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ORIGINAL PAPER

Possible involvement of melatonin in tooth development: expression of melatonin 1a receptor in human and mouse tooth germs

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Abstract Melatonin is known to regulate a variety of physiological processes including control of circadian rhythms, regulation of seasonal reproductive function, regulation of body temperature, free radical scavenging, and so forth. Accumulating evidence from in vitro and in vivo experiments has also suggested that melatonin may have an influence on skeletal growth and bone formation. However, little is known about the effects of melatonin on tooth development and growth, which thus remain to be elucidated. This study was performed to examine the possibility that melatonin might exert its influence on tooth development as well as skeletal growth. Immunohistochemical analysis revealed that melatonin 1a receptor (Mel1aR) was expressed in secretory ameloblasts, the cells of the stratum intermedium and stellate reticulum, external dental epithelial cells, odontoblasts, and dental sac cells. Reverse transcription-polymerase chain reaction and Western blot analysis showed that HAT-7, a rat dental epithelial cell line, expressed Mel1aR and its expression levels increased after the cells reached confluence. These results strongly suggest that melatonin may play a physiological role in tooth development/growth by regulating the cellular function of odontogenic cells in tooth germs.

Keywords Melatonin · Melatonin 1a receptor · Tooth development · Immunohistochemistry · Gene expression

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Introduction

Melatonin was discovered by Lerner et al. (1958), and is secreted by the pineal gland primarily during the night. Its secretion is controlled by the light:dark cycle in such a manner that it is released in large amounts during the night, but only minimally during the day (Lynch et al. 1987; Reiter 1991; Reiter et al. 1996). Melatonin has a variety of physiological actions such as control of circadian rhythms (Redman et al. 1983; McArthur et al. 1997), regulation of body temperature (Dollins et al. 1994), hormone secretion influencing sexual development (Esquifino et al. 1987; Batmanabane and Ramesh 1996) and the reproductive cycle in seasonally breeding animals (Kennaway and Rowe 1995), activation of the immune system (Garcia-Maurino et al. 2000; Raghavendra et al. 2001; Guerrero and Reiter 2002), and anti-tumor activity (Hill and Blask 1988; Martínez-Campa et al. 2006; Sánchez-Barceló et al. 2005). In addition, melatonin and its metabolites potentially exert anti-aging effects due to its ability to function as a free

radical scavenger and antioxidant (León et al. 2005; Tan et al. 2007). Moreover, there are some reports that melatonin controls the secretion of human growth hormone (Forsling et al. 1999) and adrenocortical hormones (Kostoglou-Athanassiou et al. 1998; Cagnacci et al. 1995). Furthermore, it has been reported that melatonin enhances the differentiation of osteoblasts in vitro and promotes bone formation in vivo (Roth et al. 1999; Koyama et al. 2002; Turgut et al. 2005; Machida et al. 2006; Nakade et al. 1999; Satomura et al. 2007). These data strongly suggest that melatonin is involved in the development and/or growth of other hard tissues as well as bone tissue. However, there are no reports on the involvement of melatonin in tooth development or growth.

Two genes encoding membrane melatonin receptors have been identified in mammals including humans: one is the melatonin 1a receptor (Mel1aR) (Reppert et al. 1994) and the other is the 1b receptor (Mel1bR) (Reppert et al. 1995). These receptors are characterized by seven transmembrane-spanning domains and coupling to a trimeric G protein. Both receptors inhibit adenylate cyclase activity via a Gi protein (Reppert et al. 1994, 1995). Of the two melatonin receptors, Mel1aR was originally identified in the suprachiasmatic nucleus and pituitary, but recently its expression was confirmed in other organs and cells including the cerebellum (Al-Ghoul et al. 1998), kidney (Drew et al. 1998), leptomeninges of the brain and spinal cord (Drew et al. 1997), monocytes (Raghavendra et al. 2001), and lymphocytes derived from the thymus or spleen (Pozo et al. 1997). However, there are no reports on the expression of melatonin receptors in teeth.

During mammalian tooth development, a complex series of interactions occur between the oral epithelium and neural crest-derived ectomesenchyme (Gilbert 2000; Jernvall and Thesleff 2000; Thesleff and Mikkola 2002). In the initial stage of tooth development, the process begins with odontogenic differentiation of ectomesenchyme, which is induced by signals from the oral epithelium. Thereafter, tooth morphogenesis is regulated by sequential and reciprocal interactions between the epithelial and mesenchymal tissues (Ten Cate 1994). Such interactive processes between oral epithelium and ectomesenchyme are mediated by a variety of macromolecules of the extracellular matrix together with diffusible signaling molecules, and continue until a tooth is formed with organ-specific mesenchymal cells and organ-specific epithelial cells (Jernvall and Thesleff 2000; Thesleff and Mikkola 2002; Thesleff et al. 1991). Accumulating evidence from in vitro and in vivo experiments has demonstrated that paracrine signaling molecules, including bone morphogenetic proteins (BMPs), fibroblast growth factors (FGFs), sonic hedgehog (Shh), Wnts, tumor necrosis factors (TNFs) and activin, play important roles in the sophisticated epithelial–mesenchymal interaction of the tooth development (Jernvall and Thesleff 2000; Thesleff and Mikkola 2002).

Development of the tooth germ comprises extensive tissue growth and expansion in the developing jaw bones. It is well known that skeletal growth is regulated not only by local factors such as BMPs (Yamaguchi et al. 1991), IGFs (Shinar et al. 1993) and FGFs (Nakamura et al. 1995) but also by systemic hormones including growth hormone (Kassem et al. 1994), parathyroid hormone (Isogai et al. 1996) and estrogen (Jilka et al. 1998). Recently, melatonin was proven to enhance the differentiation of human osteoblasts in vitro, promoting mouse bone formation in vivo (Satomura et al. 2007). Cells of the tooth germ have to form the tooth in harmony with the growth of the surrounding jaw bone. This raises the possibility that systemic hormones such as melatonin could regulate tooth development as well as skeletal development.

From this point of view, it is conceivable that melatonin would involve in tooth development as well as in bone formation. In this study, to confirm this possibility, we examined immunohistochemically whether the odontogenic cells in human and mouse tooth germs express Mel1aR, and also investigated the expression of Mel1aR in a rat dental epithelial cell line, HAT-7.

Materials and methods

Human tissue samples

The tooth germs of the mandibular third molar in the late bell stage, extracted from five Japanese boys and girls (9 or 10 years of age) during orthodontic treatment, were used in this study. The experimental protocol was approved by the Ethical Review Committee of the Tokushima University Hospital, and informed consent was obtained for the use of the tooth germ. Extirpated tooth germs were fixed with 10 N Mildform® (Wako Pure Chemical Industries, Ltd, Osaka Japan), decalcified in 10% EDTA, and embedded in paraffin. Sections were cut 5 mm thick, deparaffinized, and stained with hematoxylin and eosin (Fig. 1). The data presented herein are representative of reproducible results from five different donors.

Mouse tissue samples

The housing care and experimental protocol were approved by the Animal Care and Use Committee of the University of Tokushima School of Dentistry. The mandibles of neonatal mice (ICR, male) were extirpated, fixed with 10 N Mildform® (Wako), decalcified briefly in 10% EDTA, and embedded in paraffin. Sections were cut 5 mm thick, deparaffinized, and stained with hematoxylin and eosin.

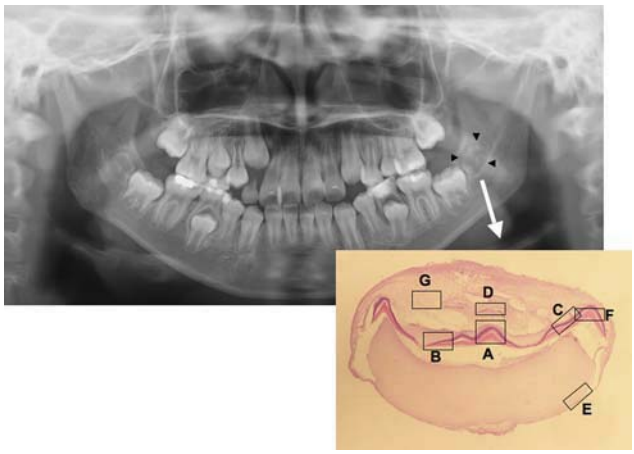

Fig. 1 (*a*) Panoramic radiograph showing a representative case of a 10-year-old girl with tooth germs of the mandibular third molars. The tooth germs (*arrowheads*) were extirpated under local anesthesia and used for histological analysis. (*b*) Transverse sections of a human tooth germ at the late bell stage (hematoxylin and eosin staining). Higher magnification views of several portions indicated by alphabetized squares are shown in Fig. 2. (FW = 10 mm)

Immunohistochemistry

Some sections of human tooth germs and mouse mandibles were transferred onto poly-L-lysine-coated glass slides. After deparaffinization with xylene and rehydration with descending concentrations of ethanol, endogenous peroxidase was blocked by treatment with 3% H_2O_2 in methanol for 1 h at room temperature (RT). After treatment with 10% normal goat serum at RT for 1 h, sections were incubated with rabbit anti-melatonin receptor 1a (MT1) polyclonal antibody (CIDtech Research Inc., Cambridge, Canada) at a concentration of 1 mg/ml in phosphate-buffered saline (PBS, pH 7.4) containing 1% bovine serum albumin at RT for 1 h. Sections were washed with PBS, and localization of Mel1aR was visualized using a Histofine SAB-PO(R) Kit (Nichirei Corporation, Tokyo, Japan) and a 3,3′-diaminobenzidine (DAB) Substrate Kit (Nichirei Corporation). Sections were counterstained with hematoxylin and mounted. The specificity of the immunoreaction was confirmed by (a) incubation with normal rabbit IgG instead of the primary antibody and (b) preabsorption of primary antibody with synthetic peptides which was used as an antigen for anti-Mel1aR antibody.

Cell culture

HAT-7, which is a dental epithelial cell line originating from a rat incisor, was a generous gift from Dr. Hidemitu Harada, Iwate Medical University, Iwate, Japan (Kawano et al. 2002). HAT-7 cells were cultured in Dulbecco's Modified Eagle's Medium Nutrient Mixture F-12 HAM (DMEM/F12HAM: Sigma Chemical Co, St. Louis, MO, USA) containing 10% FBS (Filtron, Brooklyn, Australia), 100 mg/ml ascorbic acid (Wako Pure Chemical Industries, Osaka, Japan), 1× Glutamax® (Invitrogen, Carlsbad, CA, USA), 100 U/ml penicillin (Invitrogen) and 100 mg/ml streptomycin (Invitrogen). The culture was maintained at 37°C in a humidified atmosphere of 5% CO_2 in air and medium was changed twice a week.

Analysis of expression of melatonin Mel1aR receptor

Cells were seeded at a density of 1×10^5 cells/dish in 60 mm Petri dishes and cultured until they reached confluence. The expression of Mel1aR was confirmed by reverse transcription-polymerase chain reaction (RT-PCR) and Western blot analysis. Total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA). cDNA synthesis from 1 mg total RNA was performed using the SUPERSCRIPT Pre-amplification System (Invitrogen) after DNase I treatment. The amplification of primer sequences used to detect the rat Mel1aR gene (NM_053676) were F: 5′-(515)TCAAGTT AGTGCCTTCCTAATGG(537)-3′ and R: 5′-(1058)GACG AGGAAGTGGAAAACCACCAC(1081)-3′. PCR products were analyzed by ethidium bromide staining on a 2.0% agarose gel. For Western blot analysis, the cells were lysed in RIPA buffer (10 mM Tris–HCl, 1% NP-40, 0.1% SDS, 150 mM NaCl and 1 mM EDTA) containing 1 mM phenylmethylsulfonyl fluoride, 5 mM leupeptin and 10 mM aprotinin. The samples (100 mg protein) were separated by the NuPAGE System (Invitrogen) using a 4–12% Bis–Tris Gel, and electroblotted onto a nitrocellulose membrane (Hybond-ECL, Amersham Pharmacia Biotech, Buckinghamshire, UK). After blocking with 5% skimmed milk for 1 h at 4°C, membranes were incubated with 1 mg/ml of rabbit anti-Mel1aR monoclonal antibody (CIDtech Research Inc.) overnight at 4°C. Membranes were then washed several times and incubated with horseradish peroxidase-conjugated anti-rabbit IgG antibody (Dako, Glostrup, Denmark) for 1 h at room temperature. After thorough washes, the immunocomplex was visualized using ECL Western Blotting Detection Reagents (Amersham Pharmacia Biotech) and an ECL mini-Camera (Amersham Pharmacia Biotech). The brain tissues of neonatal rats (Sprague–Dawley, male) were used as a positive control for Mel1aR expression. The housing care and experimental protocol were approved by the Animal Care and Use Committee of the University of Tokushima School of Dentistry. Extirpated brain tissues were rinsed with ice-cold PBS several times and then homogenized at on ice in RIPA buffer containing protease inhibitor cocktail (Roche Diagnostics, Mannheim Germany) for Western blot. The tissue homogenate in RIPA buffer was centrifuged for 10 min at 4,000×*g* and supernatants were used for Western blot as mentioned above.

Results

Localization of Mel1aR in human tooth germs at the late bell stage

In the tooth germs of human mandibular third molars at the late bell stage, some initial dentin and enamel were present in the area where the cusp would later be formed (Fig. 1). General histology revealed that enamel organs in the extirpated tooth germs contained mature ameloblasts secreting enamel matrix, epithelial cells in the stratum intermedium, epithelial cells in the stellate reticulum and external dental epithelium as the epithelial components of tooth germs (Fig. 2a, c). Moreover, dentin-forming odontoblasts, dental papilla cells and dental sac cells were noted as mesenchymal components of the tooth germs (Fig. 2b). Immunohistochemistry for Mel1aR showed that the secretory ameloblasts, the cells of the stratum intemedium and the external dental epithelium had pronounced immunoreactivity for Mel1aR (Fig. 2e–g). In contrast, the cells of the

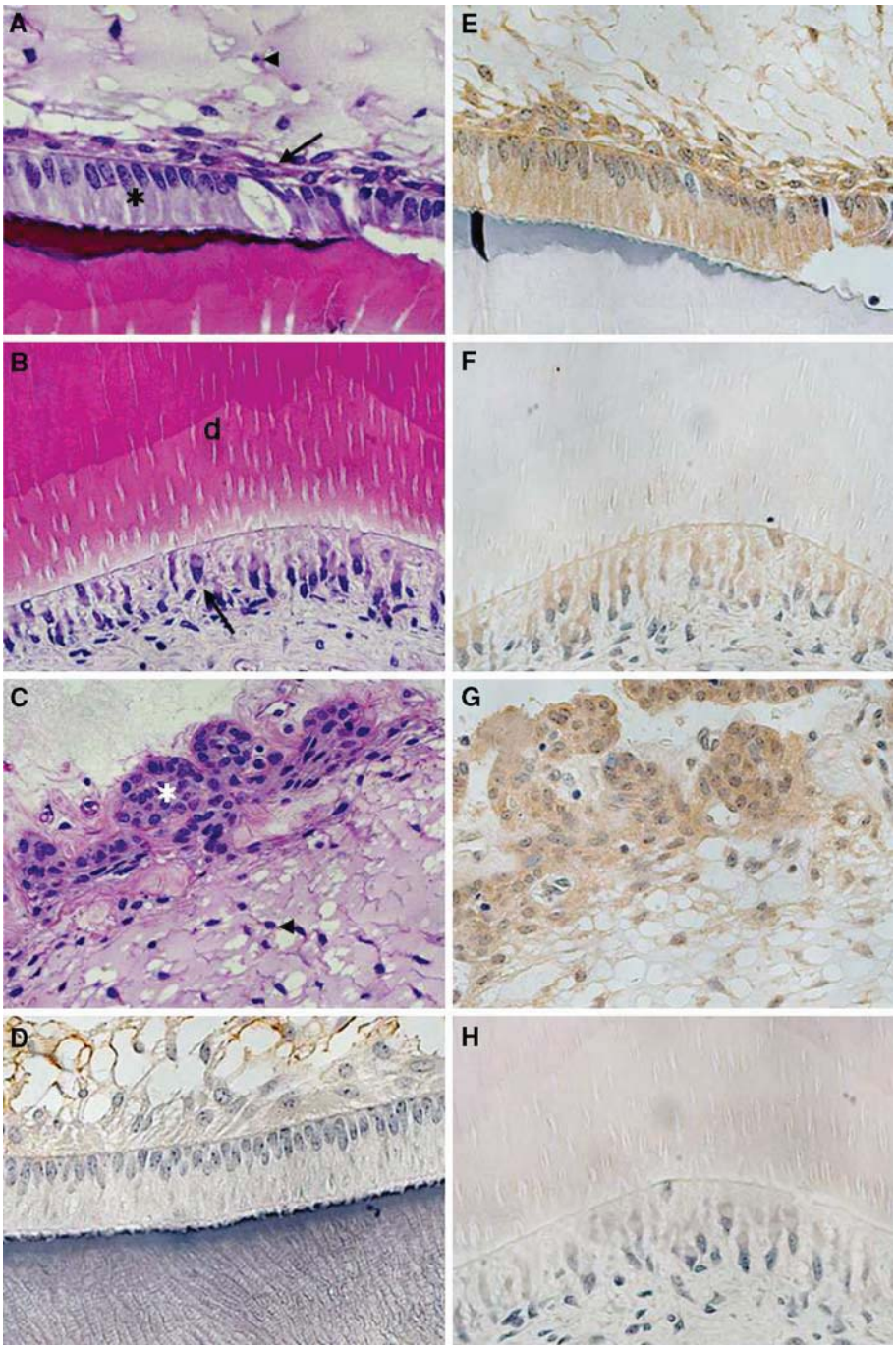

Fig. 2 Higher magnification views of characteristic areas of the tooth germ (hematoxylin and eosin staining). **a** Mature ameloblasts (*asterisk*), stratum intermedium (*arrows*) and stellate reticulum (*arrowhead*). **b** Mature odontoblasts (*arrows*) and dentin (**d**). **c** External dental epithelium (*asterisk*) and stellate reticulum cells (*arrowhead*). Immunohistochemical localization of Mel1aR in human tooth germ at the late bell stage (DAB staining). **d**, **h** Negative controls. Primary antibody was preabsorbed with antigenic peptide. **e** Mature and secretory ameloblasts and stratum intermedium cells were positive for Mel1aR. The cells of the stratum intermedium and stellate reticulum were also noted to be positive for Mel1aR. **f** Odontoblasts were noted to be positive for Mel1aR. **g** External dental epithelial cells were positive for Mel1aR (**a**, **d**, **e** FW = 210 μm; **b**, **c**, **f**, **h** FW = 270 μm; **g** FW = 300 μm)

stellate reticulum showed only slight immunoreactivity for Mel1aR (Fig. 2e). Interestingly, no evident expression of Mel1aR was noted in the odontogenic epithelial cells of the cervical loop region (data not shown). Of the mesenchymal cells in the human tooth germs, the secretory odontoblasts and their processes were positive for Mel1aR (Fig. 2f). Weak positive staining for Mel1aR was found in the cells of the dental papillae. The cells of the dental sac were also moderately positive for Mel1aR (data not shown).

Localization of Mel1aR in mouse tooth germs

Similar to the human tooth germs, a positive immunoreaction for Mel1aR was observed in secretory ameloblasts, cells of the stratum intermedium, secretory odontoblasts and cells of the dental papillae (Fig. 3a, b, d, e). Moreover, osteoblasts were also noted to be positive for Mel1R (Fig. 3c, f). This staining pattern was substantially similar to that in human tooth germs.

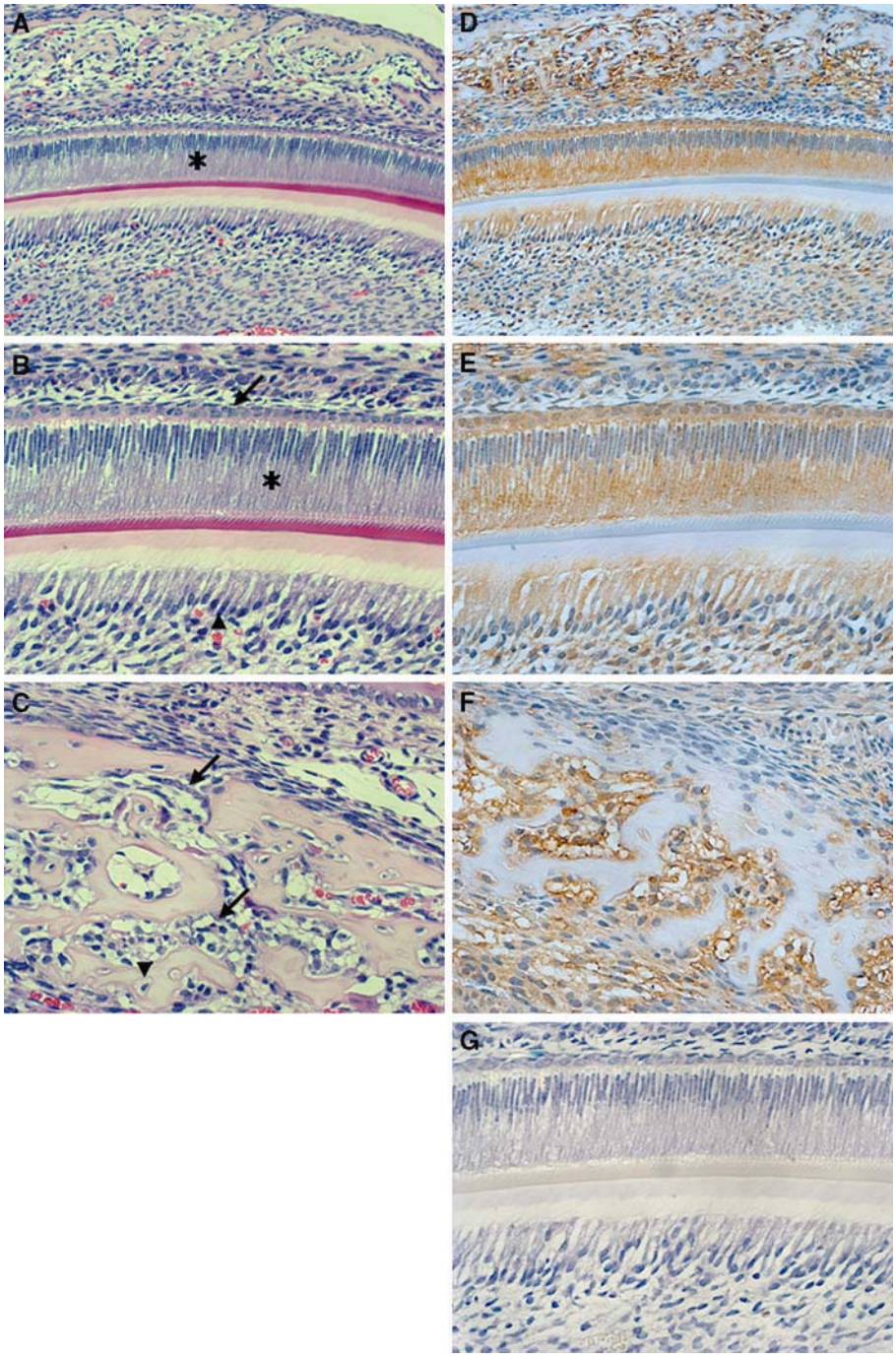

Fig. 3 Immunohistochemical localization of Mel1aR in mouse incisors. **a**, **b** Mature and secretory ameloblasts (*asterisk*), stratum intermedium (*arrow*) and mature odontoblasts (*arrowheads*) can be seen (hematoxylin and eosin staining). **a**, **c** Mandibular bone around the incisor containing osteoblasts (*arrows*) and osteocytes (*arrowheads*) was noted (hematoxylin and eosin staining). **d**, **e** Ameloblasts, cells of the stratum intermedium and odontoblasts were positive for Mel1aR. Osteoblasts of the mandibular bone were noted to be positive for Mel1aR. **a**–**c** Hematoxylin and eosin staining. **d**–**g** DAB staining, **g** negative control. Primary antibody was preabsorbed with antigenic peptide (**a**, **d**: FW = 600 μm; **b**, **c**, **e**, **f**, **g**: FW = 300 μm)

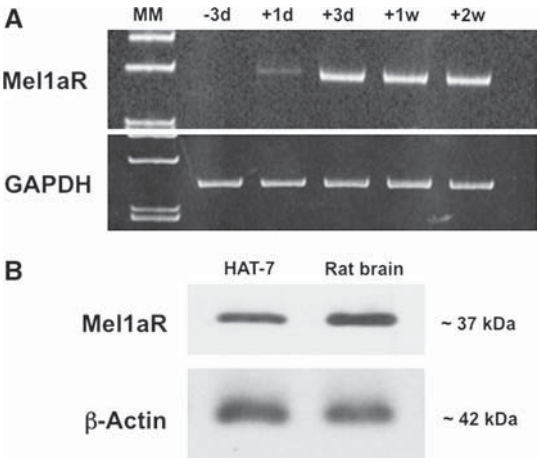

Fig. 4 **a** The expression of mRNA for Mel1aR in HAT-7 cells. Significant expression of Mel1aR was not detected before confluence, whereas Mel1aR expression increased after the cells reached confluence. *−3d* 3 days of culture before confluence, and *+1d*, *+3d*, *+1w* and *+2w* 1 day, 3 days, 1 week and 2 weeks, respectively, of culture after reaching confluence. *MM* molecular marker (ϕX174/*Hae*III). **b** Expression of Mel1aR protein in HAT-7 cells. HAT-7 cells were cultured for 2 weeks after reaching confluence. Thereafter, the expression of Mel1aR protein was analyzed by Western blot. A major band for Mel1aR was observed at ~37 kDa. The amount of protein loaded for HAT-7 and rat brain was 80 and 100 μg, respectively

Expression of Mel1aR in rat dental epithelial cells

Using semi-quantitative RT-PCR, we studied the mRNA expression of Mel1aR in HAT-7 cells, a rat dental epithelial cell line. The expression levels of Mel1aR mRNA increased significantly after the cells reached confluence (Fig. 4a). Western blot analysis also confirmed the expression of Mel1aR protein in HAT-7 cells (Fig. 4b).

Discussion

There are many previous studies showing that melatonin has a variety of physiological actions such as control of circadian rhythms (Redman et al. 1983; McArthur et al. 1997), regulation of body temperature (Dollins et al. 1994), activation of the immune system (Garcia-Maurino et al. 2000; Raghavendra et al. 2001; Guerrero and Reiter 2002), anti-tumor activity (Hill and Blask 1988; Martínez-Campa et al. 2006; Sánchez-Barceló et al. 2005), function as a free radical scavenger and antioxidant (León et al. 2005; Tan et al. 2007) and anabolic effects on bone formation (Roth et al. 1999; Koyama et al. 2002; Turgut et al. 2005; Machida et al. 2006; Nakade et al. 1999; Satomura et al. 2007). However, no data are available about its effect on tooth development. In this study, to investigate the possibility that melatonin plays a physiological role in tooth development as well as in skeletal development, we examined immunohistochemically whether Mel1aR, which is the most potent transmembrane receptor for melatonin, was expressed in human and mouse tooth germs. The present study demonstrates for the first time that Mel1aR is expressed in a variety of human and mouse dental structures during tooth development. Mel1aR is also localized in osteoblasts which are forming bone of the jaws around the tooth germ. The expression pattern of Mel1aR in the developing mouse tooth germs is consistent with the pattern observed in human tooth development. We also confirmed immunohistochemically that Mel1aR was expressed in tooth germs of another strain of mouse (C57Bl/6J) and F344 rat (data not shown). In addition, HAT-7, a rat dental epithelial cell line, was also confirmed to express Mel1aR at the protein level by Western blot analysis. Interestingly, the expression levels of mRNA for Mel1aR in HAT-7 cells increased significantly after the cells reached confluence. This phenomenon may suggest that melatonin exerts its influence not on the proliferation but on the differentiation and/or function of odontogenic cells and be consistent with the findings that Mel1aR was not expressed in the cervical loop.

The physiological or developmental significance of the presence of Mel1aR in the tooth germs remains unknown. The issue of whether Mel1aR expressed in tooth germs has normal function should be addressed in the future. The physiological role of melatonin itself in tooth development also remains unknown in the present study. Nevertheless, our observations strongly suggest that melatonin might exert its influence on tooth development at least partly through Mel1aR, and that tooth development and growth may be under regulation by systemic hormones in the same way as skeletal growth. It is possible that melatonin may regulate the proliferation, differentiation and/or function of odontogenic cells in the tooth germs in order to maintain the expanding tooth morphology. In fact, it is known that ameloblasts slowly form prismatic enamel with cross striation (4–8 μm) under the regulation of circadian clock (Smith 2006). The effects of melatonin could be conceivably related to this secretory behavior. Another possibility is that melatonin could harmonize the growth of the tooth with the growth of the surrounding jaw bone through physiological regulation of odontogenic cells, judging from the fact that skeletal growth is also controlled by melatonin. To elucidate these issues, in vitro studies using odontogenic cells would be very helpful, and should be performed in the near future.

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