Cell Proliferation and Migration Mechanism of Caffeoylserotonin and Serotonin via Serotonin 2B receptor in Human Keratinocyte HaCaT cells

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Supplementary Materials and Methods

Reagents

A FITC BrdU Flow kit assay kit was purchased from BD Pharmingen™ (San Jose, CA, USA). CaS was synthesized by mixing activated esters of hydroxycinnamic acids with serotonin hydrochloride in an alkaline solution as described previously (4). 5-HT and all other reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise specified. Anti-cyclin D1, cyclin E, p-Rb, c-Myc, c-Fos, 5HTR2B, MMP9, anti-β-actin, and horseradish peroxidase (HRP)-conjugated anti-goat, anti-mouse, and anti-rabbit IgG antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). p-GSK3 and GSK3 antibodies were bought from BioVison (Milpitas, CA, USA). Anti-p-Akt, Akt, p-ERK1/2, ERK1/2, p-p38, p38, p-JUK, and JNK antibodies were obtained from Cell Signaling Technology (Beverly, MA, USA). For RNA isolation, TRI reagent was purchased from MRC Inc. (Cincinnati, OH, USA). Maxime RT-PCR PreMix Kit was purchased from iNtRON (Sungnam, Korea).

Cell culture

Human keratinocyte HaCaT cells were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS), penicillin (100 U/mL),

and streptomycin (100 μg/mL) at 37°C and 5% CO_2 atmosphere.

Cell cycle analysis

To induce cell cycle synchronization, cells were brought to quiescence by serum starvation (medium without FBS) for at least 48 h or 0.1 M hydroxyurea for G1 arrest and 100 ng/ml Nocodazole for G2/M arrest. Cultured cells were trypsinized and fixed with 70% ethanol at 4°C overnight followed by staining with propidium iodide (PI). DNA contents were analyzed by using Attune acoustic focusing cytometer (Applied Biosystems, CA, USA). Flow cytometry histogram was further analyzed using Flowjo (Ashland, OR, USA) to obtain fitting for cell cycle fraction data.

BrdU incorporation

To confirm that CaS could stimulate S phase entry, DNA synthesis was measured using a FITC BrdU Flow Kit (BD Pharmingen™, San Jose, CA, USA) according to the manufacturer's instructions. Briefly, G1 synchronized cells were stimulated with 10 μM CaS or 5-HT for 24 h. Every 4 hours after the stimulation, cells were labeled with bromo-deoxy-uridine (BrdU) for 30 min before harvesting. Cell samples were subjected to FACS analysis of DNA content and BrdU incorporation.

Semi-quantitative reverse transcription polymerase chain reaction (semi qRT-PCR)

Quiescent HaCaT cells were treated with CaS or 5-HT for 1 h and subjected to total RNA isolation using TRI reagent (MRC Inc., Cincinnati, OH, USA) according to the manufacturer's instructions. To analyze c-myc, c-fos, cyclin D1, CDK4, CDK6, cyclin E, CDK2 and 5-HT2BR mRNA levels, reverse transcription and cDNA amplification

were carried out using 25 ng of isolated total RNA and Maxime RT-PCR PreMix. RT-PCR was performed in 40 cycles of 94°C for 30s, 60°C for 30s, and 72°C for 60s for c-myc, c-fos, cyclin D1, CDK6, CDK4, cyclin E, CDK2, and 5-HT2BR. For β-actin amplification, PCR was performed in 30 cycles of 94°C for 30s, 57°C for 30s, and 72°C for 60s. Oligonucleotide primers used for semi RT-PCR are listed in Supplemntary data 1-2.

Western blotting

G1-arrested HaCaT cells were pre-incubated with appropriate inhibitors for 30 min as necessary and then stimulated with CaS or 5-HT for 1 h. These cells were lysed in cold RIPA buffer (pH 7.4) containing protease inhibitor cocktail. Whole-cell lysate containing 20 μg of protein was subjected to SDS-PAGE and transferred to PVDF membranes. After blocking, these membranes were incubated with primary antibodies at 4°C overnight. After being washed, membranes were incubated with secondary antibodies and subsequently visualized using an enhanced chemiluminescence kit (Amersham Biosciences, Little Chalfont, UK). Equal loading was assessed using anti-β-actin antibody to normalize the amount of total protein.

Cell migration assay

For cell migration assay, HaCaT cells were grown to confluence in a 12-well plate, placed in serum free medium for 24 h, and scratched using 1 mL pipette tip. After incubating cells with 10 μM SB212205, a selective inhibitor of 5-HT2BR, cells were washed with PBS and refreshed with medium containing 0.5% FBS and CaS or 5-HT. To prevent apoptosis, cells were incubated in DMEM media containing 0.5% FBS. Cell

migration was recorded using a Cannon Power shot G12 (Japan). The area of wound sealing was calculated using NIH ImageJ software.

Transient transfection and luciferase assay

HaCaT cells were plated into 12-well plates at a density of 1.5×10^5 cells/mL at 4 hours before transfection. Transient transfection was performed using Hily Max method (Dojindo, Japan) with NF-κB dependent luciferase reporter plasmid pNF-κB-Luc (1 μg) or AP-1 dependent luciferase reporter plasmid pAP-12-Luc (1 μg) and 20 ng *Renillar* luciferase expression vector (pRL-TK). pRL-TK was co-transfected to normalize the transfection efficiency as control. DNA/Hily Max ratio was 1:3 (μg/μL). Cells were maintained in the presence of this mixture for 4 h and then washed. Cells were then treated with inhibitors for 30 min and incubated with 10 μM CaS or 5-HT for 24 h. After drug treatments, cells were lysed with 250 μL Reporter Lysis Buffer (Promega, Madison, WI, USA). Luciferase activity from 20 μL of lysate was measured using a luminometer (Molecular Devices, Sunnyvale, CA, USA).

Statistical analysis

Values are expressed as mean ± SD for three independent experiments. Statistical significance in pairwise comparison was evaluated using Student's *t*-test. Statistical differences were determined with analysis of variance (ANOVA) using SPSS 12.0 K (Chicago, IL, USA). *P* values < 0.05 were considered statistically significant.

Supplementary data

S1. List of primers used for semi RT-PCR of cell cycle related genes in HaCaT cells

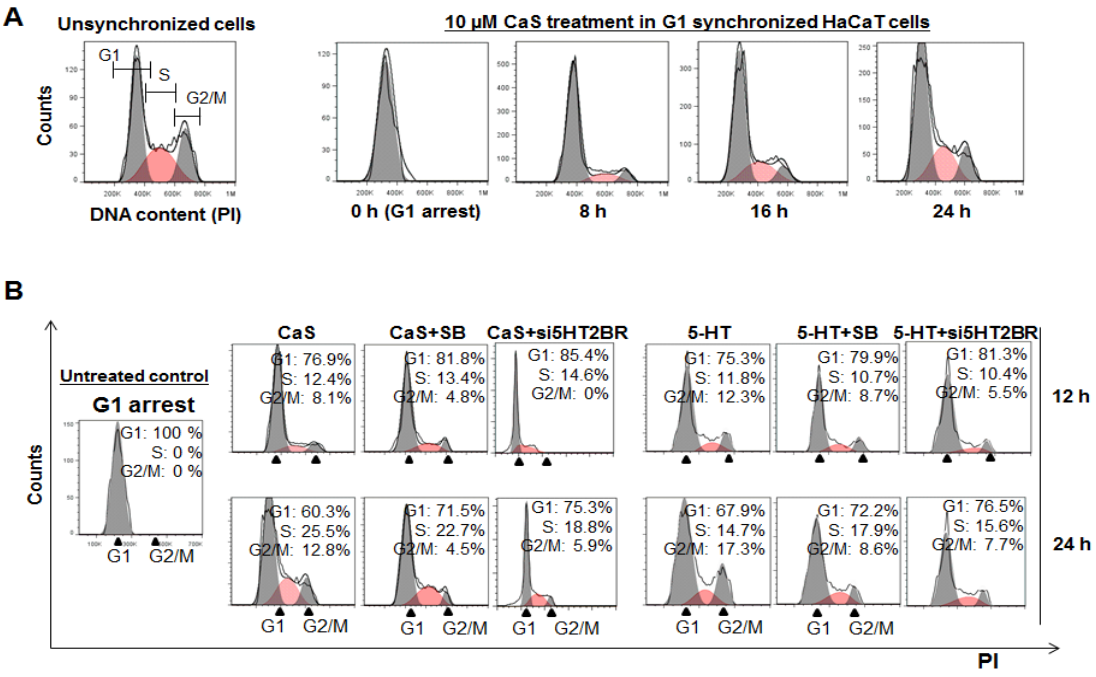
Gene	Forward (5'→3')	Reverse (5'→3')	Product size (bp)
c-myc	GATTCTCTGCTCTCCTCGACGGAG	GCGCTGCGTAGTTGTGCTGATGTG	274
c-fos	TTACTACCACTCACCCGCAGACTC	TGGAGTGTATCAGTCAGCTCCCTC	414
Cyclin D1	ACCTGGATGCTGGAGGTCTG	GAACTTCACATCTGTGGCACA	402
CDK4	TGCTGCAGAGCTCGAAAGGCA	CCTGTGGACATGTGGAGTGTTGGC	296
CDK6	CGAGGTGTTCTGGCTGGGCG	TGGACGTGATTGGACTCCCAGGA	269
Cyclin E	GGTGAGGAGCCCACTGGGGA	ACTTGCTGCTTCGGCCTTGT	292
CDK2	CTCACTGGAATTCCTCTTCCC	AACTTTACTAAAATCTTGCCG	468
β-actin	GGACTTCGAGCAAGAGATGG	AGCACTGTGTTGGCGTACAG	243

Human keratinocyte HaCaT cells were cultured in 10% DMEM for 24 h and then subjected to total RNA isolation using TRI reagent according to the manufacturer's instructions. To confirm mRNA expression levels of cell cycle related genes, reverse transcription and cDNA amplification were carried out with 25 ng of isolated total RNA using a Maxime RT-PCR PreMix. The reaction was cycled 40 times for c-myc, c-fos, cyclin D1, CDK6, CDK4, cyclin E, CDK2, and 5-HT2B with the following program: 30s at 94°C, 30s at 60°C, and 60s at 72°C. The reaction for β-actin was cycled 30 times with the following PCR program: 30s at 94°C, 30s at 57°C, and 60s at 72°C.

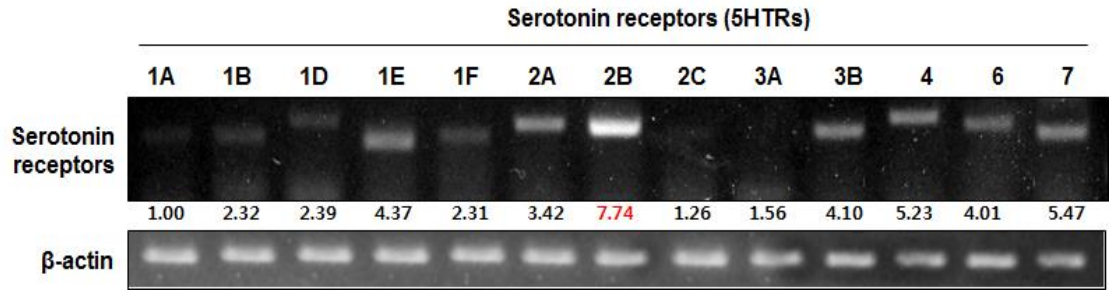
S2. List of primers used for semi RT-PCR of 13 HTRs in HaCaT cells

Gene	Forward (5'→3')	Reverse (5'→3')	Product size (bp)
5HTR1A	CCGCCTCTTTCGAGAGGAAA	GTTGGAGTAGCCCAGCCAAT	224
5HTR1B	AGTCAAAGTGCGAGTCTCCG	GGAGTTGAGATAGCCCAGCC	220
5HTR1D	CCCTGGAACGCAAGAGGATT	GCCTTCCGGAAAGGGACAAT	274
5HTR1E	CTCCACCTCAGACCCTACCA	TCCGAGGACACGGTGTAGAT	231
5HTR1F	GAGGTGAATGGCCAAGTCCT	TGGCTGCTTTCCGTTCTCTT	202
5HTR2A	GTTTGTGGTGATGTGGTGCC	CTTGTAGGCCAAAGCCGGTA	268
5HTR2B	ACATTTCGGGATGCATTTGGC	ATGGTTGAACTTCGGAGCCT	253
5HTR2C	CCGAAGGGCATTCTCCAACT	TTCGCTAACCACACTGGAGG	235
5HTR3A	GGAGAGAATCGCCTGGCTAC	AATTGCCGGATGGAGGACAG	240
5HTR3B	TGCATGGCCTTCTTGGTTCT	CCTGTTGGTCTGTCTGGTCC	280
5HTR4	AGGACAGAGACCAAAGCAGC	TCGGTAGCGCTCATCATCAC	303
5HTR6	CCACTCTTCATGCGGGACTT	GAAGAAATTGACGGCGGCAG	252
5HTR7	TGTGCTGGCTGCCATTTTTC	AGGTCTCTCTGGCCTCTCAG	272

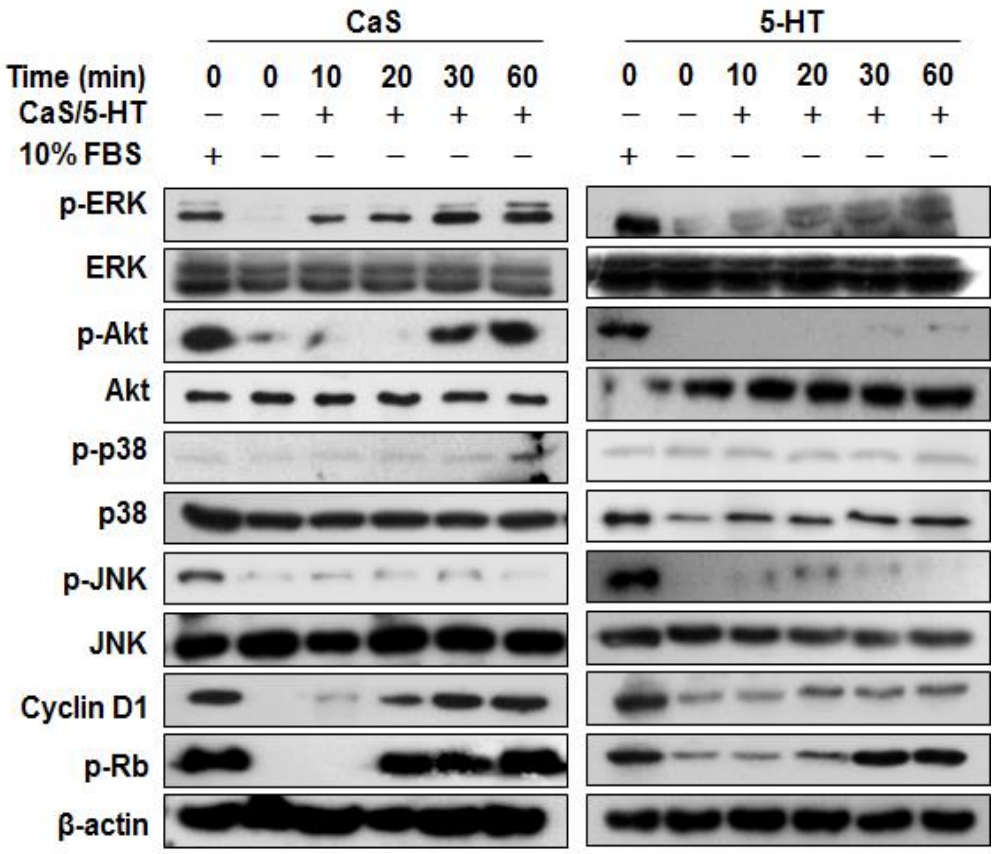
Human keratinocyte HaCaT cells were cultured in 10% DMEM for 24 h and then subjected to total RNA isolation using TRI reagent according to the manufacturer's instructions. To confirm mRNA expression levels of 13 serotonin receptors, reverse transcription and cDNA amplification were carried out with 25 ng of isolated total RNA using a maxime RT-PCR PreMix. The reaction was cycled 40 times for 13 serotonin receptors with the following PCR program: 30s at 94°C, 30s at 60°C, and 60s at 72°C. The reaction for β-actin was cycled 30 times with the following PCR program: 30s at 94°C, 30s at 57°C, and 60s at 72°C.



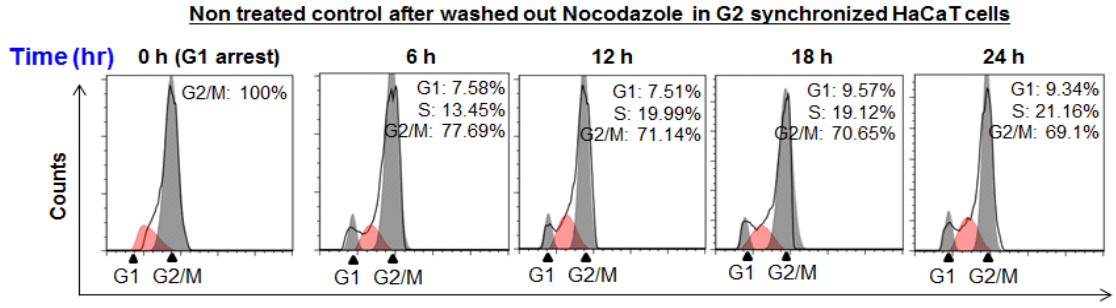
S3. Cell cycle analysis of HaCaT cells under various conditions. (A) Cell cycle analysis of HaCaT cells treated with CaS. FACS analysis of DNA content in HaCaT after PI staining. DNA profile of unsynchronized cells is presented in the left panel. Gates used to define cells in G1, S, or G2/M are indicated in this panel. Cells were synchronized in G1 phase (G1 arrest, 0 h) and released in the presence of CaS. Cell were then harvested for FACS analysis at different time points. (B) Effect of inhibitor and siRNA for 5-HT2BR on G1 progression induced by CaS or 5-HT. HaCaT cells synchronized at G1 arrest by serum starvation were pretreated with 10 μM SB215505 before treatment with CaS or 5-HT for 24 h. CaS or 5-HT treated cells were fixed and stained with PI and analyzed by FACS.



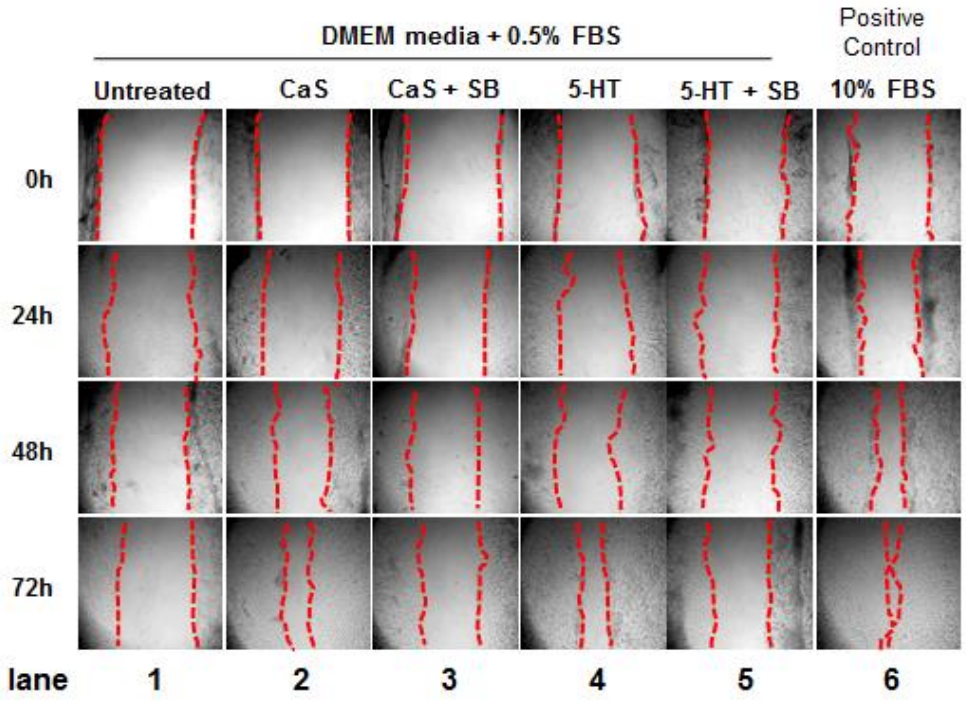
S4. RT-PCR analyses for 13 serotonin receptors for indicated time. Oligonucleotide primers and semi RT-PCR conditions which used in this experiment were the same as those explained in S2. β-actin was used as a loading control. Serotonin (5-HT) actions are mediated that through interactions with membrane-bound receptors that can be categorized into seven families with 13 subtypes, including 5-HT2B and 5-HT7 receptors that are generally detected in normal and pathologic human keratinocytes (16). In the current study, 5-HT2B mRNA was found in the majority of HaCaT cells at basal level.



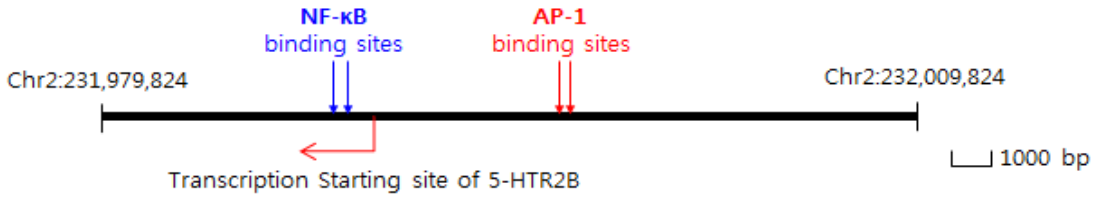
S5. Western blotting analysis for various proteins related to cell signaling pathways. CaS stimulated both p-ERK and p-Akt. However, 5-HT only stimulated p-ERK. p-Akt was observed only at detectable level. Moreover, there was no significant alteration in JNK or p38 pathway after 5-HT or CaS treatment. Protein expression levels of cyclin D1 and p-Rb in this figure provided clear comparison. . β-actin was used as a loading control.



S6. HaCaT cells arrested at G2/M were released simply by washing of nocodazole even without FBS, CaS or 5-HT. However, the portions of G1 was less than 10% of whole cells even after 24 h, and percent of the G2/M remained about 70% until 24 h after remove out nocodazole.

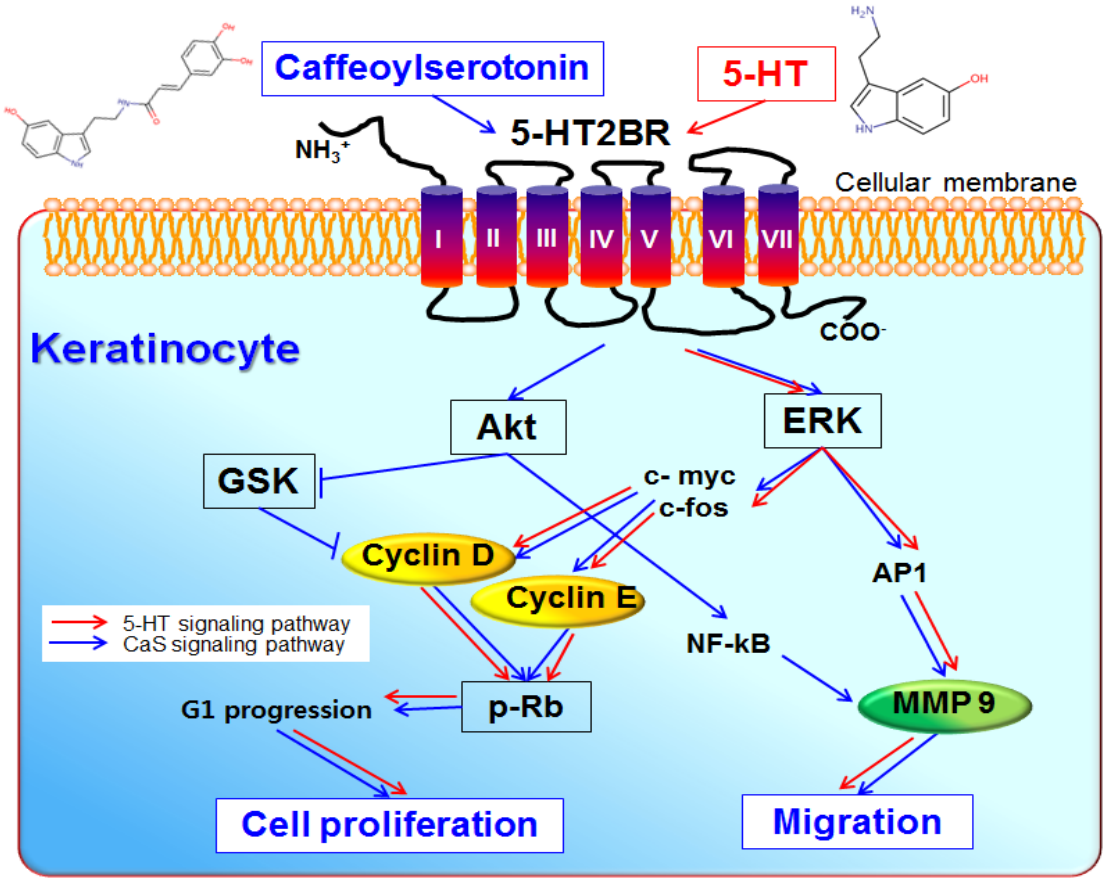


S7. Time-lapse analysis of wound healing in HaCaT cells. HaCaT cells were pre-treated with 10 μM SB215505. HaCaT monolayer was then scratched (dotted line) with a 1 mL pipette tip and cultured with 5-HT or CaS for indicated time period. HaCaT cells were cultured in DMEM media containing 0.5% FBS to avoid serum starvation induced cell apoptosis. Magnification, x20.



Transcription factor	Binding position	Strand	Binding Sequence
NF-kB	chr2: 231988258-231988270	–	AGGGTATTTCCAC
NF-kB	chr2: 231988832-231988844	–	CAGAGAGTTCCCC
AP-1	chr2: 231997033-231997042	+	TGTGAGTCAG
AP-1	chr2: 231996656-231996669	+	AGAATGTGTCAGAG

S8. The putative AP-1 and NF-κB motifs in the promoter region of the human 5-HT2BR gene. 5-HT increased both mRNA and protein expression of 5-HT2BR in human keratinocyte HaCaT cells. Both CaS and 5-HT significantly increased AP-1 levels. Moreover, CaS increased NF-κB level as well as AP-1 (Fig. 4). This diagram and table depicts the putative AP-1 and NF-κB motifs in the promoter region of the human 5-HT2BR gene. Therefore we suggest that 5-HT can stimulate the expression of 5-HT2BR via an AP-1-dependent mechanism. In addition, CaS may stimulate 5-HT2BR expression through both only AP-1 also NF-κB.



S9. Schematic representation describing putative pathway involved in CaS or 5-HT induced G1 progression and cell migration via 5-HT2BR. Both cell proliferation and cell migration induced by CaS or 5-HT are regulated by 5-HT2BR. However, CaS could stimulate cell proliferation and cell migration through both ERK and Akt pathways whereas 5-HT could stimulate cell proliferation and cell migration mainly through ERK pathway.