

Melatonin receptor heterodimerization in a photoreceptor-like cell line endogenously expressing melatonin receptors

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Purpose: Melatonin signaling plays an important role in the modulation of retinal physiology and photoreceptor viability during aging. In this study, we investigated whether 661W cells—a photoreceptor-like cell that endogenously expresses melatonin receptor type 1 (MT₁) and melatonin receptor type 2 (MT₂) receptors—represent a useful model for studying the biology of heterodimerization and signaling of MT_{1/2} receptors.

Methods: 661W cells were cultured, and MT₁/MT₂ heterodimerization in 661W cells was assessed with proximity ligation assay. MT₂ was removed from the 661W cells using the MT₂-CRISPR/Cas9 system. Melatonin receptor signaling was investigated by measuring cAMP levels and activation of the AKT-FoxO1 pathway.

Results: The results demonstrated that heterodimerization of MT₁ and MT₂ receptors occurs in 661W cells. The pathways activated by MT₁/MT₂ heterodimer (MT_{1/2h}) in 661W cells are similar to those previously reported in mouse photoreceptors. Disruption of the heterodimer formation by genetically ablating MT₂ from 661W cells abolished the activation of melatonin signaling in these cells.

Conclusions: The data indicated that in 661W cells, MT₁ and MT₂ receptors are functional only when they are associated in a heteromeric complex, as occurs in mouse photoreceptors. 661W cells represent a useful model for studying the mechanism underlying MT₁/MT₂ heterodimerization.

Retinal melatonin is synthesized by the photoreceptors of many vertebrate species via a well-defined biosynthetic pathway [1]. In the retina, as in the pineal gland, melatonin synthesis occurs during the night [2] and is under direct control of the circadian clock [3,4]. Transcriptional and post-translational mechanisms ensure that melatonin levels are maintained at extremely low levels during the light phase of the light-dark cycle [5,6], and appear to be important, as high melatonin levels during the light phase may be deleterious for photoreceptor cells [7,8]. Retinal melatonin acts mainly via the activation of two different G protein-coupled receptors (GPCRs) known as melatonin receptor type 1 (MT₁) and MT₂ [9-13] which are present in the retina [14-17].

A previous study [14] reported that in murine photoreceptors MT₁ and MT₂ appear to form functional heterodimers (MT_{1/2h}). This MT_{1/2h} signals via the activation of the Phospholipase C (PLC)- Protein Kinase- ζ (PKC- ζ ; Life technologies, Carlsbad, CA) pathway [14,18], and accumulating experimental evidence indicates that, at least in the mouse, MT_{1/2h} modulates the daily rhythm in the a- and b-waves of the scotopic electroretinogram and thus, light sensitivity [14,18]. Furthermore, it has been reported that the nocturnal activation of MT_{1/2h} signaling in photoreceptors protects rods

and cones during aging by modulating the AKT-FOXO1 survival pathway [19]. However, a significant barrier to full understanding of MT_{1/2h} signaling is the lack of a cell line in which the specific signaling of each melatonin receptor (i.e., MT₁, MT₂, and MT_{1/2h}) can be studied.

Recently, a cone-like photoreceptor cell line (661W cells) was developed [20], and has been recognized as a useful model for studying photoreceptor cell biology [21-25]. Several studies using 661W cells have partially elucidated the molecular mechanisms underlying photoreceptor cell metabolism [24-26] and cell death following light exposure [27-31] or oxidative stress [32-34]. Our laboratory recently showed that 661W cells express functional MT₁ and MT₂ receptors, and the activation of these receptors by exogenous melatonin can partially protect them from H₂O₂-induced cell death by inhibiting the AKT-FOXO1-Fas/FasL-caspase-3 pathway [35].

Although the formation and signaling of MT_{1/2h} have been previously studied, those experiments used overexpression of MT₁/MT₂ in human embryonic kidney (HEK) 293T cells [36,37] or mouse photoreceptors [14]. In this study, we investigated whether endogenous MT₁ and MT₂ receptors form MT_{1/2h} in 661W cells and studied the intracellular pathways activated by this GPCR heterodimer.

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METHODS

Cell culture: 661W cells were grown in Dulbecco's Modified Eagle's medium (DMEM; Gibco, Life Technologies, Carlsbad, CA) supplemented with 5% fetal bovine serum (Gibco) and 1% penicillin/streptomycin, at 37 °C in a 5% CO₂ humidified atmosphere [35]. Cells were seeded in six-well plates or 75-mm flasks at a concentration of 1×10^5 cells in a volume of 3–10 ml of media and expanded to approximately 50% to 90%, depending on the experiment.

In the proximity ligation assay (PLA) and immunofluorescence experiments, cells were seeded in sterile glass chamber slides and allowed to expand to 50% confluence. The genetic profile of the cells was established by IDEXX Bioresearch (Columbia, MO). Nine short tandem repeat (STR) loci including a gender-determining locus were analyzed (Appendix 1) [35].

Drug stock preparation: Melatonin (MEL; 8 mg/ml, Sigma, St. Louis, MO) and forskolin (1 μM; Sigma) stock solutions were prepared in absolute ethanol and then diluted to the working concentration in DMEM. Forskolin (25 mM; Sigma), luzindole (LUZ; 10 mM; Tocris, Ellisville, MO) and IIK7 (30 mg/ml; Sigma) stock solutions were prepared in dimethyl sulfoxide (DMSO) and diluted to working solutions in DMEM.

Colocalization of MT₁ and MT₂ in 661W cells: Cells were directly washed in PBS (1X; KCl 2.67 mM, KH₂PO₄ 1.47 mM, NaCl 139.9 mM, Na₂HPO₄ 8.1 mM, pH 7.4) and fixed in cold acetone for 2 min. Once fixed, the cells were washed in PBS twice for 10 min and incubated in BLOXALL (Vector Laboratories, Burlingame, CA) to block endogenous peroxidases and phosphatases for 10 min. Then, samples were washed twice with PBS for 10 min, blocked with PBS containing 0.02% Triton and 5% bovine serum albumin (BSA; 1 h at room temperature), and incubated with the primary antibodies MT₁ (0.004 mg/ml; rabbit, AMR-031; Alomone Laboratories, Jerusalem, Israel) and MT₂ (0.004 mg/ml; goat, SC13177; Santa Cruz Biotechnology, Santa Cruz, CA) for 3 h at room temperature. After incubation with the primary antibodies, the samples were washed three times in PBS and subsequently incubated with the secondary antibodies (0.1 mg/ml; anti-rabbit Alexa 488 and anti-mouse Alexa 594; Abcam, Cambridge, MA) for 1 h at room temperature. Finally, after two washes in PBS, the chambers were detached from the slide and mounted with VECTASHIELD mounting medium (Vector Laboratories). The slides were examined with a confocal microscope (Zeiss LSM700; Göttingen, Germany). Images were processed with Zeiss Cell software. Micrographs were generated in TIFF format and adjusted linearly for light and contrast before being assembled on plates using

Adobe Photoshop CS6 (San Jose, CA). To validate the MT₁ and MT₂ receptor antibodies, a secondary antibody control was used without the primary antibody, as well as an absorption control (by preincubating the antibody with its specific blocking peptide).

Proximity ligation assay: Cells were washed in PBS and fixed in cold acetone for 2 min. Then, the cells were washed in PBS twice for 10 min and incubated in BLOXALL (Vector Laboratories) to block endogenous peroxidases and phosphatases, for 10 min. Samples were then washed twice in PBS for 10 min, blocked with PBS containing 0.02% Triton and 5% BSA (1 h at room temperature), and incubated with previously validated primary antibodies [37], MT₁ (0.004 mg/ml; rabbit, AMR-031; Alomone) and MT₂ (0.004 mg/ml; goat, SC13177; Santa Cruz Biotechnology), overnight at 4 °C. After incubation with primary antibodies, PLA was performed following the manufacturer's instructions (Sigma) with minor modifications. Briefly, samples were washed two times in PBS for 10 min and one time in PBS with 0.02% Triton for 5 min. Subsequently, samples were incubated with the PLA probes (1:5 in blocking solution) for 1 h at 37 °C. Then after two washes in wash buffer A for 5 min, samples were incubated with ligase solution for 30 min at 37 °C. After two washes in wash buffer A for 2 min, samples were incubated with polymerase solution for 100 min at 37 °C. After washing in wash buffer B for 10 min, samples were incubated with SYTOX green (1:10,000) for 1 min to stain cell nuclei. Finally, after washing in wash buffer B for 10 min and wash buffer B diluted (0.01X) for 1 min, samples were mounted with VECTASHIELD mounting medium. Slides were examined with a confocal microscope (Zeiss LSM700). Images were processed with Zeiss Cell software. Micrographs were generated in the TIFF format and adjusted linearly for light and contrast before being assembled on plates using Photoshop CS6. To validate MT₁ and MT₂ receptor antibodies, a secondary antibody control was used (staining without the primary antibody) as well as an absorption control (by preincubating the antibody with its specific blocking peptide).

Establishment of MT₂^{-/-} cell lines using the CRISPR/Cas9 system: A construct to knock out MT₂ receptor was purchased from Santa Cruz Biotechnology, Inc. (MEL-1B-R CRISPR/Cas9 KO plasmid). The MEL-1B-R CRISPR/Cas9 KO plasmid is a pool of three different gRNA plasmids with the following sequences: sc-434093 A: Sense: 5'-AAC CGC AAG CTG CGG AAC GC-3'; sc-434093 B: Sense: 5'-GCA GCG CCT AGT GTT CCA TA-3'; sc-434093 C: Sense: 5'-CGT CAC GGA TAA TGG CCA CA-3'. The 661W cells cultured on a 12-well plate were cotransfected with 0.5 μg of the MEL-1B-R CRISPR/Cas9 plasmid and 0.5 μg of the MEL-1B-R

HDR plasmid, which included a puromycin resistance gene (used for selection of colonies) and red fluorescent protein (RFP) to detect the correct insertion of the plasmid in the genome (Santa Cruz Biotechnology, Inc.). After selection in medium containing puromycin (4 µg/ml), only cells with RFP signals were isolated.

Detection of MT_2 mRNA: RNA extraction (TRI® Reagent method, DNase treatment (Promega, Madison, WI), cDNA synthesis, and conventional PCR (One Step SYBR® Prime-Script™ RT-PCR kit II, Takara, Mountain View, CA) reactions were performed following the manufacturer's instructions. About 200 ng of RNA obtained from 30,000 to 40,000 cells was used to perform the one-step Reverse transcription polymerase chain reaction (RT-PCR) reactions that were developed in a final volume of 50 µl. The resulting PCR product (MT_1 primers, F: 5'-ATC GTG GTG GAC ATT CTG GG-3'; R: 5'-TGC CAC AGC TAA ACT CAC CA-3', expected size of 105 bp; MT_2 primers, F: 5'-GAT GAC AAG CAG GTT CCC CA-3'; R: 5'-GCG CAC CTT GTG CAT CTA TC-3', expected size of 109 bp) was run in an agarose 1.5% Tris-Acetate-EDTA buffer (TAE) gel and visualized by using the EZ-Vision kit (Amresco, VWR, Solon, OH) in an ultraviolet (UV) transilluminator.

cAMP formation assay: To test the functionality of the melatonin receptors, 661W and 661W- $MT_2^{-/-}$ cells were treated with forskolin (1 µM) alone or in combination with MEL (10, 100, or 1,000 nM) or IIK7 (1, 10, 100, or 1,000 nM) for 15 min. The cAMP levels were determined with enzyme-linked immunosorbent assay (ELISA) using a commercial kit (Enzo Life Sciences, St. Petersburg, FL) following the manufacturer's instructions. The limit of detection calculated in our laboratory was 4.09 ng/ml.

AKT and ERK1/2 activation assay: To test the intracellular pathways activated by MEL, 661W and 661W- $MT_2^{-/-}$ cells were treated with MEL (100 nM) for 90 min. Total AKT, phospho-AKT (S473; P-AKT), total FoxO1, phospho-FoxO1 (S256; P-FoxO1), total ERK1/2, and phospho-ERK1/2 (T202/Y204; P-ERK1/2) were measured with ELISA using commercial kits (RayBiotech, Peachtree Corners, GA; LSBio and Life Technologies, respectively), following the manufacturer's instructions with minor modifications. After the proteins were extracted, the samples were loaded in the specific ELISA plates to measure the AKT/FoxO1 and ERK1/2 pathways, respectively, and the manufacturer's guidelines were followed.

Data analysis: A one-way ANOVA followed by the post-hoc Student-Newman-Keuls (SNK) test was performed for data from the viability and gene expression experiments. A p value

of less than 0.05 was considered statistically significant in all tests.

RESULTS

MT_1 and MT_2 receptors heterodimerize in 661W cells: MT_1 and MT_2 were detected in 661W cells, and colocalization was found (Figure 1, upper panels). To determine the presence of $MT_{1/2h}$, we performed PLA [14]. As shown in the lower panels in Figure 1, a PLA positive signal was detected in 661W cells, thus suggesting an interaction between these endogenously expressed receptors. To confirm that this signal was specific to heterodimer formation, the CRISPR/Cas9 system (Figure 2) was used to disrupt the heterodimer formation by knocking out the MT_2 receptor in the 661W cells (661W- $MT_2^{-/-}$). MT_2 mRNA and immunoreactivity were completely ablated in 661W- $MT_2^{-/-}$ cells (Figure 3, upper panels) whereas MT_1 receptor mRNA and immunoreactivity were still present at levels comparable to those observed in the 661W cells (Figure 3, middle panels). As expected, we could not detect $MT_{1/2h}$ formation with PLA in 661W- $MT_2^{-/-}$ cells (Figure 3, lower panels), confirming the specificity of this assay for the heterodimer. Thus, the data suggest that endogenously expressed MT_1 and MT_2 form heterodimers.

Administration of exogenous melatonin activates cAMP signaling in 661W cells: To determine whether 661W cells respond to melatonin, we tested whether administration of exogenous MEL was able to inhibit the forskolin-induced cAMP production in 661W cells [14]. As expected, MEL was able to suppress the forskolin-induced cAMP production in a dose-dependent manner (Figure 4A, $p < 0.05$). When the MEL agonist, IIK7 was used, the effect was observed only at 1,000 nM (Figure 4B), a concentration in which MT_1 and MT_2 receptors are activated [14]. The suppression of cAMP production by MEL (100 nM) was blocked in a dose-dependent manner when the 661W cells were pretreated with the melatonin antagonist LUZ (1 and 10 µM; Figure 4C, $p > 0.05$). Similarly, the reduction in the forskolin-induced cAMP production by MEL was abolished in the 661W- $MT_2^{-/-}$ cells (Figure 4D, $p > 0.05$).

MEL signaling in 661W cells is comparable to that observed in the murine retina: Previous work by our laboratory showed that MEL induces phosphorylation of AKT and FoxO1 in murine photoreceptors. Likewise, we found that administration of exogenous MEL to 661W cells increased the phosphorylation of AKT/FoxO1 (Figure 5A, $p < 0.05$) and ERK1/2 (Figure 5B; $p < 0.05$). AKT/FoxO1 and ERK1/2 were also phosphorylated in response to IIK7 administration (Figure 5, $p < 0.05$), but only at a concentration of 1,000 nM which activates MT_1 and MT_2 receptors [14].

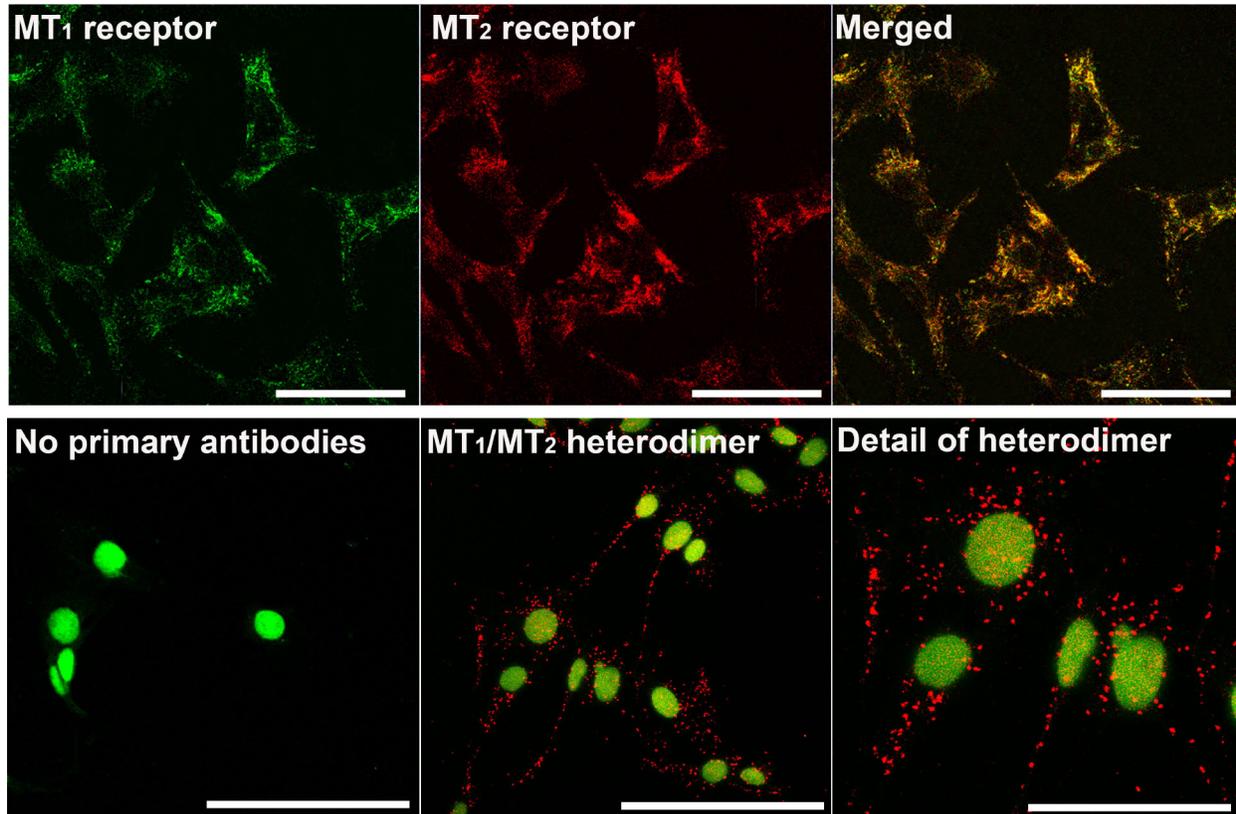


Figure 1. MT₁ and MT₂ form heterodimers in 661W cells. Upper panels: Immunofluorescence for melatonin receptor type 1 (MT₁) and MT₂ receptors and colocalization. Scale bars = 100 μ m. Lower panels: Proximity ligation assay (PLA) showing the MT₁/MT₂ interaction (positive signal shown as red dots in the right panel). Cell nuclei are shown in green. Scale bars: left and center = 100 μ m, right = 50 μ m.

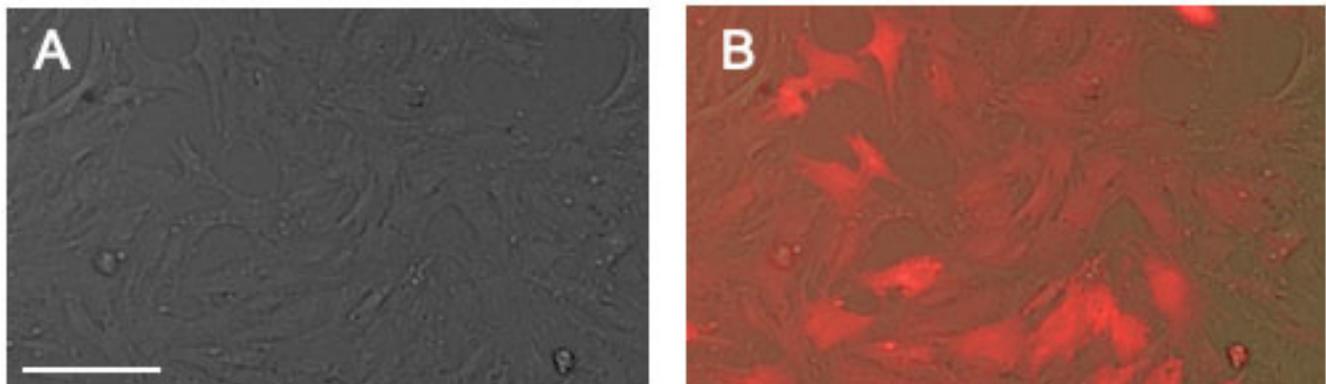


Figure 2. RFP signal in 661W cells transfected with the MEL-1B-R HDR plasmid. 661W cells were cotransfected with MEL-1B-R HDR and MEL-1B-R CRISPR/Cas9 KO. After 2–3 weeks of selection in medium containing puromycin, colonies were picked to become individual cell lines. Expression of red fluorescence protein indicates the successful homology directed insertion at the melatonin receptor type 2 (MT₂) locus. **A:** Bright-field. **B:** Bright-field plus red fluorescent protein (RFP). Scale bar = 100 μ m.

However, removal of MT₂ receptors in 661W-MT₂-KO cells completely blocked these effects, as exogenous MEL could no longer induce phosphorylation of AKT/FoxO1 (Figure 5A,B, $p > 0.05$).

DISCUSSION

Heterodimerization of GPCRs is a new and important topic of investigation as GPCR heteromers may represent an important therapeutic target for the development of new drugs [12]. Previous studies have shown that melatonin receptors

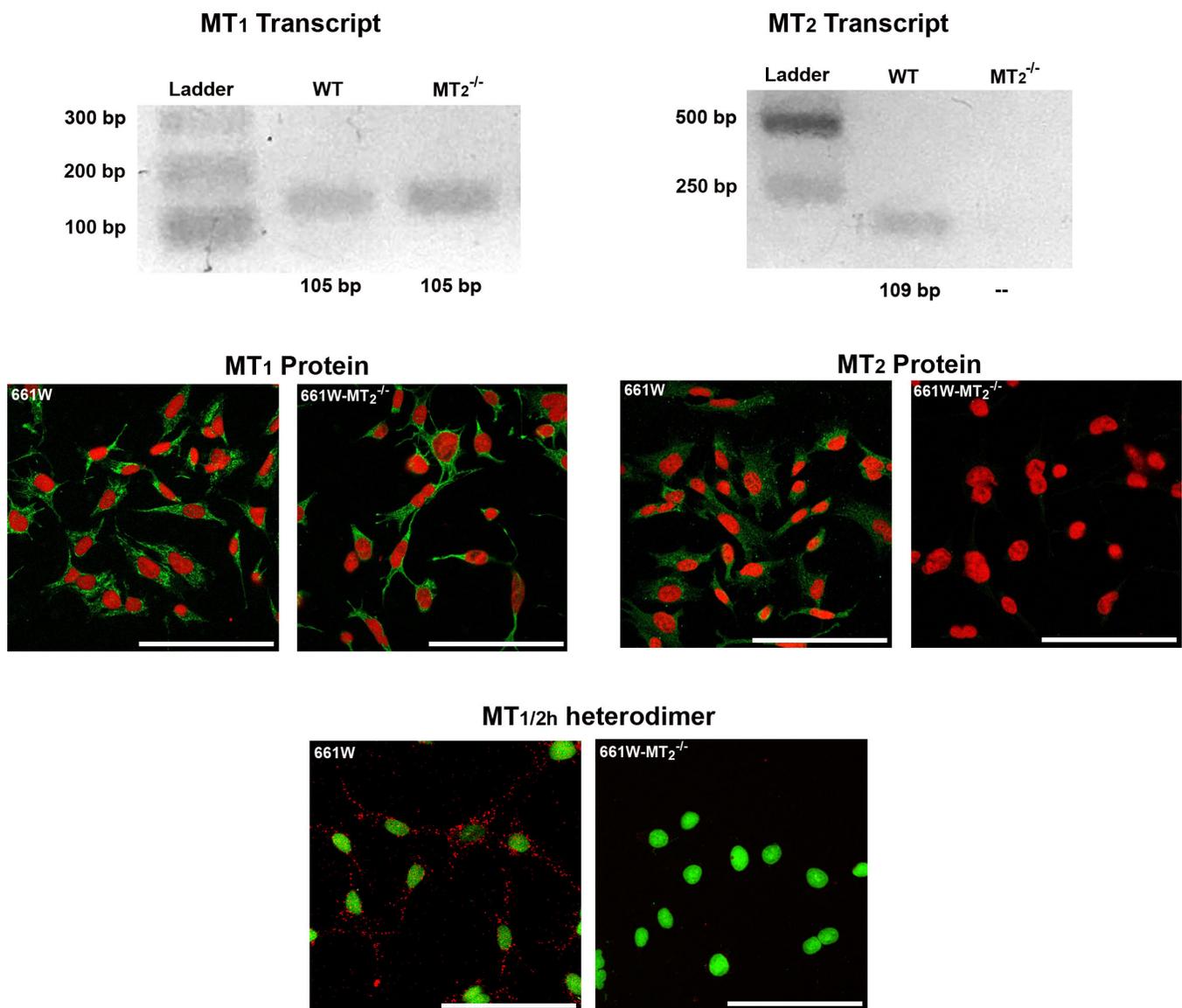


Figure 3. Genetic ablation of MT₂ with CRISPR/Cas9 system does not affect MT₁ mRNA or protein. Top panels: cDNA from 661W and 661W-MT₂^{-/-} cells was analyzed with reverse transcriptase PCR (RT-PCR) to detect MT₁ (left) and MT₂ (right) expression. Middle panels: Immunofluorescence was performed in 661W and 661W-melatonin receptor type 2 (MT₂)^{-/-} cells to detect MT₁ (left) and MT₂ (right) proteins. The positive signal of each receptor is shown in green, and nuclei are counterstained in red. Lower panel: The presence or absence of MT₁/MT₂ heterodimer in 661W and 661W-MT₂^{-/-} cells was assessed with proximity ligation assay (PLA; positive signal in red), and nuclei were counterstained in green. MT₁ expression (transcript and protein) was detected in 661W and 661W-MT₂^{-/-} cells, whereas MT₂ expression (transcript and protein) was detected only in 661W cells. Accordingly, MT_{1/2h} was found only in 661W cells. Scale bars = 100 μ m.

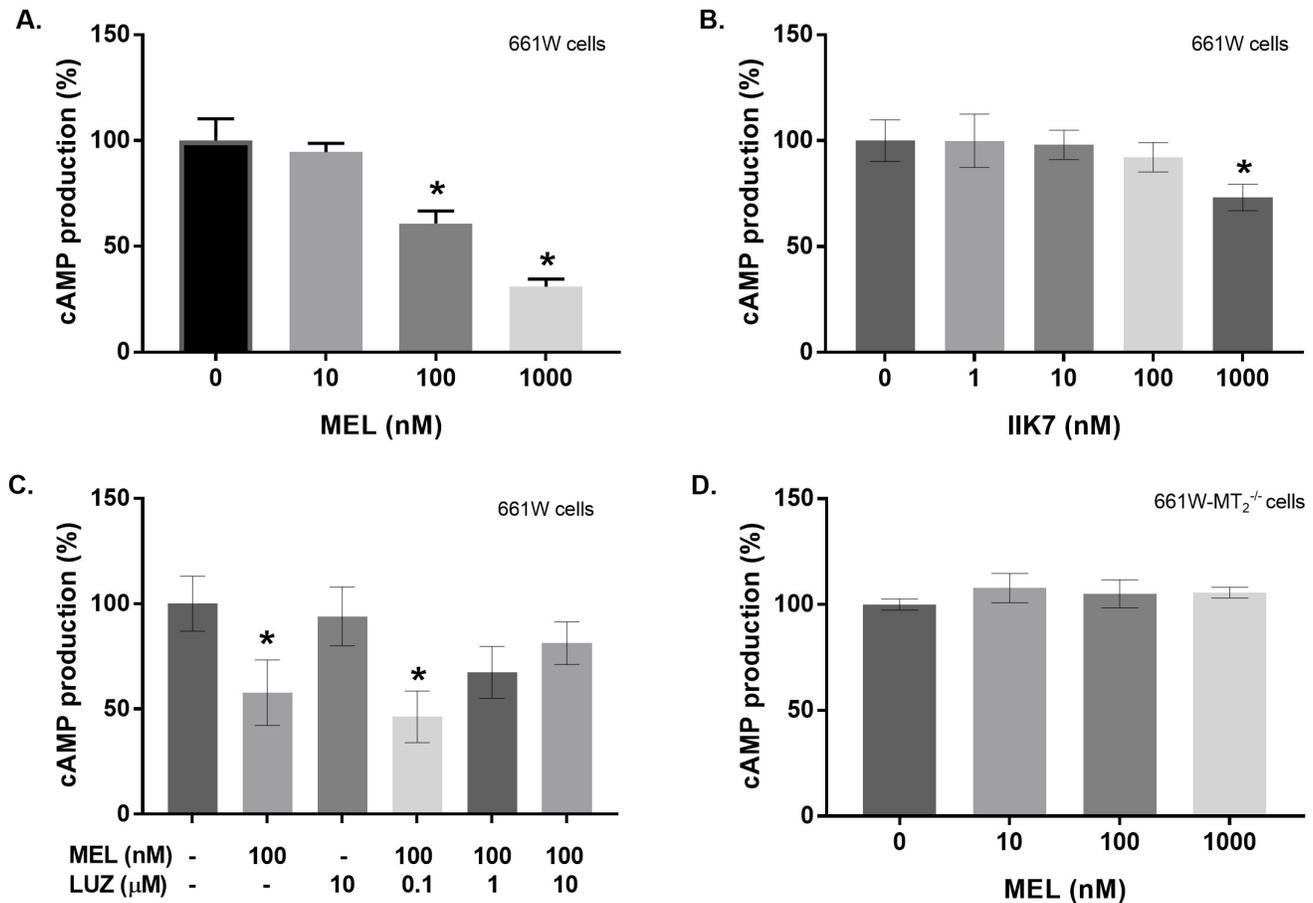


Figure 4. MEL and IIK7 inhibit forskolin-induced cAMP production only in the presence of MT_{1/2h}. **A:** Effect of melatonin (MEL) on forskolin-induced cAMP production in 661W cells. **B:** IIK7 effect on forskolin-induced cAMP production in 661W cells. **C:** Effect of luzindole (LUZ) on the effect of MEL on forskolin-induced cAMP production. **D:** Effect of MEL on forskolin-cAMP in 661W-melatonin receptor type 2 (MT₂)^{-/-} cells. In all the cases, results (n = 6) are shown as mean \pm standard error of the mean (SEM). Asterisks indicate statistically significant differences compared to the control group with one-way ANOVA (Student–Newman–Keuls (SNK) post-hoc test; $p < 0.05$).

can form MT_{1/2h} heterodimers when overexpressed in HEK 293 cells [36,37] or mouse photoreceptors [14]. However, although the formation of MT_{1/2h} was detected, these data were obtained by overexpressing MT₁ and MT₂ in either the HEK 293 or photoreceptor cells (myc/flag system), and therefore, it is still questionable whether MT₁ and MT₂ can form MT_{1/2h} under physiologic conditions.

The results in 661W cells, which endogenously express melatonin receptors [35], suggest these receptors form functional MT_{1/2h} under a physiologic setting. Intriguingly, the prevention of the formation of heterodimers by removal of MT₂ receptors alone completely abolished the activation of intracellular pathways normally activated by melatonin, thus suggesting that in these cells (as reported for mouse photoreceptors [14]) melatonin signaling can be activated only when both receptors are present.

The present data provide experimental evidence supporting the formation of MT_{1/2h} in 661W cells. We showed that the PLA signal is present only when both receptors are present in these cells (Figure 1), and the results obtained with 661W-MT₂ KO indicate that the presence of MT₂ is required to respond to melatonin stimulation (Figure 3, Figure 4, Figure 5). Finally, melatonin action can be blocked only when the agonist LUZ is present at a concentration that blocks both receptors [14] (Figure 4), and the melatonin agonist IIK7 can activate melatonin signaling only at a concentration that activates both receptors [14].

In this context, although MT₁ receptors mRNA and immunoreactivity were unaffected in 661W-MT₂^{-/-} cells 1 (Figure 3), administration of exogenous MEL did not activate any of the intracellular signaling pathways known to be activated by melatonin [38]. Although we have not tested

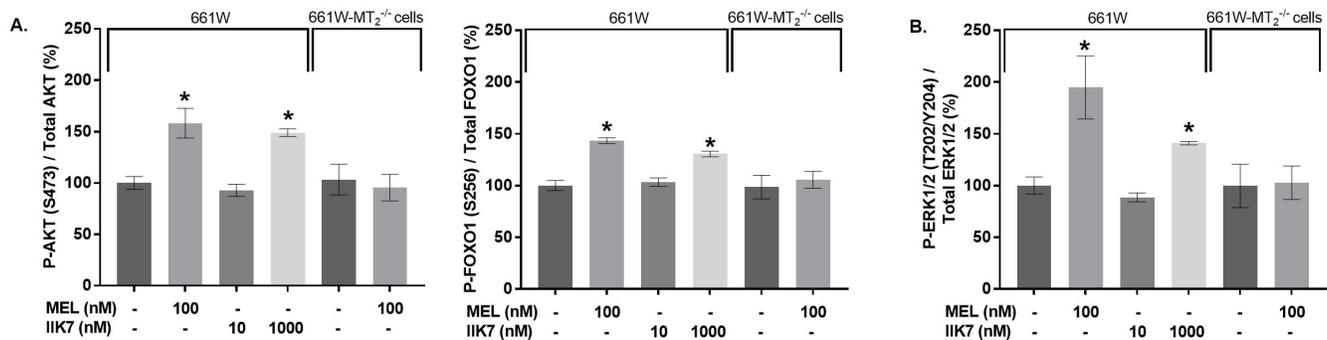


Figure 5. MEL activates AKT/FoxO1 and ERK1/2 only in the presence of the MT_{1/2h}. **A:** Effect of melatonin (MEL; 100 nM) or IIK7 (1,000 nM) 90-min treatment on the phosphorylation of AKT and FoxO1 in 661W cells and 661W-melatonin receptor type 2(MT₂)^{-/-} cells. **B:** Effect of MEL (100 nM) or IIK7 (1,000 nM) 90-min treatment on the phosphorylation of ERK1/2 in 661W cells and 661W-MT₂^{-/-} cells. In all cases, results (n = 5) are shown as mean ± standard error of the mean (SEM). Asterisks indicate statistically significant differences compared to the control group with one-way ANOVA (Student–Newman–Keuls (SNK) post-hoc test; p<0.05).

whether removal of MT₁ from these cells would produce results similar to those observed in 661W-MT₂^{-/-} cells, we believe that removal of the MT₁ also abolishes melatonin signaling. This expectation is based on our previous in vivo study [14] in which we showed that in murine photoreceptors the lack of either MT₁ or MT₂ completely prevents the action of melatonin on scotopic ERGs [14,39] and the protective action of melatonin on photoreceptors during aging [19].

Thus, it seems that in cells where MT₁ and MT₂ are coexpressed in the same cells under natural conditions, the receptors form obligate heterodimers where if one receptor is removed or mutated the other receptor cannot activate the intracellular signaling in response to the ligand. Thus, such a result suggests that the specific cellular environment may have an important role in determining the response of GPCR heterodimers.

Melatonin receptors are distributed in many organs and tissues where they mediate many different physiologic functions [11,12]. However, to date, the heterodimerization of these receptors has been reported in only retinal photoreceptors [14], and because 661W cells are derived from mouse photoreceptors, it is not surprising that they also express MT_{1/2h}. A recent study reported that 661W cells may represent retinal ganglion precursors, with characteristics of ganglion and photoreceptor cells [38]. The present data showing that MT_{1/2} are in these cells further suggest that the cellular identity of the 661W cells must be similar to murine photoreceptors.

Taken together, these results indicate the presence of MT_{1/2h} in 661W cells demonstrates that these heterodimers can form under physiologic conditions. As previously observed in mouse photoreceptors, melatonin signaling in 661W cells can

occur only when both receptors are present. Thus, the present study results indicate that 661W cells may represent a new and important tool for studying MT_{1/2h}, and more generally, the biology of GPCR heterodimerization.

APPENDIX 1. STR ANALYSIS.

To access the data, click or select the words “[Appendix 1.](#)”

ACKNOWLEDGMENTS

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