope. Representative photographs of fluorescence imaging of ZsGreen1 protein (left panels), the merged images with brightfield imaging (middle panels) of SAS-A3 cells and their enlarged images (right panels) are shown. Scale bar: 100 μm. (B, C) Chemical structures of isoxsuprine hydrochloride (B) and isoprenaline hydrochloride (C) were drawn with ChemDraw 19.1 Software (PerkinElmer, Waltham, MA, USA).

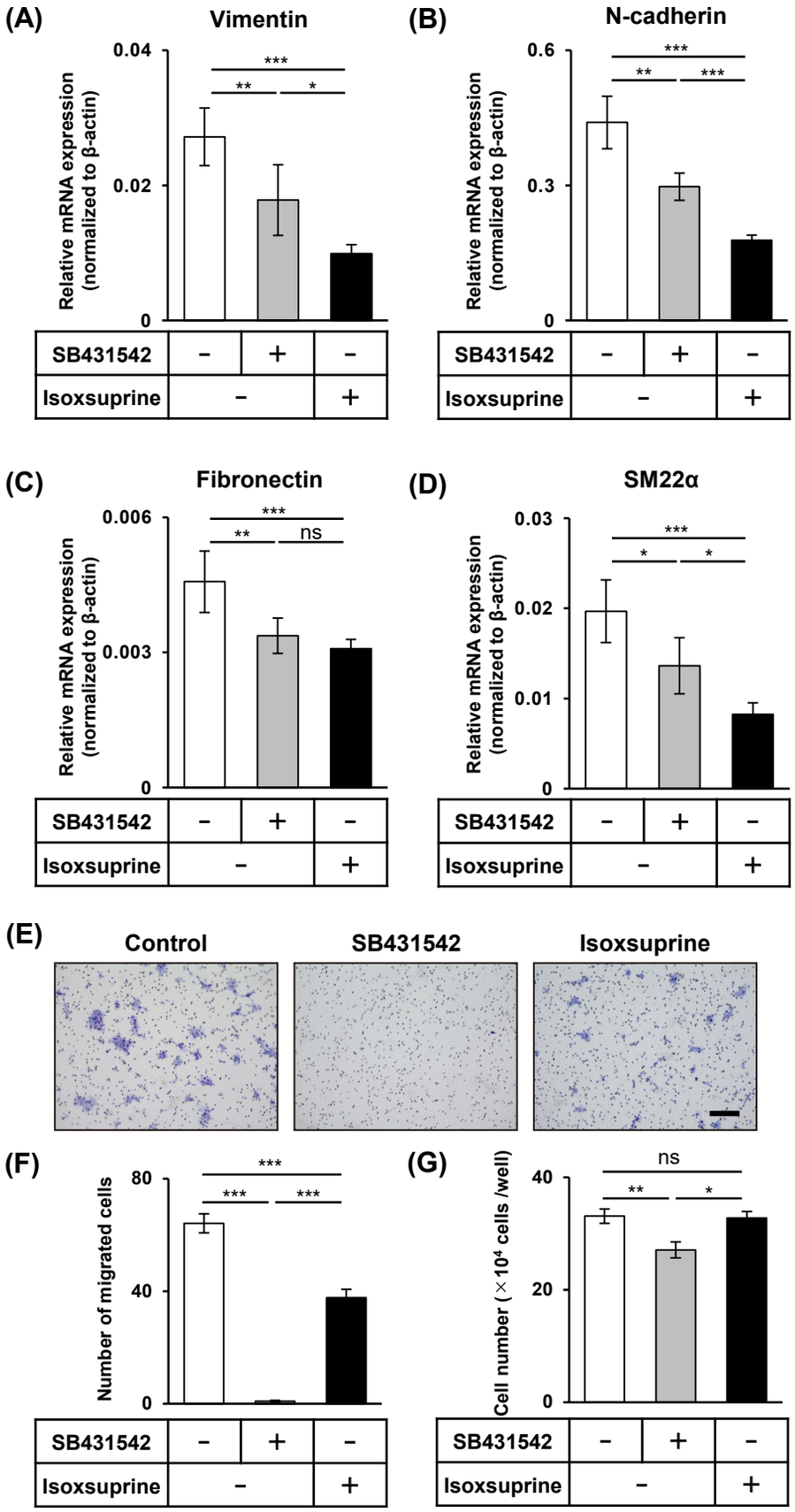


FIGURE S3 Effects of SB431542 and isoxsuprine on the mesenchymal characteristics, migration and proliferation of HSC-4 oral cancer cells. HSC-4 cells were cultured in the absence (-/control) or presence of 10 μM SB431542 or 10 μM isoxsuprine for 72 h, followed by the qRT-PCR analyses for the expression of vimentin (A), N-cadherin (B), fibronectin (C), SM22α (D), and chamber migration assay (E, F). The cells migrated for 48 h were stained (E) and counted (F). Scale bar: 200 μm. (G) The SAS cells were grown for 72 h in the absence (-) or presence of SB431542 or isoxsuprine and counted. Data are represented as mean ± S.D. *P<0.05, **P<0.01, ***P<0.001; ns, not significant.

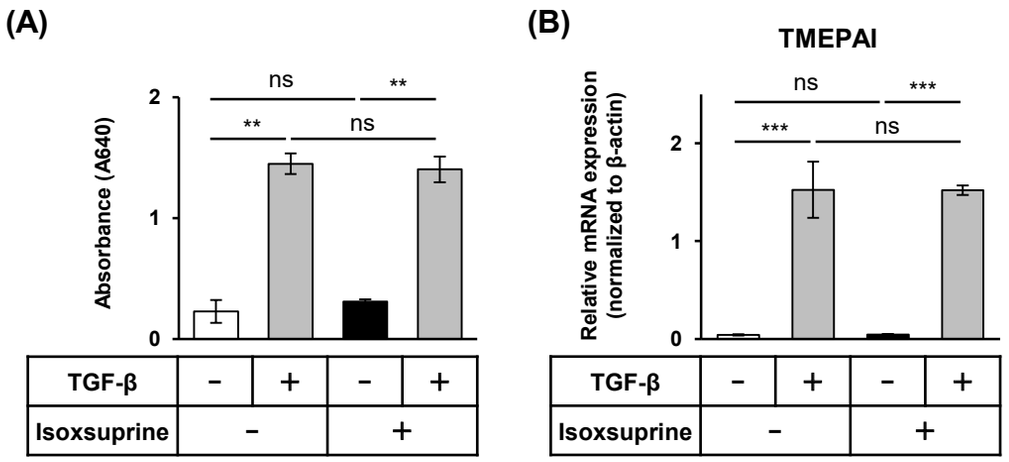


FIGURE S4 Effect of isoxsuprine on the activation of TGF-β signal. HEK-Blue TGF-β reporter (A) and SAS (B) cells were cultured in the absence (-) or presence of 1 ng/mL TGF-β1 in combination with 10 μM isoxsuprine, followed by measurement of activation of TGF-β signals (A) and qRT-PCR analysis for the expression of TMEPAI (B). The values represent TGF-β signal activation corresponding to the colorimetric changes of the Quanti-Blue substrate by SEAP at 640 nm. Data are represented as mean ± S.D. **P<0.01, ***P<0.001; ns, not significant.

(A)

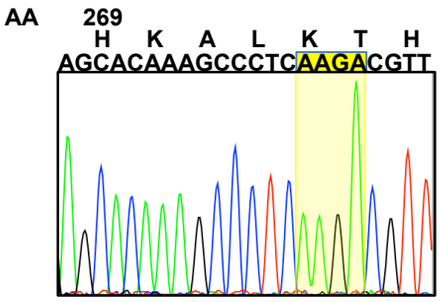
ADRB2 gene locus (chr5, NC_000005.10)

...AGCACAAAGCCCTCAAGACGTTAGGCA...

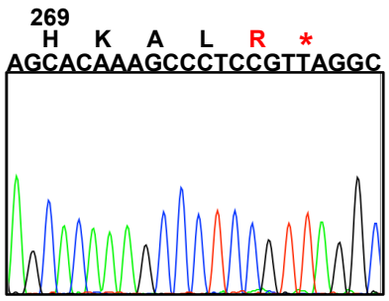
gRNA target

ADRB2 KO #1 ...AGCACAAAGCCCTCCGTTAGGCA...

(B) WT



(C) *ADRB2* KO #1



(D)

ADRB2 gene locus (chr5, NC_000005.10)

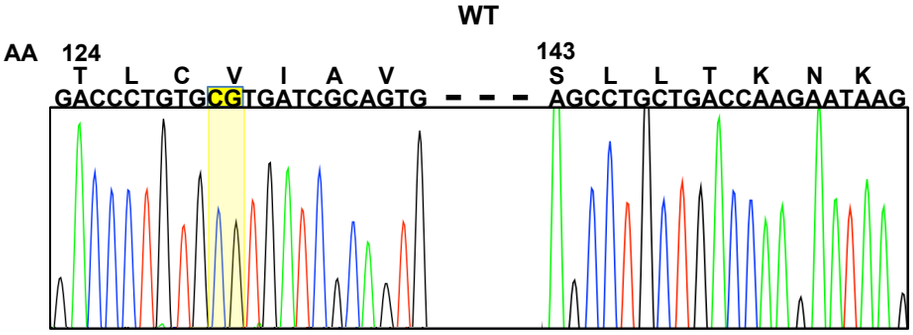
...GACCCTGTGCGTGATCGCAGTGGATCG...

gRNA target

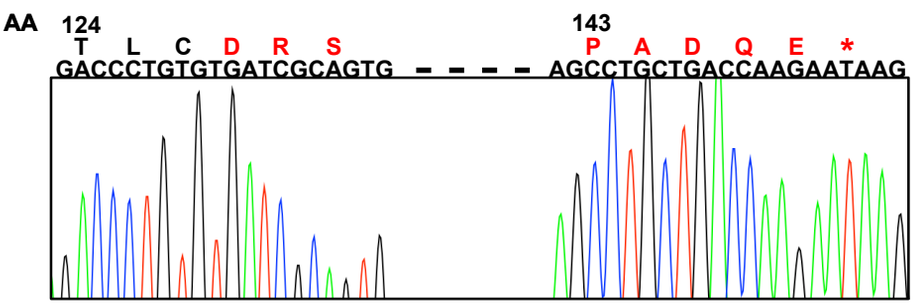
ADRB2 KO #2 ...GACCCTGTGTGATCGCAGTGGATCG...

...GACCCTGTGGCGTGATCGCAGTGGATCG...

(E) WT



(F) *ADRB2* KO #2 allele-1



(G) *ADRB2* KO #2 allele-2

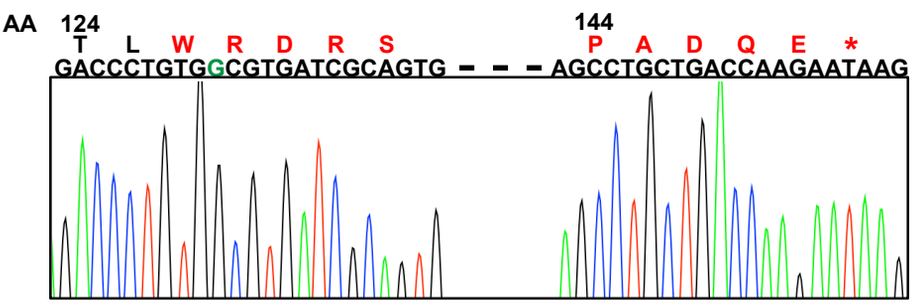


FIGURE S5 Establishment of β2-AR gene (*ADRB2*) knockout SAS cells using CRISPR/Cas9 system. (A, D) Experimental design of *ADRB2* targeting. The scheme represents gRNA #1 (A) and gRNA #2 (D) target sequences (underlined) with PAM (boxed) and Cas9 cleavage site indicated by arrowhead. Representative sequence electrograms of control (wild type: WT) (B, E) and *ADRB2* knockout (KO) clones, *ADRB2* KO #1 (C) and *ADRB2* KO #2 (F, G) at the target sites. *ADRB2* KO #2 cells have differential mutations referred as allele-1 (F) and allele-2 (G). Sequences highlighted in yellow in electrograms from WT cells (B, E) are deleted in *ADRB2* KO #1 (C) and *ADRB2* KO #2 allele-1 (F) cells. In *ADRB2* KO #2 allele-2 genome editing led to insertion of guanine residue (marked in green) (G). The amino acid (AA) sequences of WT and *ADRB2* KO cells are shown. The changes in amino acid sequence resulted from the frameshift mutations introduced by genome editing are marked in red. Premature STOP codons introduced by frameshift mutations are shown (*). Mutations in *ADRB2* KO #2 allele-1 (F) and allele-2 (G) cells resulted in the identical premature STOP codons.

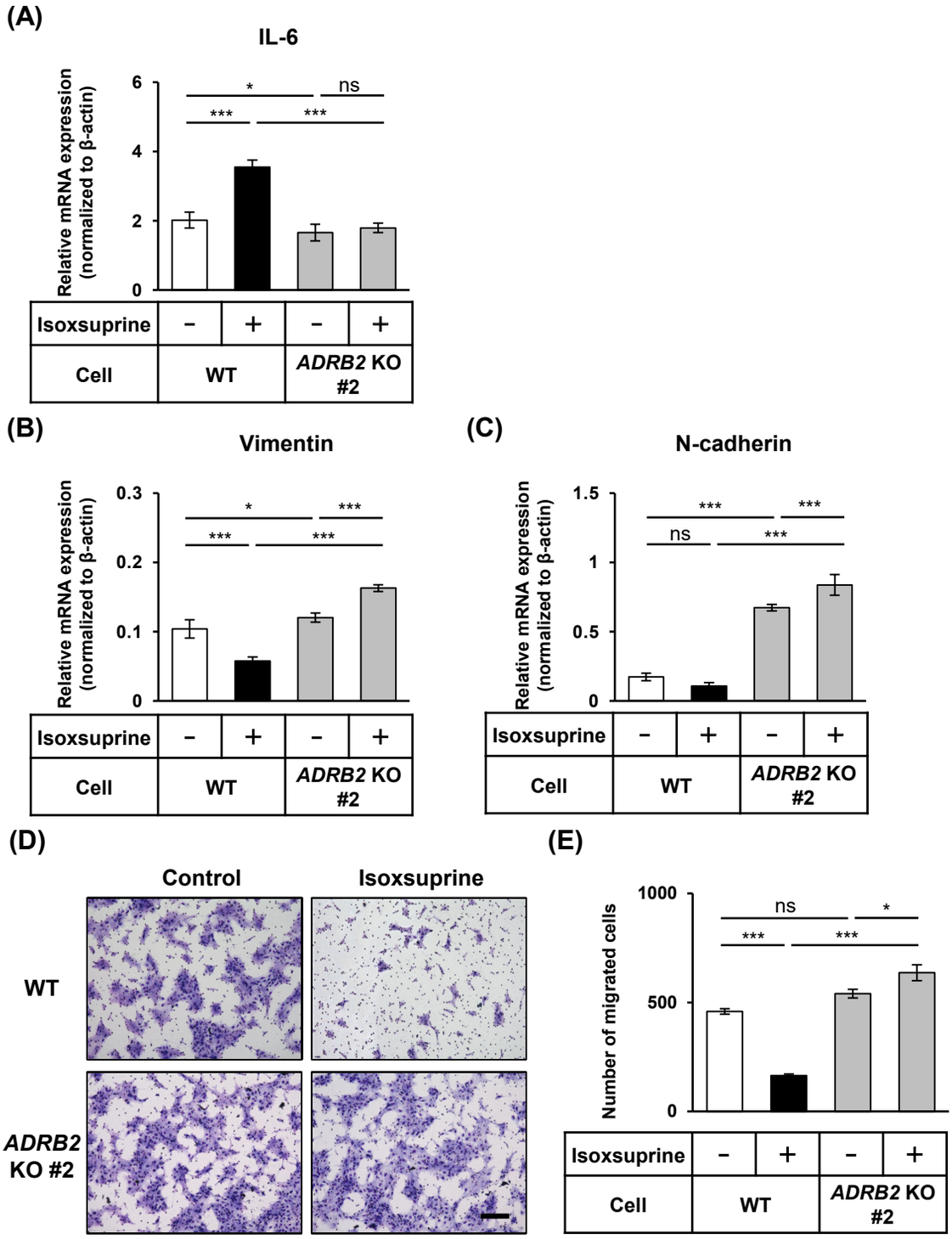


FIGURE S6 Roles of β2-adrenergic receptor in isoxsuprine-induced inhibition of mesenchymal traits. (A-C) The control (wild type: WT) and *ADRB2* knockout (*ADRB2* KO #2) SAS cells were cultured in the absence (-) or presence of 10 μM isoxsuprine for 72 h, followed by the qRT-PCR analyses for the expression of IL-6 (A), vimentin (B) and N-cadherin (C). (D, E) The WT and *ADRB2* KO #2 cells were cultured in the absence (-/control) or presence of 10 μM isoxsuprine for 72 h. The cells migrated for 48 h were stained (D) and counted (E). Scale bar: 200 μm. Data are represented as mean ± S.D. *P<0.05, ***P<0.001; ns, not significant.