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# Melatonin Receptor MT1 is Expressed in Mouse Skin and *In vitro* Melatonin Treatment Enhances Whisker Growth

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## Abstract

Melatonin is an amine derivative produced in the pineal body and retina, and is present in all vertebrates. Melatonin production and secretion are regulated by sunlight sensed by the eyes and control circadian rhythms. It mediates its effects through the melatonin receptors MT1 and MT2, expressed in the central nervous system and retina. Recent studies describing the expression of melatonin in mouse skin, binding to epidermal cells and epithelial hair follicles, and positive melatonin receptor antibody staining in human epidermis suggest the presence of melatonin receptors in the skin. However, the exact localization of melatonin receptor expression in the epidermal tissue and hair follicles is unclear. The aim of this study was to determine the precise location of MT1 in mouse skin, hair and whiskers, and to study the growth effects of melatonin on mouse whiskers *in vitro*. MT1 localization in mouse skin and whiskers was examined immunohistochemically. In addition, organ cultures of mouse whiskers were grown in media containing different concentrations of melatonin, and its effects on whisker growth were evaluated. MT1 was present in the granular layer of mouse epidermal keratinocytes and in the outer root sheath, and *in vitro* treatment of melatonin enhanced whisker growth. Thus, the effects of melatonin on keratinization suggest it may be useful as a drug for promoting hair growth.

**Keywords:** Melatonin; MT1; Keratinization; Hair follicles; Whiskers

## Introduction

Melatonin is a physiologically active amine derivative first isolated from the bovine pineal body in 1958 [1]. Melatonin is produced in the pineal body and retina, and is present in all vertebrates. Since its production and secretion are regulated by the duration of sunlight radiation sensed by the eyes of mammals, melatonin is considered to control circadian rhythms. It also mediates sleep hypnotic effects and has inhibitory effects on reproduction, and was recently shown to have antioxidant effects [2-4].

Melatonin is both fat- and water-soluble and can pass through biological barriers and readily enter cells and therefore is present in various types of humors such as the blood, saliva, cerebrospinal fluid, and liquor folliculi [5,6].

Melatonin receptors are classified as high affinity receptors (ML-1) and low affinity receptors (ML-2). The details of ML-2 are still unclear. ML-1 is further sub-classified to Mel1a, Mel1b, and Mel1c, where Mel1a and Mel1b are present in mammals. The Committee of International Union of Pharmacology proposed changing Mel1a and Mel1b to mt1 and mt2, respectively, and subsequently, changed this to MT1 and MT2. With this change in notation, the conventionally named ML-2 began to be called MT3 [7]. According to this notation, the unified term "MT1" should be used. However, in this study, the name of the commercially available antibody used in immunohistostaining was Mel-1a-R antibody. Therefore, in the text, both terms are used when necessary.

MT1 and MT2 were initially considered present in the central nervous system and central nervous system/retina, respectively [8]. However, recent studies have shown the presence of MT1 in the human retina and studies investigating the locations of MT1 and MT2 expression are continuing [9-11].

A study in 1994 used 3H-malatonin to determine the presence of melatonin in mouse skin, and showed binding to epidermal cells and epithelial hair follicles, probably the external root sheath. However, its exact localization in the epidermal tissue and hair follicles was unclear [12]. The absence of melatonin receptors in goat hair follicles was reported in 1996 [13]. Recently it was shown that melatonin receptor antibody staining was positive in the human epidermis [14]. However, the detailed localization of melatonin receptors in human skin is also unclear. In 1996, Slominski et al. were reported 'Complete metabolism of serotonin in mammal skin *in vitro*' [15]. It turned out that serotonin and melatonin may be metabolized on the skin, and the announcement showed a possibility that serotonin and melatonin were carrying out a certain work on the skin.

The aim of this study was to clarify the effects of the external use of melatonin on mouse hair. Since it has been shown that the external use of melatonin can change mouse body hair, we speculated that melatonin receptors are present in mouse skin. In this study, MT1 localization in mouse skin and whiskers was examined immunohistochemically. In addition, organ cultures of mouse whiskers were grown in media containing melatonin at different concentrations, and the effects of melatonin on whisker growth were evaluated.

## Materials and Methods

### Immunohistochemical examination

Male C57BL/6 mice aged 7-8 weeks were used. Experiments were performed according to the animal experiment principles of the Faculty

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of Medicine, Yamagata University. Mouse dorsal skin was depilated using a depilation tape for the synchronization of the hair cycle, and the anagen phase was induced. Subsequently, a skin incision was made in parallel to the paravertebral line, and skin tissue was collected. Without depilation of the skin, tissue in the telogen phase was also collected. Collected tissue specimens were fixed in 10% formalin solution and embedded in paraffin. The streptavidin/biotin method was performed using Mel-1a-R (MT1) polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA), and color was developed with aminoethylcarbazole. In addition, serial sections were stained with hematoxylin and eosin. Mouse whisker specimens were fixed and stained using the same methods as above. Positive control was granular layer of mouse retina. Negative control was used PBS instead of MT1 polyclonal antibody.

### Organ culture

Male C57BL/6 mice aged 7-8 weeks were used. Mouse whiskers were isolated and cultured for 1 day in Dulbecco's Modified Eagle's Medium (DMEM, Sigma, St Louis, MO, USA) containing 25 mM glucose and 20% fetal bovine serum (FBS), and only whiskers showing elongation were used. Whiskers showing elongation were divided into control and melatonin addition groups by blinded staff members who did not perform experimental measurements. To prevent arbitrary manipulation of measurements, the whisker groups were blinded until the end of the measurement period. The melatonin concentration in the culture medium was adjusted to 0 M (n=67) in the control group and $10^{-16}$ M (n=34), $10^{-14}$ M (n=67), $10^{-12}$ M (n=67), $10^{-10}$ M (n=67), $10^{-8}$ M (n=12), or $10^{-3}$ M (n=11) in the melatonin groups. Specimens were cultured with rotation, in room air containing 5% $CO_2$ at 98.6F for 7 days, and whisker elongation was measured. The results of the measurement of hair length were expressed as the mean ± SEM (standard error of the mean).

Additional experiments on whisker organ culture were performed. The composition of medium used was the same as that in the main experiments. The melatonin concentration was 0 M in the control group and $10^{-14}$ M, $10^{-13}$ M, $10^{-12}$ M, $10^{-11}$ M, or $10^{-10}$ M in the melatonin groups. Sample numbers were 27 in all groups. The culture conditions, duration, and statistical methods were the same as those used for the main experiments.

### Statistical analyses

Statistical differences were analyzed by ANOVA, and $p<0.05$ was regarded as significant. The control group and each melatonin group were compared using the Tukey method.

## Results

### MT1 is expressed in mouse skin and hair bulbs

Immunohistostaining revealed MT1 antibody-positive cells in the granular layer of mouse epidermal keratinocytes and in the outer root sheath (Figures 1 and 2). In the untreated dorsal skins in the telogen phase without induction of the anagen phase by depilation, MT1 antibody-positive cells were also present in the granular layer (Figure 3). In both the anagen and telogen phases, MT1 antibody-positive cells were observed in the external root sheath superior to the opening of the sebaceous glands and in the hair infundibulum showing differentiation similar to that of the epidermis. MT1 localization in the outer root sheath was further clarified by observation of cross-sections of skin (Figure 2). No MT1 antibody-positive cells were observed in any other epidermal layer or hair bulbs. In mouse whiskers, MT1 antibody-positive cells were also present only in the outer root sheath (Figure 4).

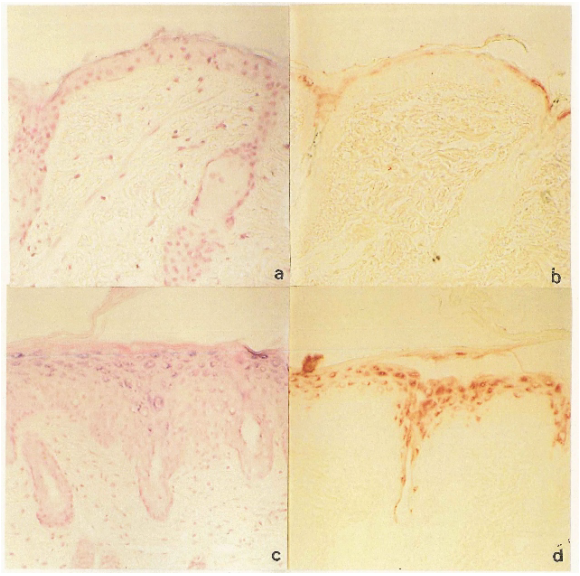


**Figure 1:** Histopathologic features of mouse dorsal skin. **a)** Anagen: One day after depilation, epidermis is thin (hematoxylin-eosin). **b)** Anagen: One day after depilation, single granular layer is stained (MT1-antibody). **c)** Anagen: Five days after depilation, epidermis is thick, especially granular layer (hematoxylin-eosin). **d)** Anagen: Five days after depilation, stained lesion is granular layer only (MT1-antibody).

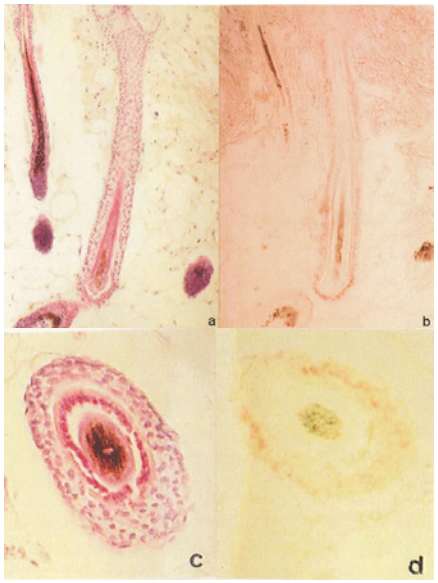


**Figure 2:** Histopathologic features of mouse dorsal skin. **a)** Anagen: After depilation 12days, hair has reached the depths of fat tissue (hematoxylin-eosin). **b)** Anagen: After depilation 12days, stained layer is outer root sheath, not hair bulb (MT1-antibody). **c)** Anagen: After depilation 8 days, a thick layer of outer root sheath is observed on hair cross section (hematoxylin-eosin). **d)** Anagen: After depilation 8 days, it is stained in accordance with the thick outer root sheath (MT1-antibody).

### Melatonin enhanced whisker growth *in vitro*

After whisker organ culture in medium supplemented with melatonin, significant whisker elongation was observed in the $10^{-12}$ M melatonin group compared with the control group (Figure 5).

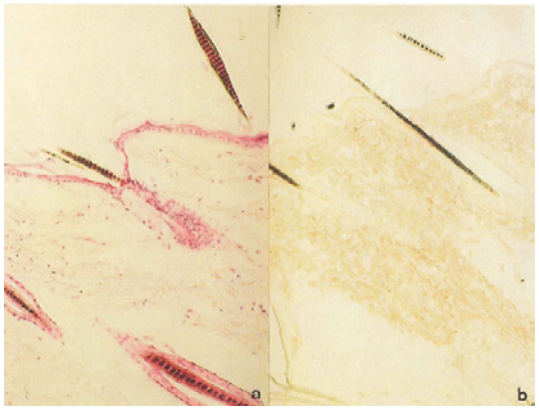


**Figure 3:** Histopathologic features of mouse dorsal skin: **a)** Telogen: No depilation, epidermis is very thin. And thin hair has grown (hematoxylin-eosin). **b)** Telogen: It is stained in accordance with the thin granular layer (MT1-antibody).

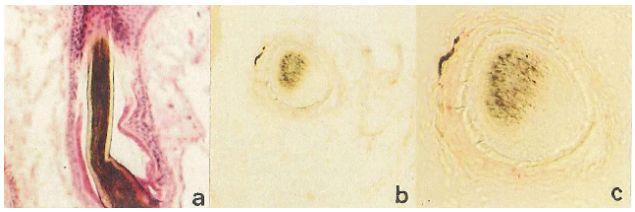


**Figure 4:** Histopathologic features of mouse whisker. **a)** Vibrissa (hematoxylin-eosin). **b)** Cross section (MT1-antibody). **c)** It is stained in accordance with outer root sheath (MT1-antibody, high magnification).

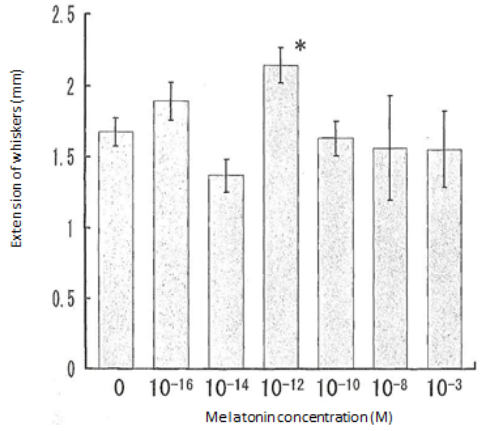


**Figure 5:** Effect of melatonin treatment in mouse whiskers elongation with control (vehicle) or melatonin for 1 week. *P<.05 vs control.

## Discussion

To date, the detailed localization of melatonin receptors in the skin is unclear. This study demonstrated the presence of MT1 in the granular layer of epidermal keratinocytes and outer root sheath of mice.

Melatonin discolors melanophores of amphibians [16]. *In vitro* cultures of mammalian cells have shown that melatonin can inhibit hair melanin synthesis and the proliferation of cultured melanoma cells [17,18]. However, there is less *in vivo* data reported and 1 study has shown improvement in pigmentation of skin after oral melatonin administration in a patient with an untreated adrenogenital syndrome [19]. In addition, the study used similar melatonin administration for patients with idiopathic pigmentation or Addison's disease, but no changes in the skin color were observed. Other studies have shown the influence of melatonin administration in seasonally reproductive animals such as deer and goats, producing seasonal changes in hair [20,21]. These changes in hair replacement after melatonin administration are due to recognition of the season by seasonally reproductive animals, which can affect prolactin secretion. Thus, in previous studies, the action of melatonin on the skin and hair in mammals was mediated by other endocrine actions. Interestingly, when melatonin was administered to patients with endocrine abnormalities no effects of melatonin on epidermal melanocytes were observed based on patients showing no influence of melatonin on skin color [18].

Melatonin was observed in part of the melanocytes surrounding the lesions, and light reactivity differed between melatonin-positive and -negative melanocytes [21].

Although the influence of melatonin on skin has been demonstrated its actions were likely mediated by other endocrine substances. However, whether melatonin acts directly on the skin is still unknown. In the clinical dermatological field, abnormal blood melatonin levels have been reported in patients with atopic dermatitis psoriasis and melanoma [22-24]. These findings together with the high absorbability and transferability of melatonin suggest the involvement of melatonin in these skin diseases.

Recent dermatological studies have shown the antioxidation effects of melatonin such as a decrease in X-ray-induced oxidation stress by melatonin in cultured human fibroblasts and the rapid disappearance of UV-induced erythema after external melatonin use [25,26]. Many studies have suggested that melatonin has antioxidant effects at pharmacological concentrations while others have suggested these effects occur at physiological concentrations [27,28]. Even if melatonin is effective only at pharmacological concentrations, melatonin with high cell transferability may be applicable in the future to percutaneous administration as a drug with antioxidant effects.

In this study, we observed the presence of MT1 in the epidermal granular layer and outer root sheath of mouse skin, both of which are closely involved in keratinization. In epidermal keratinocytes, all of the basal layer, prickle cell layer, granular layer, and horny layer are involved in keratinization during differentiation. The granular layer plays an important role in keratinization; after the appearance of numerous keratohyalin granules, nuclei and organelles disappear, and keratin formation is initiated. The relationship between the granular layer and keratinization has also been shown in several clinical disorders. In patients with vitiligo, a low blood melatonin concentration present during the night, the absence of peak formation and indefinite intra-day variations has been reported [23]. These findings strongly suggest an association between melatonin and vitiligo, which requires further study.

In hair, acute keratinization occurs in the outer root sheath. In outer root sheaths, keratinization is observed in the anagen, catagen and telogen phases although keratohyalin granules are absent, which differs from epidermal keratinization. In hair keratinization, the inner root sheath does not contain keratohyalin granules, but eosinophilic trichohyalin granules appear that resemble keratohyalin granules but which have different staining characteristics. Thus, keratinization differs between the hair and epidermis, and the role of the epidermal granular layer and that of the outer root sheath in keratinization may also differ.

However, each layer plays a part in the final stages of keratinization and considered important. MT1 antibody is positive in the normal human epidermal granular layer in areas both with and without hair and also in the intraepidermal sweat ducts of the eccrine gland in a hairless area (palm). Cells around the intraepidermal sweat ducts of the eccrine gland are considered to resemble epidermal keratinocytes. These cells produce small keratohyalin granules and undergo keratinization earlier than the surrounding epidermis, forming sweat duct walls. These findings also suggest an association between keratinization and melatonin as well as MT1.

The outer root sheath contains epidermal components such as melanocytes, Langerhans cells and Merkel cells and is considered to be continuous with the dermis but not an extension of the dermis based on its function [29-31]. For example, melanocytes contained in the basal layer of the outer root sheath are usually maintained in an inactive state, but are activated when the dermis is injured, and migrate to the regenerating dermis [32]. In addition, the bulge (or area slightly inferior to the bulge) that supplies hair stem cells is in the outer root sheath which may be involved in the supply of hair stem cells [33,34]. MT1 is localized in these areas and together with various actions of melatonin has many potential roles in hair growth.

MT1-deficient mice have been used in studies on central circadian rhythm but there has been no description of their skin or hair. Studies on the circadian rhythm mediated by MT-1 have been performed, but not on the skin which also has a circadian rhythm [35-37]. Hair rhythmically repeats growth and retrogression in the hair cycle. Since peripheral circadian rhythms have been studied in recent years, the circadian rhythm of the skin will also be extensively studied in the future.

In this study, we demonstrated that MT1 was present in the outer root sheath of whiskers as well as hair. This study may provide useful data to studies on the skin, melatonin, and melatonin receptors.

Isolated mouse whiskers can be grown for a certain period in organ cultures, and therefore, have been used in various experiments on hair growth. In this study, we performed organ culture of mouse whiskers in media containing different concentrations of melatonin and measured their elongation. Compared with the control group, the melatonin $10^{-12}$ M group showed significant elongation. Therefore, $10^{-12}$ M may be the closest to the normal blood concentration in C57BL/6 mice. Interestingly, mouse blood melatonin concentrations differ among mouse species and shows intra-day variation. In general, melatonin has a monophasic secretion pattern, reaching a peak value during the night [38]. However, in C57BL/6 mice, its pattern is unclear showing no peaks. Considering the negligible intra-day variations of melatonin levels in C57BL/6 mice, whisker elongation induced by melatonin ($10^{-12}$ M), similar to physiological concentrations, may be mediated by actions other than the circadian rhythm. Since the actions of melatonin remain unclear, discussion regarding its effects on whisker elongation is difficult. However, melatonin can stimulate enzymes such as superoxide dismutase, glutathione peroxidase, and glucose-6-phosphate dehydrogenase [39,40]. *In vitro* organ culture is a limited environment and the medium composition affects the state of cells. In the current study, medium supplemented with serum was used, which is not always used in organ cultures of hair and whiskers [41,42]. The essential components of the growth medium are glucose, calcium, and amino acids. If mice do not eat, whisker growth stops, and resumption of eating initiates whisker growth again [43]. This suggests that glucose is the most essential of all components. In addition, glucose was reported to increase division of epidermal keratinocytes at an optimal concentration but inhibit their division at a high concentration [44]. Therefore, glucose metabolism at a physiological concentration is important. However, medium containing glucose at a high concentration is widely used for cultures, and such medium was also used in this study. Thus, we speculate that the whisker elongation observed in the melatonin $10^{-12}$ M group is due to enhancement of the activity of glucose-6-phosphate dehydrogenase by melatonin. This enzyme is a rate-limiting enzyme of the oxidation pathway of the pentose phosphate pathway. Since all substrates in the oxidation pathway of the pentose phosphate pathway are metabolites of the glycolytic pathway, the pentose phosphate pathway is a shunt of glycolysis. It is possible that melatonin at a concentration close to the physiological concentration enhanced glucose in the high glucose concentration environment, promoting whisker growth.

This study is clarified *in vivo* MT1 localization by immunohistochemistry. In addition, *in vitro* melatonin treatment at a physiological concentration promoted whisker elongation. This suggested the direct effects of melatonin on whiskers, which are not mediated by actions of other hormones. The observed MT1 localization suggested a close association between melatonin and whisker elongation. This finding, together with the results of organ culture suggests that melatonin is necessary for hair growth.

In conclusion, the effects of melatonin on keratinization suggest its potential use as a drug for the promotion of hair growth and keratosis, particularly for psoriasis.

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