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A role of melatonin in neuroectodermal-mesodermal interactions: the hair follicle synthesizes melatonin and expresses functional melatonin receptors

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ABSTRACT

Since mammalian skin expresses the enzymatic apparatus for melatonin synthesis, it may be an extrapineal site of melatonin synthesis. However, evidence is still lacking that this is really the case in situ. Here, we demonstrate melatonin-like immunoreactivity (IR) in the outer root sheath (ORS) of mouse and human hair follicles (HFs), which corresponds to melatonin, as shown by radioimmunoassay and liquid chromatography/tandem mass spectrometry (LC/MS/MS). The melatonin concentration in organ-cultured mouse skin, mouse vibrissae follicles, and human scalp HFs far exceeds the respective melatonin serum level and is significantly increased *ex vivo* by stimulation with norepinephrine (NE), the key stimulus for pineal melatonin synthesis. By real-time PCR, transcripts for the melatonin membrane receptor MT₂ and for the nuclear mediator of melatonin signaling, retinoid orphan receptor α (ROR α), are detectable in murine back skin. Transcript levels for these receptors fluctuate in a hair cycle-dependent manner, and are maximal during apoptosis-driven HF regression (catagen). Melatonin may play a role in hair cycle regulation, since its receptors (MT₂ and ROR α) are expressed in murine skin in a hair cycle-dependent manner, and because it inhibits keratinocyte apoptosis and down-regulates ER α expression. Therefore, the HF is both, a prominent extrapineal melatonin source, and an important peripheral melatonin target tissue. Regulated intrafollicular melatonin synthesis and signaling may play a previously unrecognized role in the endogenous controls of hair growth, for example, by modulating keratinocyte apoptosis during catagen and by desensitizing the HF to estrogen signaling. As a prototypic neuroectodermal-mesodermal interaction model, the HF can

be exploited for dissecting the obscure role of melatonin in such interactions in peripheral tissues.

Key words: melatonin • hair follicles • MT2 • ROR α • estrogen receptors • C57BL/6 mice • apoptosis

Melatonin (5-methoxy-*N*-acetyltryptamine), the hallmark hormone of the pineal gland, exerts a puzzlingly wide range of physiological functions such as the bioregulation of pigmentation, sleep, circadian rhythms, and cellular growth (1–5). As a potent free radical scavenger, it also has a important cell-protective functions (6–9), and has found increasing attention as an immunomodulator and putative antiaging agent (10–12).

In mammalian skin and/or its isolated cell populations in culture, melatonin has been ascribed many different functions, such as the inhibition of melanogenesis (13–15) and melanocyte growth (14, 16, 17), and protection from UV light-induced damage (18–21). However, the mechanisms underlying the cutaneous effects claimed for melatonin are unknown, the *in vitro* literature on the cellular effects of melatonin is full of contradictions, and the physiological functions of melatonin in skin biology and pathology remain very ill-defined. Therefore, instructive and easily handled model systems are urgently needed that allow us to characterize and dissect cutaneous melatonin biology under physiologically relevant tissue interaction conditions.

As a classical neuroectodermal-mesodermal interaction system (22–25), the hair follicle (HF) is ideally suited to serve as such a melatonin research model. Also, melatonin reportedly stimulates wool HF growth *in vitro* (26), alters seasonal fur growth in mink (1–3), goat (27), and ferret (28), and may increase the anagen hair rate in women with hair loss (29). This suggests that the HF is a melatonin-sensitive tissue, which expresses cognate receptors, while the HF expression profile for melatonin receptors remains to be characterized.

The major membrane-bound, G protein-coupled melatonin receptors, MT1 and MT2 (30, 31), are thought to be expressed primarily in the central nervous system (32, 33), where they engage most prominently in the control of circadian rhythms and sleep (34–36). MT1 transcripts have also been found in murine heart, kidney, liver, and lung tissue, while MT2 mRNA was also detected in mouse lung (37). Interestingly, the nuclear receptor ROR α (38) has been shown to act as a mediator of nuclear melatonin signaling (39, 40), whose stimulation down-regulates for example, human 5-lipoxygenase expression (41).

ROR α appears to be ubiquitously expressed, with the highest levels found in leukocytes and skin (42). By autoradiography, melatonin binding-sites have been detected in mouse skin epidermis and HFs (43) and weak mRNA expression of the high-affinity membrane melatonin receptors MT1 and MT2 was found in cultured human skin cells (44). Reportedly, normal mouse skin expresses MT1B, while human skin predominantly expresses MT1A (45). Therefore, it is reasonable to assume that mouse and/or human HF express one or several of these melatonin receptors.

Because the HF has surfaced as a major neuroendocrine organ (46–48) and since mammalian skin has the enzymatic equipment required for melatonin synthesis (see below), it is reasonable to ask whether the HF can even synthesize melatonin. Melatonin is synthesized primarily in the

pineal gland after stimulation with NE derived from sympathetic nerve fibers (49–51). However, several extrapineal sites of melatonin production are now well recognized (52), including retina (53), gut (54–57), liver, kidney, spleen (55), ovary (58), bone marrow (59), leukocytes (60), and lymphocytes (12). Mammalian skin and isolated cultured keratinocytes (including HF keratinocytes), melanocytes, dermal fibroblasts, and dermal papilla fibroblasts, indeed, express the key enzymes required for melatonin synthesis (61–64). However, it still remains to be demonstrated that mouse or human skin really synthesizes melatonin in situ.

On this background, we have developed simple *ex vivo* assays for probing the effects of melatonin on intimately interacting neuroectoderm- and mesoderm-derived cell populations (keratinocytes, melanocytes, Merkel cells, fibroblasts, immunocytes) under physiologically relevant conditions. In mouse skin organ culture and in microdissected, organ-cultured mouse vibrissae HFs and human scalp HFs, we explored whether mammalian skin and/or its appendages actually synthesize melatonin in situ, and whether this can be stimulated by NE. In parallel, we analyzed which melatonin receptors (MT1, MT2, ROR α) are expressed by murine HFs and whether these are functional. The latter was assessed exemplarily by checking the effect of melatonin on HF keratinocyte apoptosis in mouse skin organ culture (melatonin is a suppressor of apoptosis in several systems (65–70)), and on the expression of ER α (in vitro, ER α transcription is down-regulated via MT1 or MT2 signaling (22, 71), and ER α stimulation plays an important role in the endogenous controls of hair growth (72, 73)).

MATERIALS AND METHODS

Animals

Six- to nine-week-old, female mice (C57BL/6J, bred at the University Hospital Hamburg-Eppendorf animal facility) in the telogen stage of the hair cycle were housed in community cages with 12-h light periods and fed *ad libitum*. The growth phase of the hair cycle (anagen) was induced in the dorsal skin by depilation, as described previously (74). Dorsal skin with all HFs in defined stages of induced anagen, spontaneously developed catagen, and telogen was harvested for further analysis at the indicated days postdepilation.

Immunohistochemistry

Immunohistochemistry was performed on sections of mouse HFs (melatonin, ROR α) and microdissected human scalp HFs (melatonin). Immunohistochemistry for ER α was performed on organ-cultured mouse skin after stimulation with melatonin or vehicle.

The ABC (avidin-biotin-peroxidase complex) method (75) was used for the detection of melatonin-like IR. Briefly, tissue fixation was performed in Bouin's fluid (picric acid: 35% formaldehyde: acetic acid=15: 5: 1, pH 7.2). After deparaffinization, 4- μ m tissue sections were equilibrated in tris-buffered saline (TBS), and blocked with 10% normal porcine serum in TBS and incubated for 1 h at 37°C with a polyclonal rabbit anti-melatonin antibody (126–6295, Immune Systems Ltd, Paigton, UK), diluted 1:5 in TBS containing 0.02% Tween 20. After washing, the samples were incubated for 45 min with biotinylated porcine anti-rabbit IgG (DAKO, Hamburg, Germany), diluted 1:200, at room temperature, further washed and then incubated in 1:100 streptavidin/alkaline phosphatase conjugate (ABC-AP kit, Vector Laboratories, Burlingame, CA). FAST RED (Sigma-Aldrich, Deisenhofen, Germany) was used

as the chromogen and Mayer's hemalaun was used to counterstain. Microdissected mouse pineal gland was used as a positive control. Omission of primary antibody was used as a negative control.

ROR α -like IR was detected by immunofluorescence (76). Briefly, 4- μ m cryosections were fixed in acetone for 10 min at -20°C , rehydrated in phosphate-buffered saline (PBS), and then blocked with 10% normal goat serum and incubated overnight at 4°C with a polyclonal goat anti-ROR α antibody (sc-6062, Santa Cruz Biotechnology, Santa Cruz, CA), diluted 1:25. After washing, the samples were incubated with a rhodamine-conjugated rabbit anti-goat secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, PA), diluted 1:200 for 45 min at room temperature. Sections were counterstained with 4', 6-diamidino-2'-phenylindol-dihydrochloride (DAPI, Roche Diagnostics, Mannheim, Germany). Mouse Purkinje cells (42), were used as a positive control. As a negative control, the antibody was preincubated overnight at 4°C with 10 times higher concentration of blocking peptide. The mean fluorescence intensity was measured at the DP and middle part of outer root sheath (ORS) by Scion image-analysis (Scion Corp., Frederick, MD), and the average mean fluorescence intensity was calculated ($n=30$ HFs/stage).

The ABC method was used for ER α immunodetection. Briefly, 4- μ m cryosections were fixed in acetone for 10 min at -20°C , rehydrated in TBS, and blocked with 3% H_2O_2 for 15 min and then for 20 min with avidin, biotin (BLOCKING KIT, Vector Laboratories) and 10% nonimmunized goat serum supplemented with 0.3% Triton-X100 at room temperature. Subsequently, the sections were incubated overnight at 4°C with anti-ER α antibody (MC20, Santa Cruz Biotechnology) (77), diluted 1:2000. The samples were then incubated for 45 min at room temperature with biotinylated goat anti-rabbit antibody (Jackson ImmunoResearch), diluted 1:200, together with 2 or 4% of normal goat or mouse serum, followed by incubation for 30 min at room temperature with streptavidin:horseradish peroxidase conjugate (ABC kit, Vector Laboratories), diluted 1:100. The signal was visualized by using diaminobenzadine (Vector Laboratories). Sections were counterstained by methylene green (DAKO). Mouse uterus was used as a positive control (78).

Mouse skin organ culture

Mouse dorsal skin with all HFs in telogen, anagen or catagen (day 0, 12, or 19 after anagen induction by depilation) was harvested and 3×10 mm skin fragments were cultured as described before (79, 80). Three skin fragments with 5 ml of Dulbecco's modified Eagle's medium, 2 mM L-glutamate, 10% fetal bovine serum, and 1% antibiotic/antimycotic mixture were incubated in a 6-well culture plate for 6 to 48 h after stimulation of 0.01 nM or 1 nM melatonin (Sigma-Aldrich) (13, 14, 26, 43, 81) at 37°C in 5% CO_2 and 100% humidity. Cryosections were prepared and double immunodetection of terminal dUTP nick-end labeling (TUNEL) and Ki67-positive cells were determined (76, 82).

To test the effects of NE, 14 mouse dorsal skin fragments (3×10 mm from 10 mice), were cultured for 24 h with 50 nM or 5000 nM NE or vehicle (50). After freezing in liquid nitrogen, the skin samples were homogenized in 0.1 M PBS, pH 6.8, supplemented with 1% Triton X-100, followed by centrifugation at 10,000 g for 15 min at 4°C (83). Melatonin concentration was analyzed by radioimmunoassay and LC/MS/MS.

Mouse vibrissae follicle culture

Mouse vibrissae follicles were isolated as described before (84). Briefly, whisker pads were cut off the face of mouse and transferred to a Petri dish with PBS, in the presence of 1% antibiotic mixture. Each vibrissae follicle that was in anagen was isolated from the whisker pads and cultured in William's E medium (Biochrom AG, Berlin, Germany), supplemented with insulin (10 µg/ml, Sigma-Aldrich), hydrocortisone (10 ng/ml, Sigma-Aldrich), 1% penicillin streptomycin antibiotic mixture and L-glutamine (2 mM), using 24-well culture plates (3 vibrissae follicles per well) and incubated at 37°C, in 5% CO₂ and 100% humidity, with 50 nM or 5000 nM of NE or vehicle. After 24 h incubation, vibrissae follicles were frozen and prepared to analyze melatonin concentration by radioimmunoassay and LC/MS/MS, as described previously.

Human HF organ culture

Human scalp skin biopsies from healthy individuals undergoing cosmetic surgery were obtained after informed consent. The microdissected HFs were cultured according to the well-established Philpott model (85), briefly in William's E medium, supplemented with 10 µg/ml insulin, 10 ng/ml hydrocortisone, 1% antibiotic mixture, and 2 mM L-glutamine. HFs ($n=30-40$ per each concentration of melatonin from eight females and two males, aged 44-65 years (mean 55 years)) were cultured with or without 0.001, 0.1, 10, or 1000 nM melatonin (13, 14, 26, 43, 86) for 48 h (for the examination of IR of ER α) (81) or 6 days (for the examination of proliferating or apoptotic cells and Masson-Fontana staining). Furthermore, isolated human HFs (90 HFs per each group from five females, aged 50-60 years (mean 56 years)) were cultured with 50 nM or 5000 nM NE or vehicle for 24 h and frozen by liquid nitrogen before analysis of melatonin concentration by radioimmunoassay and LC/MS/MS, as described previously.

Melatonin radioimmunoassay

The whole sample lysate of mouse skin, vibrissae follicles, and human HFs was extracted in chloroform and centrifuged at 16,000 *g* for 10 min. The chloroform phase and mouse serum was measured (83) by a double antibody-radioimmunoassay, based on the Kennaway G280 anti-melatonin antibody (Bühlmann, Schoenenbuch, Switzerland). The samples were aspirated and reconstituted by adding 1 ml incubation buffer. The samples were incubated with 100 µl of antiserum and 100 µl ¹²⁵I-labeled melatonin for 20 h at 4°C, subsequently, with 100 µl of secondary antibody for 15 min at 4°C. After centrifugation at 2000 *g* for 2 min at 4°C, counts per min (cpm) were measured in a gamma counter. Melatonin concentrations in the samples were calculated by applying a standard curve fitted by a four-parameter logistic algorithm.

Melatonin LC/MS/MS

Sample supernatants (200 µl) were mixed with 20 µl NaOH (0.5 M), 10 µl of *N*-acetyltryptamine (50 ng/ml, Sigma-Aldrich), and 1 ml ethyl acetate and were extracted by lateral shaking for 10 min. After centrifugation at 2000 *g* for 5 min, the organic layer was transferred into glass tube and evaporated under vacuum. The dried samples were dissolved in the mobile phase (30 µl), and 10 µl were injected into the LC/MS/MS system consisting of a Micromass Quattro II triple-quadrupole tandem mass spectrometer (Waters, Milford, MA) (87), equipped with an electrospray interface operated in the positive-ion mode. A Nanospace SI-1 HPLC system

(Shiseido, Tokyo, Japan) was used. Chromatography was performed on a C18 Capcell Pak UG120 (Shiseido, 1.5×150 mm, 5 μm) using isocratic elution with acetonitrile/10 mM ammonium acetate (pH 4.5, 35/65, v/v) with a flow rate of 100 μl/min. Melatonin and the internal standard *N*-acetyltryptamine (87) were detected by monitoring the following transitions: m/z 233 > m/z 174 for melatonin and m/z 203 > m/z 144 for *N*-acetyltryptamine. The capillary voltage and source temperature were set to 3 kV and 60°C. Cone voltage was 25 V with collision energy of 15 eV for each compound. Quantitative determination of melatonin was made by using the Micromass MassLynx software (Waters) (87).

Semiquantitative histomorphometry

Hematoxylin- and eosin-stained cryosections of human organ cultured HF were screened for fully longitudinally cut HF sections. At least 20 follicles per group were counted and the hair-cycle stage of each follicle was assessed and classified by morphological criteria and assigned to their respective hair-cycle stages following our previously published guidelines (88, 89). The hair-cycle score was assessed for the catagen induction study and calculated as described (88, 89).

Real-time PCR

Total RNA was isolated from frozen mouse dorsal skin samples as described previously (76). Briefly, the RNA was extracted from homogenized tissue samples using RNeasy Mini columns (Qiagen, Hilden, Germany) according to the manufacturer's instructions. RNA concentration was measured spectrophotometrically at wavelength 260 nm. First-strand cDNA was generated from 1 μg of each RNA preparation by reverse transcription using the first-strand cDNA synthesis kit (Roche Diagnostics) in the presence of oligo-dT₁₂₋₁₈ primers, according to the manufacturer's instructions. Real-time quantitative PCR for the analysis of mouse ARP0, MT1, MT2, RORα and ERα gene expression was performed with an IQ-cycler (BioRad, Hercules, CA) using the dye SYBR Green (Molecular Probes, Leiden, The Netherlands). In all PCR reactions, MgCl₂ at a final concentration of 3 mM was used. The PCR cycling conditions (40 cycles) were for 30 s at 95°C; for 30 s at 58°C (MT1 and RORα), 60°C (MT2), and 62°C (ERα); and for 30 s at 72°C. The respective primer pairs are described in [Table 1](#). The fold change in expression (relative to day 0 levels) of each gene was calculated using the Eq. $2^{-(\Delta\Delta Ct)}$, where $\Delta\Delta Ct$ is the $\Delta Ct_{(day X)} - \Delta Ct_{(day 0)}$, ΔCt is $Ct_{(test\ gene)} - Ct_{(control\ gene)}$ and Ct is the cycle at which the threshold is crossed. PCR product quality was monitored using post-PCR melting curve analysis at the end of the amplification cycles. Additionally, PCR products were run onto 2% agarose, 1 × TBE (90 mM Tris, 90 mM boric acid, 2 mM EDTA, pH 8.3) buffered electrophoresis gels to confirm PCR product sizes. Limited-cycle PCR was performed to obtain representative exponential phase amplifications for each gene. Products were then run on 2% agarose, 1 × TBE buffered gels. The numbers of cycles performed for each gene were 17, 40, 32, or 25 cycles for the ARP0, MT1, MT2, or RORα, respectively.

Double immunodetection of TUNEL and Ki67-positive cells

For double-immunofluorescence detection of apoptotic cells and Ki67-IR, the protocol for the TUNEL kit (ApopTag, Oncor, Gaithersburg, MD) (82) was combined with that for Ki67-immunohistochemistry (90, 91). Briefly, 6 μm cryosections were incubated with rabbit anti-Ki67 antiserum followed by incubation with digoxigenin-dUTP in the presence of TdT.

Subsequently, TUNEL-positive cells were visualized by anti-digoxigenin fluorescein isothiocyanate-conjugated F(ab)₂ fragments, Ki67-IR was detected by goat anti-rabbit tetramethylrhodamine B isothiocyanate-conjugated antibody and the sections were counterstained with DAPI. The rate of TUNEL positive cells in the HF was then measured.

Statistical analysis

Data were expressed as means \pm SD and assessed for statistical significance ($P < 0.05$). Mann-Whitney *U* tests were performed to compare the obtained values of staining intensity of ROR α and ER α , TUNEL-positive cells in skin organ culture, morphological hair-cycle score, and Student's *t* test were performed to statistically test the results of MT2, ROR α , and ER α mRNA transcription by real-time PCR.

RESULTS

Melatonin-like IR can be detected in murine and human HFs

To explore whether HFs express melatonin and whether its levels change in relation to the HF cycle, melatonin-like IR was assessed during the depilation-induced mouse hair cycle and in scalp human HF. In murine back skin sections, prominent and widespread melatonin-like IR, which corresponded well to the positive control (mouse pineal gland) ([Fig. 1E](#)), was detected in keratinocytes of the ORS and the lower part of the inner root sheath (IRS) as well as in the sebaceous gland (SG) ([Fig. 1A](#)). Melatonin-like IR was found to change in a hair cycle-dependent manner with an apparent maximum in anagen and catagen HFs ([Fig. 1A, G](#)). In human scalp HFs, prominent melatonin-like IR was detected in the ORS and in the lower part of the IRS keratinocytes, and dermal papilla (DP) fibroblasts were also slightly positive ([Fig. 1C](#)).

The melatonin content of mouse skin and vibrissae follicles and of human HFs is higher than the serum level

To probe these immunohistochemical findings with independent biochemical methods, the melatonin concentration was measured by radioimmunoassay in mouse skin, vibrissae follicles, as well as in human HFs. These samples were all separated from their normal vascular and neural connections, thus dissociating them from possible extracutaneous/extrafollicular melatonin sources. This was compared with the melatonin concentration of mouse serum.

In organ-cultured back skin of C57BL/6 mice, the melatonin level was more than 10 times higher than the serum level (0.5 ± 0.6 pg per mg total protein, [Fig. 2A](#)). This is surprising, because this mouse strain has been claimed to be a “natural melatonin knockout” due to an enzymatic deficiency that results in insufficient pineal melatonin production (92).

NE stimulates the intrafollicular synthesis of melatonin

The key pineal stimulus for melatonin production is NE (49–51). Therefore, we next examined whether NE potentiated the levels of melatonin in our samples. Stimulation of organ-cultured mouse skin with NE *in vitro* increased the melatonin content compared with vehicle controls ~2.5-fold ([Fig. 2A](#)). In microdissected mouse vibrissae follicles, the melatonin level was even ~100 times higher than the peak serum level, and NE stimulation could further increase this level by a factor of 1.5 ([Fig. 2B](#)).

Similar results were obtained from microdissected human scalp HFs. Although the serum peak level of melatonin ranges in humans between 40 and 260 pM (8), which is equal to 0.1–0.5 pg/mg total protein, human scalp HFs contained ~50 pg of melatonin per mg of total protein. Therefore, microdissected human scalp HFs are also enriched in melatonin compared with basal serum levels. Finally, NE treatment could potentiate this level by a fivefold ([Fig. 2C](#)). The latter finding was independently confirmed by yet another biochemical technique for melatonin detection (quantitative LC/MS/MS), which detected a prominent peak of melatonin in extracts from NE-stimulated human HFs ([Fig. 2E](#)) compared with vehicle control ([Fig. 2D](#)).

Taken together with the immunohistochemical findings, these independent biochemical findings suggest that both C57BL/6 mouse and human skin, and in particular their HFs, are important extrapineal sites of melatonin synthesis. Additionally, we find that the levels of melatonin can be further enhanced by the key stimulus for pineal synthesis NE.

The melatonin receptors MT2 and ROR α are expressed in mouse skin and their transcription is hair cycle-dependent

To study whether mouse HFs express the two membrane-bound G protein-linked receptors (MT1, MT2) and/or the orphan nuclear receptor, ROR α , quantitative real-time PCR was performed on mouse whole skin cDNA from C57BL/6 mice in unmanipulated telogen skin (day 0) and at various time points after hair cycle induction by depilation. This revealed that mouse skin transcribes ROR α and MT2, but not MT1 receptors ([Fig. 3](#)).

Importantly, both the MT2 and the ROR α mRNA steady-state levels showed significant variations during the hair cycle. MT2 expression was found to be up-regulated in late anagen, was maximal in catagen and decreased in telogen ([Fig. 3A](#)). In contrast, the profile of the ROR α expression showed a slight but significant down-regulation in the late anagen phase, up-regulation in late catagen phase, and a decrease in telogen ([Fig. 3B](#)). In contrast to mouse liver (positive control), mouse skin did not show any expression of the MT1 gene ([Fig. 3C](#)).

In summary, our data suggest that MT2 and/or ROR α , but not MT1, are involved mediating the actions of melatonin in mouse skin. Additionally, this is likely to occur in a hair-cycle-dependent manner, with the sensitivity to melatonin stimulation changing during the hair cycle.

ROR α protein expression in mouse HFs is hair cycle-dependent

Next, we attempted to localize ROR α protein-related IR by immunohistochemistry ([Fig. 4A](#)). ROR α -like IR, well above the (substantial) background levels ([Fig. 4B](#)), was indeed detected, suggesting that ROR α mRNA is also translated in murine HFs. ROR α -like IR was most prominent in the mesenchymal DP of mid-anagen HFs (anagen IV) and was also seen in the IRS and ORS in the upper part of the HF epithelium, as well as in the epidermis from mid-anagen to telogen ([Fig. 4A](#)). As a positive control, Purkinje cells in mouse cerebellum showed the expected prominent ROR α -like IR ([Fig. 4C](#)). ROR α -like IR in DP and ORS was also found to fluctuate in a hair cycle-dependent manner ([Fig. 4E](#)). The mean fluorescence intensity in the DP was maximal in mid-anagen HFs (anagen IV) and that in middle part of ORS was maximal in catagen HF (catagen III) ([Fig. 4F](#)). This is in line with the observed hair cycle-dependent changes in ROR α transcription ([Fig. 3B](#)), shows that ROR α protein expression also underlies hair

cycle-dependent variations, and suggests that ROR α is required in different cell populations at different times during the hair cycle.

Melatonin inhibits keratinocyte apoptosis in mouse skin organ culture

As a first step toward probing whether any of the above melatonin receptors detected in mouse skin are functional, melatonin was added to organ-cultured C57BL/6 mouse skin with all HFs in defined, synchronized hair cycle stages (anagen, catagen or telogen), for 6-48 h. Double immunodetection of TUNEL- and Ki67-positive cells in cryosections was used for assessing apoptosis and proliferation in the HF epithelium.

As shown in [Fig. 5](#), in unmanipulated telogen skin organ culture treated for 6 or 12 h with 1 or 0.01 nM melatonin, quantitative immunohistomorphometry revealed that the percentage of TUNEL⁺ (i.e., apoptotic) cells in the HF epithelium was significantly lower compared with vehicle controls. In contrast, the percentage of apoptotic cells did not significantly change in either anagen or catagen skin organ culture, and HF keratinocyte proliferation did not show any significant differences between test and control samples during any hair cycle stage (data not shown).

These data offer at least one piece of evidence in support of the concept that MT1 and/or ROR α operate as functional mediators of melatonin signaling in murine skin, where melatonin inhibits the spontaneous apoptosis rate of HF keratinocytes under organ culture conditions.

Melatonin down-regulates the HF expression of estrogen receptor (ER α)

Because estrogen is a powerful mediator of HF growth and cycling in all mammalian species examined in this respect so far (72, 93–97) and given that it has been shown that melatonin down-regulates ER expression (81), we examined ER α gene expression by quantitative real-time PCR on cDNA prepared from mRNA extracts of C57/BL6 mouse back skin in different hair cycle stages. This confirmed previous findings from semiquantitative RT-PCR (72) that murine skin transcribes ER α mRNA and that ER α transcript steady state levels significantly fluctuate in hair cycle-dependent manner. By carefully controlled, quantitative PCR, ER α transcript steady state levels were down-regulated from middle-anagen to early-catagen phases, up-regulated from late catagen to telogen and maximal in late telogen ([Fig. 6A](#)).

Finally, we determined whether melatonin administration altered the follicular expression of ER α 6 h after stimulation with 0.01 nM and 1nM in C57/BL6 mouse skin organ culture with all HFs in either telogen, anagen, or catagen phase. The distribution of ER α IR agreed with a previous study (97), in which ER α expression was largely restricted to DP. Melatonin treatment down-regulated ER α -like IR in the HF matrix and in IRS keratinocytes as well as in DP fibroblasts in situ ([Fig. 6B–D](#)). By semiquantitative immunohistomorphometry (Scion image-analysis), the intensity of ER α IR in defined reference areas in the DP decreased in melatonin-treated mouse skin ([Fig. 6E–G](#)). ER α mRNA expression was also significantly decreased after melatonin treatment in telogen ([Fig. 6H](#)) and anagen ([Fig. 6I](#)) mouse skin organ culture.

Instead, by quantitative histomorphometry, no significant and reproducible effects of melatonin could be discerned when comparing vehicle controls and melatonin-treated, organ-cultured

human scalp HFs with respect to hair shaft elongation, HF cycling, or HF pigmentation (data not shown).

These data confirm the functionality of intracutaneous/intrafollicular melatonin receptors and suggest that melatonin can indirectly affect hair growth by desensitizing the HFs to ER ligands.

DISCUSSION

In this study, we demonstrate that mouse skin, murine HFs, and human scalp HFs are not only important targets for melatonin bioregulation, but also prominent extrapineal sites of melatonin synthesis. We also show that murine skin expresses *functional* melatonin receptors (MT₂, ROR α), whose expression levels fluctuate in a hair-cycle-dependent manner. This suggests that their regulated, differential expression is an integral component of the molecular controls of HF biology. Locally generated melatonin may inhibit murine HF keratinocyte apoptosis and may desensitize both mouse and human HFs to stimulation by estrogens, which operate as potent hair growth modulators.

Melatonin is thought to be mainly synthesized in the pineal gland (98, 99). However, it is now known to be synthesized also by a large variety of extrapineal tissues (52–60). Recent landmark studies have elegantly demonstrated the expression and activity of melatonin-synthesizing key enzymes in intact mammalian skin or skin cells, including epidermal and HF keratinocytes, melanocytes, and melanoma cells in vitro (44, 61–64, 100). However, it remained to be shown that mammalian skin actually synthesizes melatonin in situ. Here, we show that this is indeed the case: murine and human HFs show melatonin-like IR in situ ([Fig. 1A, C](#)). That this really corresponds to intrafollicular melatonin is demonstrated by two independent biochemical methods, which also reveal a very high melatonin content in murine and human HFs, well-above the respective serum levels ([Fig. 2A–C](#)).

Most importantly, we show that NE, the key stimulus for pineal melatonin synthesis, significantly up-regulates the already high melatonin content in organ-cultured mouse skin, mouse vibrissae follicles, and human HFs. ([Fig. 2A–E](#)). Therefore, C57BL/6 mice can no longer be considered a “natural melatonin ‘knock-down’” (92).

A high intrafollicular melatonin content might be explained by passive melatonin uptake from the serum via intrafollicular melatonin receptors. Yet, the NE-induced increase of the melatonin content renders it highly likely that there was indeed genuine intrafollicular melatonin synthesis, since the HFs had been disconnected from any extracutaneous, neural, or vascular sources of melatonin. This also shows that melatonin synthesis in the HF and the pineal gland respond to at least one common key stimulus (catecholamines). However, the remote possibility remains to be excluded that NE stimulation only inhibited the constitutive rate of melatonin degradation in organ-cultured HFs. Because the key pathways of melatonin degradation in mammalian skin and other peripheral tissues remain to be elucidated (R. J. Reiter, personal communication), we could not exclude this theoretical possibility by assaying for widely accepted key melatonin degradation products. However, NE has not been reported to inhibit melatonin degradation, and no new peaks that might have corresponded to melatonin degradation products were detected by LC/MS/MS. Therefore, our data most likely reflect a genuine stimulation of intrafollicular melatonin synthesis by NE.

The available IR data ([Fig. 1A, G](#)) already are suggestive of hair cycle-dependent changes in the melatonin content of murine HFs. However, because of the high background staining obtained with the employed melatonin antiserum (which is a well-known problem from previous studies (101, 102)), we cannot state with certainty yet to what degree melatonin like-IR in ORS and IRS keratinocytes change in a hair cycle-dependent manner. In the future, extensive RIA, LC/MS/MS, and enzymological analyses of different stages of HF cycling are needed to clarify whether intrafollicular melatonin synthesis is indeed hair cycle-dependent in mice and/or humans.

Our observations raise the question of whether melatonin plays a role in the complex endogenous controls of HF biology (22–25, 103), for example, as a potent antioxidant (110, 104, 105). Interestingly, low doses of melatonin (0.01 and 1 nM) inhibited HF keratinocyte apoptosis in short-term mouse skin organ culture ([Fig. 5](#)). Although the mechanism of this antiapoptotic effect of melatonin is not clear, this finding is in line with previous reports that melatonin inhibits apoptosis of keratinocytes (44), immune cells (70, 104), and neuronal cells (105) in vitro. Given that the dissection and preparation of mouse skin for organ culture is a highly traumatic event, which must be expected to generate large amounts of reactive oxygen species, the antiapoptotic effect of melatonin could be related to the antioxidant effects of melatonin acting as a potent free radical scavenging molecule (6, 7, 65, 106) and also as a regulator of antioxidative enzymes (107). Also, other melatonin-modulated intracellular pathways, such as binding to the calcium-calmodulin complex (66, 67), mitochondrial complexes I and IV (68), and down-regulation of cAMP via MT2 (108), must be taken into account when investigating the mechanisms that underlie melatonin's antiapoptotic protective effects ([Fig. 5](#)).

As an additional functional effect of melatonin, we provide here the first evidence that ER α protein and gene expression in situ in mouse HFs are down-regulated by melatonin ([Fig. 6](#)). This is in line with the reported antiestrogen effect of melatonin through its inhibition of ER (81) by down-regulation of cAMP through MT1 (71) and MT2 (108), and/or modulation of the Ca²⁺/calmodulin signaling pathway (109). ER signaling is involved in the control of HF cycling, and profoundly alters HF growth and cycling in all mammalian species examined so far (72, 73, 95–97). Therefore, by “desensitizing” HFs to estrogen stimulation via the down-regulation of ER α , melatonin could, in principle, exert substantial indirect hair growth-modulatory effects. This hypothesis remains to be tested, for example, by functional studies where organ-cultured HFs are stimulated with melatonin in the presence of ER agonists or antagonists.

Our failure to detect any significant effect of melatonin on microdissected human anagen VI HF growth and pigmentation *ex vivo*, does not rule out an effect of topically applied melatonin on human hair growth in vivo (29). The difference in melatonin administration (our organ culture assay imitates a systemic mode of application) and the lack of the entire distal components of the human pilosebaceous unit, the epidermis, and all extrafollicular skin compartments in our assay system raise the possibility that topical melatonin may still exert indirect effects on human scalp HFs in vivo. Also, if any analogies to the murine hair cycle can be drawn at all, the human anagen VI HF examined by us in organ culture may be relatively insensitive to melatonin stimulation via MT2 and ROR α .

The limited, but biologically important effects that we observed with 0.01 nM to 1 nM of melatonin in organ cultured mouse skin, suggest the presence of functional melatonin receptors. Although our study does not yet allow us to pinpoint which of the detected melatonin receptors is the more important one and which of the observed functional effects are mediated by which

receptor type, it is conceivable that ROR α is stimulated only by higher melatonin doses, while MT2 is stimulated in the picomolar range (40). Therefore, intracellular melatonin generated in the HF may exert intracrine effects via nuclear ROR α receptors, while low levels of secreted melatonin exert auto-, para- and/or endocrine effects primarily via the MT2 cell surface receptor.

In mammals, many tissues express melatonin receptors. For example, the existence of MT1 mRNA and protein has been demonstrated in the pars tuberalis and suprachiasmatic nucleus of the brain, liver, ovary, uterus, and prostate. In contrast, MT2 gene expression has been detected in brain, retina, ovary, uterus, prostate, and kidney (30–33). Recently, MT1 mRNA expression was detected in isolated, cultured human epidermal and HF ORS keratinocytes, in DP fibroblasts and epidermal melanocytes. MT2 mRNA was previously found to be expressed in neonatal keratinocytes and melanoma cells (44).

Our carefully controlled real-time PCR failed to detect MT1 mRNA transcripts, while these were readily detectable in the positive control. Although MT1 gene expression in isolated skin has been reported by a two-round, nested PCR approach (44), it must therefore be questioned, whether MT1-mediated signaling plays an important role in skin and hair biology. ROR α mRNA expression was demonstrated in several organs including brain, retina, heart, liver, lung, gut, ovary, testis, prostate, kidney, leukocytes, and skin (38, 42). However, the expression and localization of these receptors in the HF in situ and hair cycle-dependent changes in MT2 and ROR α expression had not been clarified yet. ROR α -like IR is strongly expressed in DP fibroblasts in middle anagen, and its expression changes in hair cycle-dependent manner (MT2-like IR could not be assessed because of the lack of a suitable antibody).

These studies, which demonstrate that the HF in mice and man is both an important target and a substantial source of melatonin, further underscore the role of the HF as an amazingly versatile and highly active (neuro-)endocrine organ (24, 25, 47, 48, 103). The simple, physiologically relevant model systems employed here are well-suited to further dissect the as yet enigmatic functions of melatonin synthesis and signaling in complex extrapineal neuroectodermal-mesodermal interaction systems *ex vivo*.

NOTE

During the revision of the current paper, we were very pleased to learn that Slominski and colleagues, using different antibodies and detection techniques, were able to independently confirm our previous meeting reports (110, 111) that human scalp HF keratinocytes display melatonin-like IR in situ. In addition, these authors detected serotonin-*N*-acetyltransferase IR in the human scalp HF epithelium (112).

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Table 1**Primers used for real-time PCR**

Gene	Reference sequence^a	Primer location^b	Primer	Sequence (5'-3')	Product size, bp
MT1	NM_008639	115-136 318-296	Forward	TTTACCATCGTGGTGGACATTC	203
			Reverse	GCTGACTTGACAGTGTAGATATC	
MT2	U57554	85-105 332-312	Forward	CTCACTCTGGTGGCTTTGGTG	247
			Reverse	CTGCGCAAATCACTCGGTCTC	
ROR α	U53228	1129-1149 1379-1362	Forward	AGAACAACACCGTGTACTTTG	250
			Reverse	CTGTAGGACGTGCTGAAG	
ER α	NM_007956	1629-1648 1932-1912	Forward	CTGGACAAGATCACAGACAC	303
			Reverse	GTAAGGAATGTGCTGAAGTGG	
ARPO	NM_007475	189-207 507-488	Forward	AGATGCAGCAGATCCGCAT	318
			Reverse	GTGGTGATGCCCAAAGCCTG	

^aAccession number of the reference sequence for PCR primer design. ^bNucleotide positions of the primers relative to the reference cDNA sequence.

Fig. 1

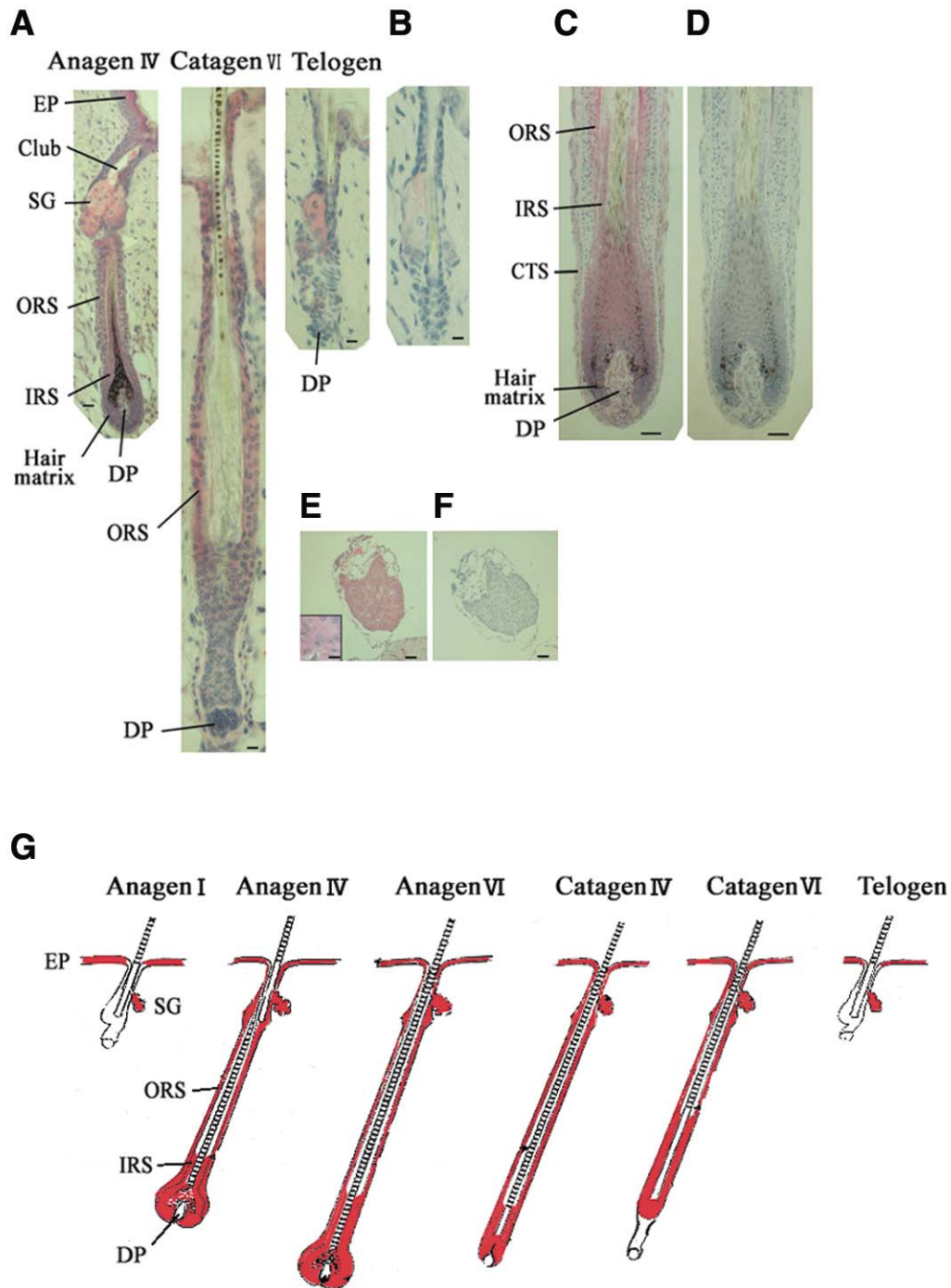


Figure 1. Melatonin-like IR in mouse and human scalp hair follicles (HFs). Melatonin-like IR during the depilation-induced mouse hair cycle (**A**), as well as human scalp HF (**C**) was stained by the ABC method using Fast Red (red) as substrate and Mayer's hemalaun for counterstaining. As a positive control, mouse pineal gland shows melatonin-like IR (**E**). Higher magnification is shown in the square. Incubation without primary antibody was used as a negative control in mouse samples (**B** and **F**) and human scalp (**D**). (**G**) Summary of hair cycle-dependent changes in melatonin-like IR in EP, lower part of IRS and ORS keratinocytes of anagen and catagen HFs and in the SG (red). EP, epidermis; Club, telogen shaft base; DP, dermal papilla; IRS, inner root sheath; ORS, outer root sheath; SG, sebaceous gland; CTS, connective tissue sheath. Scale bars: 10 μ m (**A**, **B** and small square in **E**) and 50 μ m (**C**, **D**, **E** and **F**).

Fig. 2

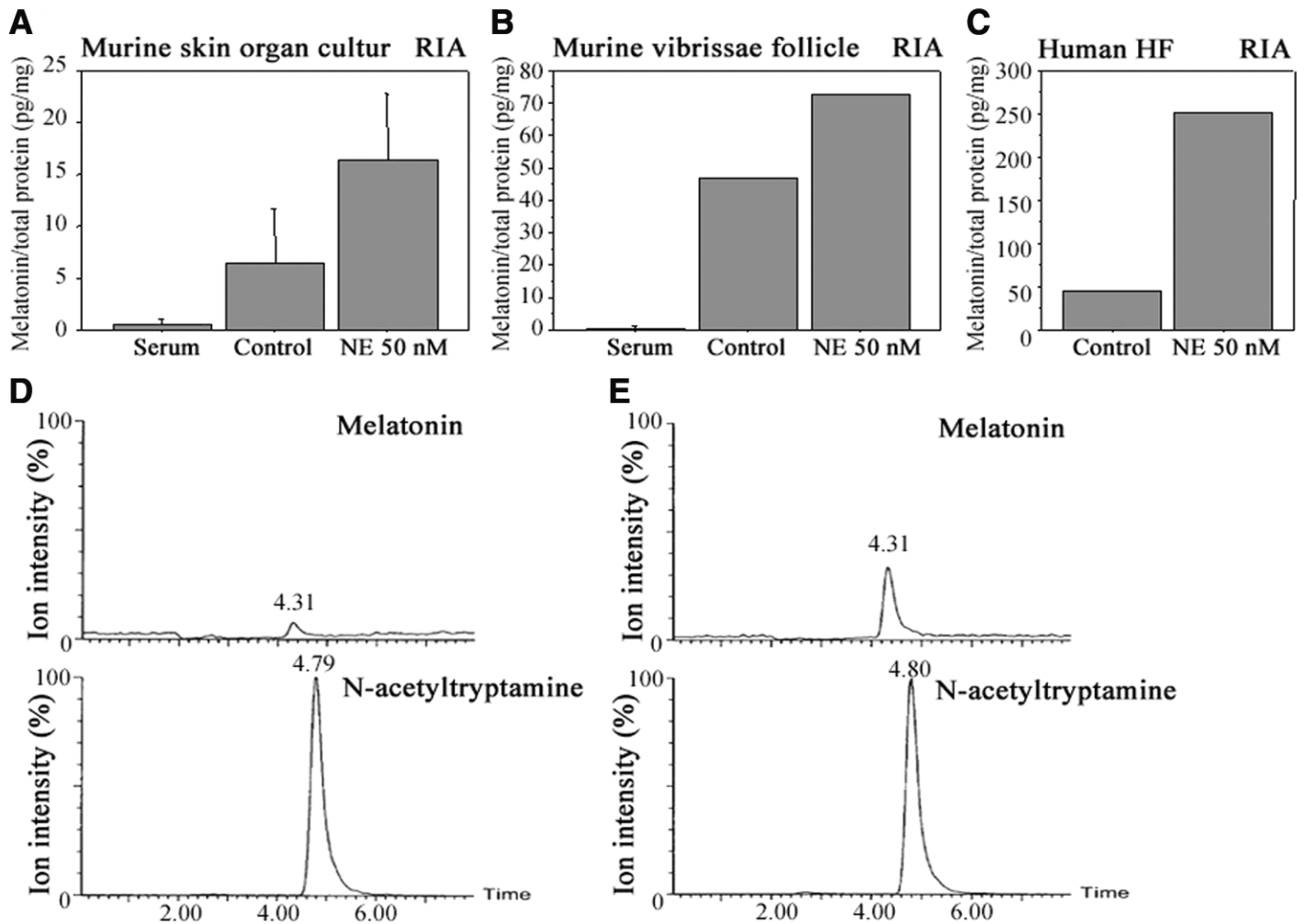


Figure 2. Mouse skin, mouse vibrissae follicles, and human scalp HFs contain melatonin. The melatonin content of mouse serum and of sample lysates of mouse skin, vibrissae follicles, and human HFs was measured by a double-antibody-radioimmunoassay using antimelatonin antibody (**A**, **B**, and **C**). Concentrations of melatonin in the samples were calculated by applying a standard curve fitted by a four parameter logistic algorithm and expressed as picogram per milligram of total protein. LC/MS/MS was used to detect the melatonin content in extracts from human HFs with (**E**) or without (**D**) stimulation with 50 nM NE. N-acetyltryptamine served as an internal standard.

Fig. 3

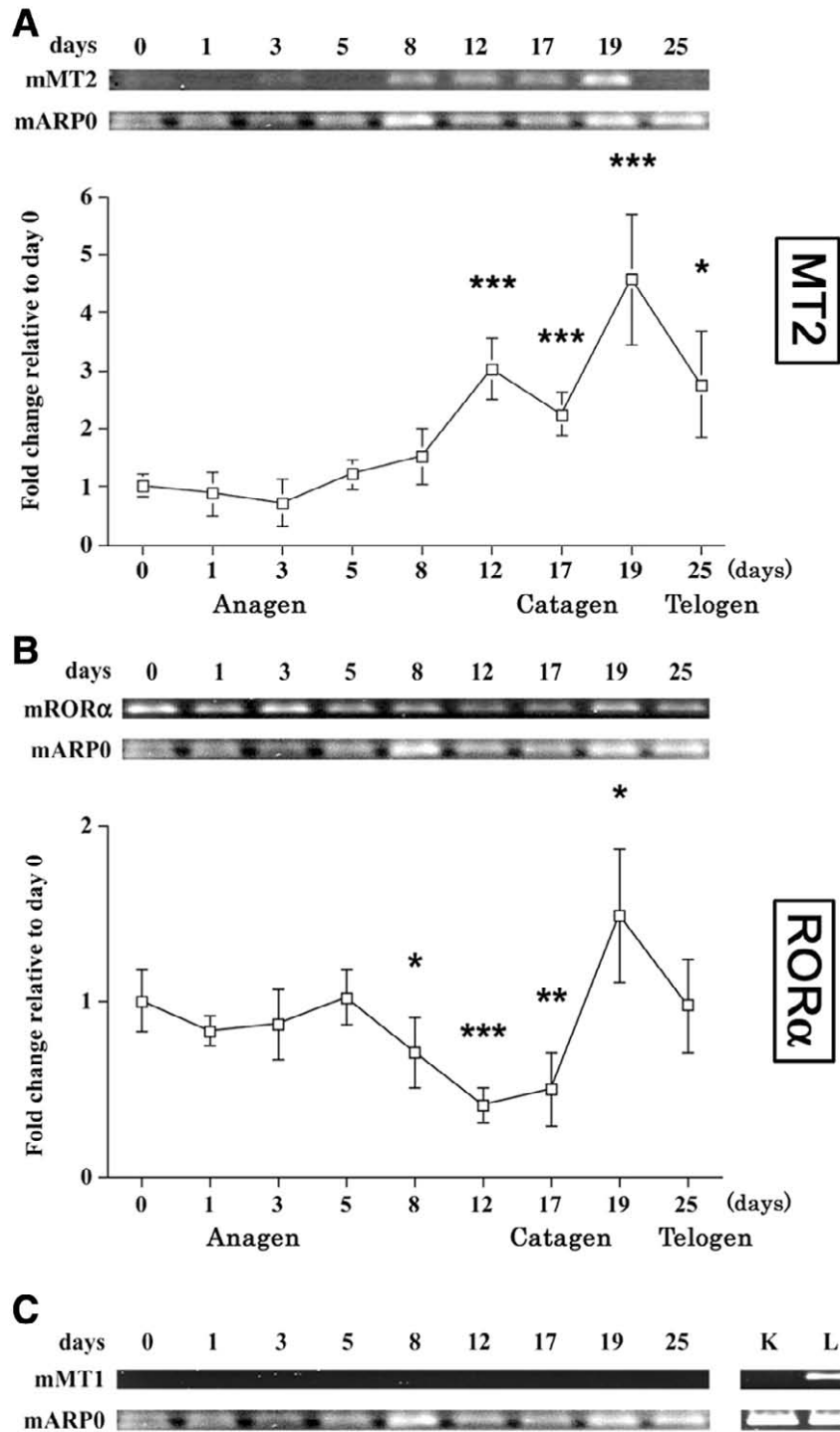


Figure 3. Hair cycle-dependent mRNA expression of MT2 and ROR α in mouse skin. Real-time PCR analysis of expression of MT2 (A), ROR α (B), and MT1 (C) in mouse skin. Amplification of product was measured by real-time PCR and the cycle numbers where the reactions crossed an arbitrary threshold fluorescence value within the exponential growth phase of the PCR were recorded. These values are compared with show fold changes in specific mRNA concentration and are normalized to the expression of ARP0 housekeeping gene. Representative gels are shown where PCR reactions were amplified to a point where reactions were within the exponential phase of amplification. Student's *t* test was performed to test statistical significance (* P <0.05, ** P <0.01, *** P <0.001). Liver (L) and kidney (K) in C represent positive and negative controls for MT1 mRNA expressing tissues, respectively.

Fig. 4

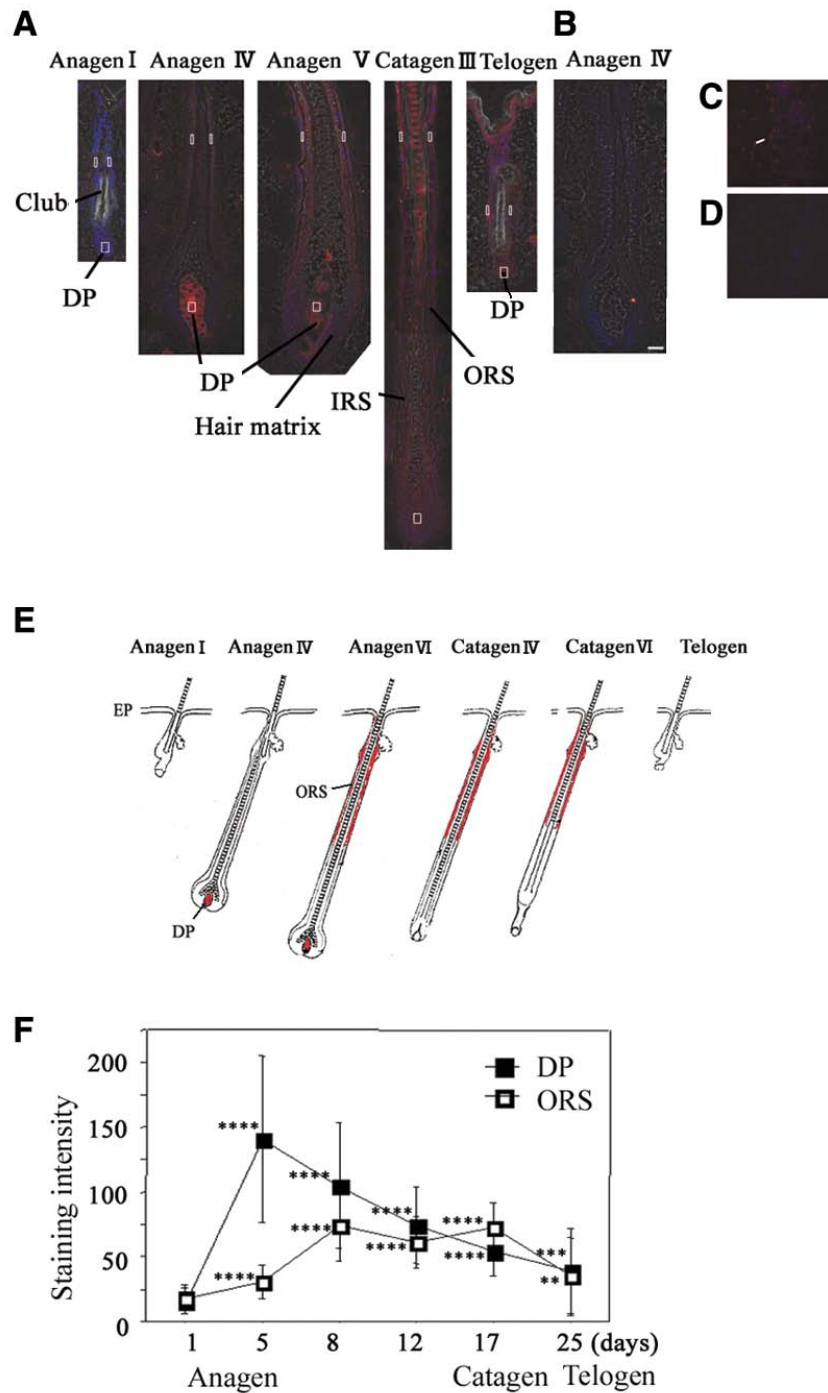


Figure 4. ROR α protein expression in mouse pelage HF in situ. ROR α -like IR during the depilation-induced mouse hair cycle was stained by immunofluorescence using rhodamine (red) and DAPI (blue) for counterstaining (A). Scale bars: 10 μ m. Preincubation of antibody with high concentration of antibody blocking peptide decreased the ROR α -like IR in mouse HF (B). Purkinje cells in the cerebellum served as a positive control (C), where also preincubation of the antibody with high concentration of antibody blocking peptide decreased the ROR α -like IR (D). (E) Summary of hair cycle-dependent changes in ROR α -like IR in DP and upper part of ORS keratinocytes of anagen and catagen HF (red). The mean fluorescence intensity was measured at a reference area in the DP and the middle part of ORS by Scion image analysis ($n=30$ HF/stage). Squares indicate mean values and bars standard deviation (F). (** $P<0.01$; *** $P<0.001$; **** $P<0.0001$ vs. vehicle control).

Fig. 5

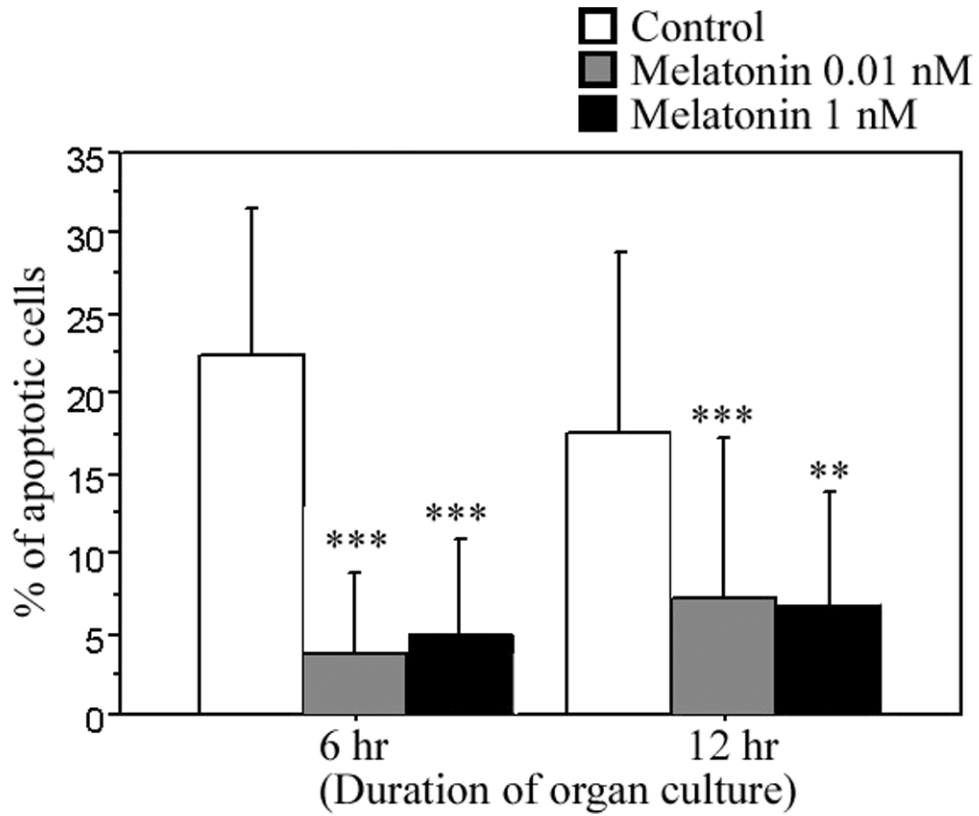


Figure 5. Melatonin reduces epithelial cell apoptosis in telogen mouse skin organ culture. The percentage of apoptotic cells was assessed by the TUNEL method in mouse telogen HF after 6 and 12 h of skin in the HF epithelium organ culture. Samples treated with melatonin at the concentration of 0.01 nM and 1 nM were compared with control samples and percentages of apoptotic cells were calculated. Columns indicate mean values and bars standard deviation (** $P < 0.01$; *** $P < 0.001$ vs. vehicle control).

Fig. 6

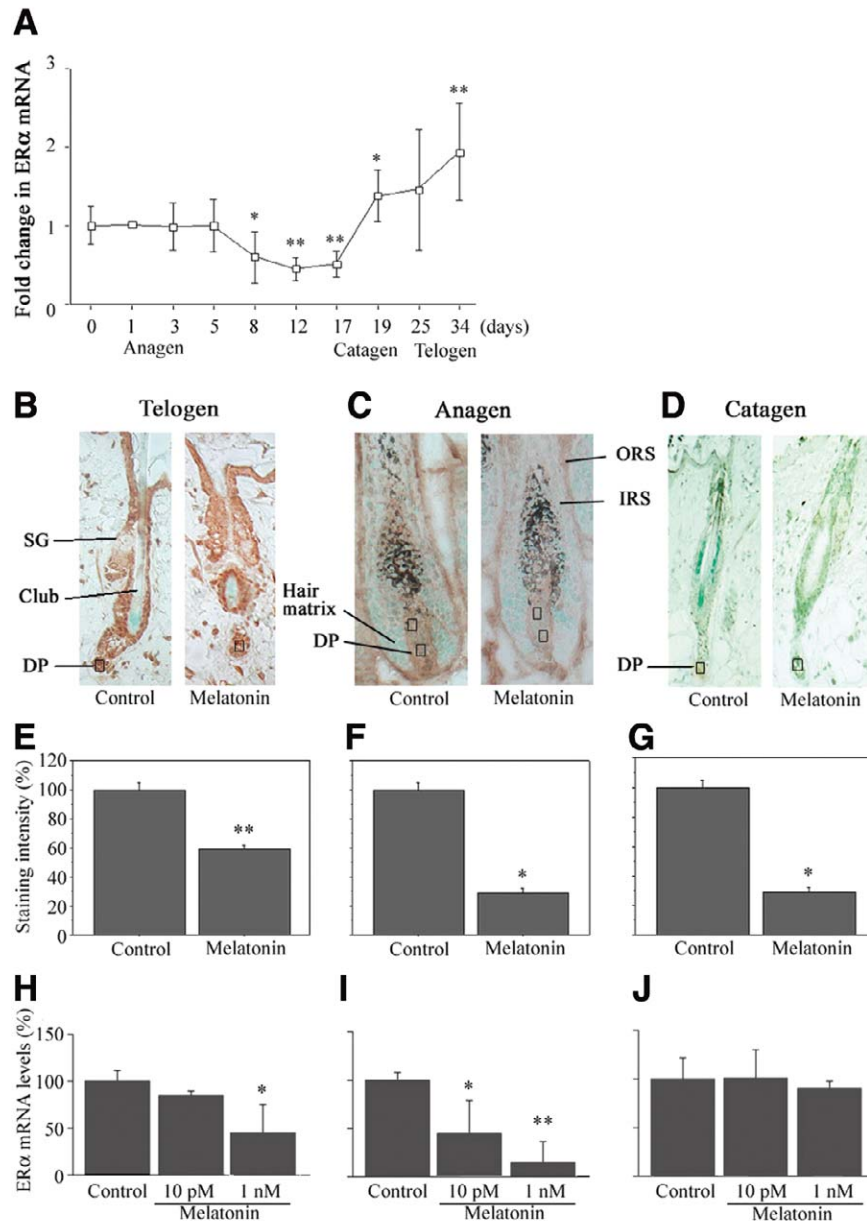


Figure 6. ERα mRNA expression changes in hair cycle-dependent manner, and melatonin decreases ERα-like IR and ERα mRNA expression in short-term mouse skin organ culture. Real-time PCR analysis of ERα expression (**A**). Product amplification was measured by real-time PCR, and the cycle numbers where the reactions crossed an arbitrary threshold fluorescence value within the exponential growth phase of the PCR were recorded. These values are compared to show fold changes in specific mRNA concentration and are normalized to the expression of ARPO housekeeping gene. Student's *t* test was performed to test statistical significance (* $P < 0.05$, ** $P < 0.01$). Immunohistochemistry of ERα was performed on organ-cultured mouse skin with all HF in telogen, anagen, or catagen in the presence or absence of 0.01 and 1 nM melatonin (incubated for 6 h). ERα-like IR was expressed in mouse HF matrix, IRS keratinocytes, and DP fibroblasts in vehicle control, and the IR was decreased in melatonin-treated mouse skin HF (**B, C, D**), and the staining intensity of ERα in DP was significantly decreased after melatonin stimulation of organ-cultured mouse skin with all HF in telogen (**E**), anagen VI (**F**), or catagen (**G**). These data are representative of three independent experiments, which showed very similar trends. The mean intensity of expression of immunoreactivity of ERα was measured at previously defined reference areas in the DP by Scion image. Columns indicate mean values and bars standard deviation (* $P < 0.05$, ** $P < 0.01$ vs. vehicle control). ERα mRNA expression was also significantly decreased after melatonin treatment in telogen (**H**) and anagen (**I**) stage mouse skin organ culture.