Published in final edited form as:

J Pineal Res. 2012 October ; 53(3): 307–318. doi:10.1111/j.1600-079X.2012.01000.x.

Impaired Mouse Mammary Gland Growth and Development is Mediated by Melatonin and its MT₁ G Protein-Coupled Receptor via Repression of ER α , Akt1, and Stat5

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Abstract

To determine if melatonin, via its MT₁ G protein-coupled receptor (GPCR), impacts mouse mammary gland development, we generated a mouse mammary tumor virus (MMTV)-MT₁-Flag-mammary gland over-expressing (MT₁-mOE) transgenic mouse. Increased expression of the MT₁-Flag transgene was observed in the mammary glands of pubescent MT₁-mOE transgenic female mice, with further significant increases during pregnancy and lactation. Mammary gland whole mounts from MT₁-mOE mice showed significant reductions in ductal growth, ductal branching, and terminal end bud (TEB) formation. Elevated MT₁ receptor expression in pregnant and lactating female MT₁-mOE mice was associated with reduced lobulo-alveolar development, inhibition of mammary epithelial cell proliferation, and significant reductions in body weights of suckling pups. Elevated MT₁ expression in pregnant and lactating MT₁-mOE mice correlated with reduced mammary gland expression of Akt1, phospho-Stat5, Wnt4, estrogen receptor alpha (ER α), progesterone receptors (PR) A and B, and milk proteins β -casein and whey acidic protein (WAP). Estrogen and progesterone stimulated mammary gland development was repressed by elevated MT₁ receptor expression and exogenous melatonin administration. These studies demonstrate that the MT₁ melatonin receptor and its ligand melatonin play an important regulatory role in mammary gland development and lactation in mice through both growth suppression and alteration of developmental paradigms.

Keywords

Melatonin; MT₁ Receptor; AKT; Stat5; Mammary Gland Development

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INTRODUCTION

The mammary gland is a unique organ that develops after birth and undergoes dynamic changes throughout the reproductive lifespan of the female. The postnatal program of mammary development occurs in two distinct stages. The first is initiated by the surge of ovarian hormones at puberty and results in elongation of the ductal tree to the edges of the mammary fat pad, along with arborization by primary, secondary, and side branches. The second major stage of mammary development occurs during pregnancy, when extensive proliferation of the epithelial compartment results in side branching and the formation of milk producing alveoli. At the onset of puberty, ovarian 17- β estradiol (E2) stimulates ductal morphogenesis during which the mammary epithelial progenitor cells differentiate and proliferate while interacting with adipocytes and stromal cells within the mammary fat pad [1, 2]. Ovarian E2, in synergy with pituitary hormones such as growth hormone (GH), stimulate stromal cells to produce local insulin-like growth factor-1 (IGF-1), providing a paracrine signal for commencing ductal morphogenesis [3]. Mice lacking ER α , GH receptor, and the IGF-1 receptor fail to undergo postnatal ductal morphogenesis [4, 5] indicating that steroid hormones and IGF-1 are common pathways for a critical developmental checkpoint. Downstream activation of Wnt proteins, and Wnt4 in particular, by ovarian steroids is a major pathway mediating ductal branching in the mammary gland [4]. Furthermore, signal transducer and activator of transcription (Stat) 5 is also a critical regulator of normal mammary development. Stat5 is reported to play important roles in cell differentiation and survival during pregnancy. Expression of Stat5 in virgin animals requires prolactin, as well as, E2 and progesterone, suggesting that it may mediate effects of these hormones. Once the arborized ductal network is established, cycles of differentiation, proliferation, and death of secretory alveolar epithelium repeat with each pregnancy [5, 6].

Melatonin (*N*-acetyl-5-methoxytryptamine) is an indolic hormone synthesized and secreted by the pineal gland [7] in response to darkness. In addition to its well-known modulatory actions on sleep and seasonal reproduction, melatonin has also been shown to suppress the *in vivo* development of spontaneous and carcinogen-induced mammary cancer and proliferation of human breast tumor cell lines *in vitro* [7–9].

The anti-tumor action of melatonin on mammary and breast cancer has been observed in numerous laboratories, including our own [10]. These studies have ascribed the anti-cancer action of melatonin to a variety of mechanisms including: (a) its repression of the mitogenic E2 signaling pathway [11, 12] via repression of E2-induced transcriptional activation of the estrogen receptor alpha (ER α), (b) its inhibition of tumor uptake of the omega-3 fatty acid linoleic acid, and its conversion to 13-HODE [13], and (c) its antioxidant properties [14], etc. Furthermore, our studies have demonstrated the ability of melatonin to modulate the activation of nuclear receptors, including repression of ER α , glucocorticoid receptor (GR), and retinoic acid-related orphan receptor alpha (ROR α), retinoic acid receptor alpha (RAR α), and retinoic acid X receptor alpha (RXR α) transcriptional activity [15, 16]. Finally, recent studies by our research team, has shown that Akt phosphorylation at serine 473 in tissue-isolated human breast tumors grown in nude rats is greatly reduced at night, when melatonin levels are elevated, as compared to daytime when melatonin levels are repressed [17].

Although melatonin is highly lipophilic and was originally proposed by Becker-André and colleagues [18] to be a ligand for the RZR β /ROR α receptors (this paper has been retracted) [19], many of its actions are mediated through the MT1 and MT2 GPCRs [20]. In fact, we have reported that the action of melatonin on nuclear receptor transcriptional activity and its suppression of breast tumor growth are mediated primarily through the MT1 receptor [21]. Previous studies by Mediavilla and co-workers [22, 23] examined the effect of melatonin on

the growth of mammary gland tissue both *in vitro* and *in vivo* and found that physiologic concentrations of melatonin decreased mammary DNA content and uptake of [³H]-thymidine in the mammary gland and decreased the development of terminal, lateral, and alveolar buds.

The ability of melatonin to suppress the *in vitro* growth and development of the mammary gland in normal mice suggests that it may play an important role in mammary development at puberty and differentiation during pregnancy and lactation. Furthermore, the reported inhibitory actions of melatonin on ER α expression and transcriptional activity suggest melatonin may inhibit E2-induced mammary gland development. To elucidate if the MT₁ receptor mediates the actions of melatonin on mammary gland development, we have developed an MT₁ transgenic mouse with expression of the MT₁-Flag tagged receptor under the control of the MMTV promoter, and, thus, conditionally expressed specifically in the mammary epithelium. Our results demonstrate that melatonin, via its MT₁ receptor, represses ductal branching, TEB formation, lobulo-alveolar development, and the proliferation of ductal epithelium to repress mammary gland development and also repress milk production during pregnancy and lactation.

MATERIALS AND METHODS

Generation of MT₁ transgenic mice

MT₁-Flag B6SJLF2 transgenic mice were generated using a 1.2 kb cDNA fragment (1085 bp of the MT₁ cDNA) containing the 3' portion of the human MT₁ gene under the control of the MMTV-LTR transcriptional promoter. The DNA fragment was isolated by agarose gel electrophoresis. The purified 1.2 kb MT₁-Flag construct was injected into the pronuclei of B6SJLF2 single-cell mouse embryos as previously described [24]. Microinjected embryos were transferred to the oviducts of pseudopregnant foster females. To identify the resulting transgenic progeny, genomic DNA isolated from tail biopsies was evaluated by real-time polymerase chain reaction (qPCR) analysis. Briefly, DNA was extracted from tail segments biopsied from 3-week-old weaned mice with Puregene Tissue Core Kit A (Qiagen, Valencia, CA) according to the manufacturer's protocol. Real-time polymerase chain reaction analysis, using 40 cycles at 95° C for 15 s, 60° C for 1 min. was performed with 0.5 μ g of tail DNA in a final volume of 20 μ l using IQ SYBR Green Supermix kit (Bio-Rad 170-8882) in a Bio-Rad (Richmond, CA) iCycler thermal cycler. Primers for the amplification of a 150 bp of Flag-MT₁ were 5' GACTACAAGGACGACGATGACAAG (corresponding to FLAG) and 3' GAAGATGAGGACGCAGGCTAGG that hybridizes to the 5' terminal 109–126 nucleotides of human MT₁ ORF. Primers for the amplification of a 130 bp mouse GAPDH fragment were 5' CAACTACATGGTCTACATG and 3' CTCGCTCCTGGAAGATG served as a qPCR internal control.

Whole mount and histological analyses

The entire left no. 4 mammary gland of each animal was removed, mounted on glass slides, fixed overnight in Carnoy's Fixative, rinsed in 70% ethanol, and then stained overnight at 4°C with carmine alum (2 g/l). The slides were rinsed in a graded series of ethanol and cleared in xylene before mounting with Permount. Images of mammary gland whole mounts were taken using a Nikon DS-Qi1MC digital camera affixed to a Nikon AZ100 multipurpose zoom microscope. For histological analysis, glands were fixed overnight in 10% neutral buffered formalin, then changed to 70% ethanol, processed, and embedded in paraffin. Sections (4 μ m thick) were prepared and stained using Harris Hematoxylin and Eosin. Images of histological sections were taken with a Nikon DXM1200 digital camera affixed to a Nikon Eclipse E800 microscope using ACT-1 software.

Ductal elongation, terminal end bud (TEB), and branch point analyses

Axiovision Release 4.5 software was used to measure ductal elongation, TEB number, and number of secondary and tertiary branch points from images of 4, 8, and 12 week-old B6SJLF2 control and B6SJLF2 MT1-mOE inguinal mammary gland whole mounts. Ductal elongation was measured as the distance from the center of the lymph node to the furthest TEB at the leading edge of the mammary epithelium. All TEBs of greater than or equivalent to 0.03 mm² were quantified. The longest primary duct directly above the lymph node was used for branch quantification from one inguinal gland per mouse (n = 10 per genotype). A secondary branch was defined as any branch that bifurcates from the primary ducts, a tertiary branch as any branch that bifurcates off a secondary branch.

BrdU analyses

B6SJLF2 control and MT1-mOE mice at 4, 8, and 12 weeks of age, 14 and 18 days of pregnancy, and day 1 of lactation (n = 5 per genotype) received an intraperitoneal injection with 10 µl/g of body weight of a BrdU solution (10 mM) [Amersham Biosciences, Piscataway, NJ] 2 h prior to euthanasia. The right side thoracic and inguinal glands were fixed in 10% neutral buffered formalin overnight then changed to 70% ethanol. Inguinal mammary glands from both wild type and MT1-mOE mice were processed, embedded in paraffin, and 4 µm thick sections were cut and stained using a BrdU Staining Kit (Zymed Laboratories, Inc., San Francisco, CA) according to the manufacturer's instructions. The proliferation index was determined by quantifying the number of BrdU-positive cells out of the total number of cells from 4 end buds and 4 ducts per mouse per unit area (mm²).

Immunohistochemical analyses

Estrogen receptor α was detected on tissue sections from B6SJLF2 control and MT1-mOE mice at 4, 8, and 12 weeks. Sections were incubated with a primary ER α antibody or a primary Akt1 antibody (Cell Signaling Technology, Danvers, MA) followed by 0.75 µg/ml goat anti-rabbit biotinylated secondary antibody. Antibody immunoreactivity was amplified using the VECTASTAIN ABC kit (Vector Laboratories, Burlingame, CA), and visualized using DAB substrate (Vector Laboratories, Burlingame, CA). The sections were counterstained in hematoxylin, dehydrated in a graded series of alcohols ending with Xylene, and mounted. All images were captured using a Nikon DXM1200 digital camera affixed to a Nikon Eclipse E800 microscope using ACT-1 software.

Western blot analyses

Mammary glands excised from developmentally staged 4, 8, and 12 week-old virgin, pregnant (day 14 and 18), and lactating (day 1) female B6SJLF2 control and MT1-mOE mice (n = 5 per genotype) were snap frozen in liquid nitrogen. Total protein was extracted by homogenization using a Dounce homogenizer in 0.6 ml extraction buffer (25 mM Tris pH 7.4, 150 mM NaCl, proteinase inhibitor cocktail, Sigma, MG, cat# P8340). Homogenates were centrifuged at 10,000 × g for 3 min at 4°C, and fat was removed from the surface by vacuum aspiration. The precipitate was resuspended by vortexing, and NP-40 was added to a final concentration of 0.5%. Samples were again homogenized using a Dounce homogenizer and centrifuged. Supernatants were decanted, and protein concentrations determined using the Coomassie protein assay (Pierce Biotechnology, Rockford, IL). Protein solutions were stored at -80° C until use. Protein concentrations were determined using the MicroBCA Kit (Pierce Biotechnology, Rockford, IL) according to the manufacturer's instructions. Approximately 75 µg of protein were diluted in Lamelli sample buffer, boiled, and loaded onto a 10% PAGE Tris-glycine gel. Separated proteins were transferred to nitrocellulose membrane (Bio-Rad Laboratories, Richmond, CA, Cat# 162-0115). Membranes were blocked with 5% milk in Tris-buffered saline-0.1% Tween 20 (TBST). Mouse monoclonal

anti-Akt1, anti-ER α , rabbit polyclonal anti-Progesterone Receptor (PR), and rabbit polyclonal anti-Stat5 antibodies were purchased Santa Cruz Biotechnology (Santa Cruz, CA), rabbit polyclonal anti-Wnt4 antibody was purchased from Epitomics (Burlingame, CA), and rabbit monoclonal anti-phosphoStat5 (phosphor Y694) antibody was purchased from ABcam (Cambridge, MA), and used according to the manufacturer's instructions and detected using peroxidase-conjugated anti-rabbit or anti-mouse secondary antibodies (Jackson Immuno-Research, West Grove, PA). Antibody detection was performed according to the manufacturer's instructions with ECL Plus Western Blotting Detection System (Habersham Bio-sciences, Piscataway, NJ) and developed on film.

Estrogen and progesterone supplementation to induce mammary gland development

Four-week-old control and MT1-mOE mice were grouped and treated as follows: controls (diluent 0.1% ethanolic saline given i.p); melatonin (25 μ g/day i.p, 1 h before lights off); E2 [0.1 mg/pellet] + progesterone (10 mg/pellet); and E2 + progesterone + melatonin. Estradiol and progesterone were administered by implanting E2 and progesterone pellets (Innovative Research of America, Sarasota, FL) under the skin with sham pellets used as controls. After 4 weeks of treatment (8 weeks of age), mice were sacrificed and the left side inguinal mammary gland (#4) was examined by whole-mounted analysis for morphological assessment as described above.

Immunofluorescent staining

Milk proteins β -casein and whey acidic protein (WAP) were detected on tissue sections from B6SJLF2 control and B6SJLF2 MT1-mOE mice at 18 days of pregnancy and day 1 of lactation, as previously indicated. The number 4 inguinal mammary gland was then snap frozen in liquid N₂ and stored at -70°C until use. The tissues were cut into 4 – 6 μM thick frozen sections and placed on pre-cleaned slides, fixed with acetone, and blocked with 10% normal horse serum. Sections were incubated with primary antibodies (β -casein and WAP from Santa Cruz Biotechnology, Santa Cruz, CA) followed by fluorescence conjugated goat anti-rabbit secondary antibody, mounted, and viewed with fluorescent microscopy.

Statistical analysis

All values were expressed as mean $\pm$ SEM. Differences in body weight, total number of mammary ductal branchings, TEBs, BrdU staining, and expression of ER α were analyzed by Student's t test between control and MT1-mOE groups. P-values < 0.05 were considered statistically significant.

RESULTS

Previous studies by Mediavilla et al. [22] demonstrated an inhibitory effect of melatonin on ductal morphogenesis of mice mammary glands. To determine the importance of the MT₁ receptor in this process, a Flag-tagged MT₁ cDNA was placed under the control of an MMTV-LTR promoter (Fig. 1a). Transgenic mice having a B6SJLF2 genetic background were produced by injection of purified DNA fragments into the pro-nucleus of fertilized mouse oocytes. The F2 and F3 generation mice were used for phenotype analysis. Quantitative real time PCR analysis of tail DNA from potential MMTV-MT1-flag founder transgenic animals revealed four founder animals that possessed the 150 bp amplified fragment. Expression of the MT₁ transgene was determined using Western blotting and antibodies to Flag or MT₁ proteins to measure their expression in the mammary glands. Two MMTV-MT1 transgenic lines were found to express Flag or MT₁ (570-4, 566-3) proteins. All of the experiments described in this manuscript were performed with these two MMTV-MT1-mOEtransgenic lines.

To determine the temporal expression pattern of the MT₁ transgene, total cellular protein was isolated from mammary glands of developmentally staged female mice and subjected to immunoblot analysis (Fig. 1b). MT₁ transgene expression was detected as early as 4 weeks post birth (onset of puberty) with expression levels significantly increased during puberty in the mammary gland at 8 weeks and even more by 12 weeks. Expression levels of MT₁-Flag transgene protein were significantly increased from day 14 of pregnancy to day 1 of lactation (about eight-fold greater than observed within the mature virgin mammary gland).

On postnatal days 10, 20, and 30, pups from each litter were weighed and average body weights recorded. Pups born to and nursed by MT1-mOE transgenic dams showed a 36% and 25% decrease ($p = 0.05$) in average bodyweight on days 10 and 20, respectively, compared with pups nursed by control non-transgenic dams (Fig. 2). On day 30 (10 days post weaning), the body weight of pups born to and nursed by MT1-mOE transgenic mothers returned to the values of pups from non-transgenic mothers. This suggests that MT1-mOE transgenic dams have impaired, but not complete loss of milk production and may have altered mammary gland development.

To determine the effects of MT₁ expression on ductal proliferation, branching morphogenesis, and TEB formation in the pubertal virgin female mouse, we performed whole mount analysis and immunohistological analysis on paraffin-embedded sections of mammary glands from MT₁ expressing virgin mice examined at 4, 8, and 12 weeks of age (Fig. 3A). Mammary glands of mice expressing the MT₁ transgene showed a 35% repression in ductal branching at 12 weeks (Fig. 3B). Similarly, MT1 over-expression resulted in impaired TEB formation by 57% at week 12 of age (Fig. 3C).

With the onset of pregnancy, proliferation of lateral and terminal ductal epithelial buds initiate ductal side branching and increased formation of lobulo-alveolar units. This lobulo-alveolar development continues through pregnancy and is maintained through lactation. In contrast to non-transgenic control mice, a 43% reduction in lobulo-alveolar outgrowth with ducts bearing few lateral and terminal alveoli was observed in MT1-mOE transgenic female mice at day 14 of pregnancy (Fig. 4). By day 18 of pregnancy, mammary tissue continued to exhibit sparse lobulo-alveolar expansion. At day 1 post-partum (day 1 lactation), lobulo-alveolar units of control mice continued to expand to the point that the ducts were obscured by the extensive expansion of alveoli. In MT1-mOE transgenic mice, the lobulo-alveolar ducts were distended with visibly reduced numbers evident at day 1 post-partum (day 1 of lactation) compared to control mice, demonstrating that lobulo-alveolar development is adversely effected by MT₁ expression.

Histological analysis also revealed a decreasing glandular ratio of ductal epithelium to adipose in mammary glands of MT1-mOE female mice during puberty, and at 14 and 18 days of pregnancy (Fig. 5A). Although cytoplasmic lipid/milk droplets were apparent in the epithelial cells in both controls and MT1-mOE female mice, the cytoplasmic droplets were smaller and less abundant in the MT1-mOE transgenic female mice. At day 1 of lactation, the mammary glands of MT1-mOE transgenic female mice continued to show greatly reduced lobulo-alveolar development and elevated levels of adipose tissue. The mammary glands of the control non-transgenic female pregnant mice were fully occupied by well-differentiated, extensive, milk-filled alveoli with little visible adipose tissue.

To determine if the suppression of mammary gland development observed in the MT1-mOE mice is a result of slowed proliferation and growth of mammary epithelial cells, DNA synthesis was measured *in vivo* by evaluating BrdU incorporation. At 8, and 12 weeks of age, days 14 and 18 of pregnancy, and day 1 of lactation, BrdU was administered to mice and BrdU incorporation into DNA was then detected by immunohistochemistry (Fig. 5B).

When compared with mammary glands from non-transgenic control mice at the same developmental times, a significant reduction in BrdU-positive immunoreactivity was observed in mammary epithelial tissue (both ductal and TEB) from MT1-mOE transgenic mice from 8 weeks of age through day 18 of pregnancy. In mammary glands from MT1-mOE transgenic mice, the proliferation index, calculated as the BrdU-positive cells for each area (mm^2), was significantly repressed by approximately 90% ($P < 0.01$) at week 12 of puberty, 24% by day 14 of pregnancy, and 37% by day 18 of pregnancy, compared to stage matched control female mice (Fig. 5C).

The ER α is a critical regulator of epithelial cell proliferation and ductal morphogenesis during postnatal mammary gland development. We have previously demonstrated that melatonin, via its MT $_1$ receptor, can suppress ER α gene expression [11] and can significantly repress E2-induced ER α transactivation in human breast cancer cells [12]. Western blot analysis demonstrated that ER α expression in mammary gland epithelial cells was significantly reduced by 67% and 56% in MT1-mOE female transgenic mice at 8 and 12 weeks of age, respectively (Fig. 6A and B). Histological analysis showed a significant decrease in ER α immunohistochemical staining in the nuclei of luminal epithelial cells in MT1-mOE mice as compared to control mice (Fig. 6C).

Estrogen and progesterone are required for functional development of the mammary gland, and E2 can induce PR expression. Two PR isoforms (A and B) have been identified, but their specific functions are not yet clearly delineated, however, perturbation of their ratio has been reported to alter ductal morphology and lateral branching [26]. As shown in Fig. 6D, MT1-mOE mice have a reduction in PR $_A$ and B compared to controls.

Given that E2 and progesterone are essential regulators of mammary gland development, we examined the ability of E2 and progesterone to stimulate mammary gland development in mice over-expressing the MT1 receptor and in control mice in both the presence and absence of exogenous melatonin. Figure 7 shows whole mount analysis of mammary gland development from the number 4 inguinal mammary glands in control and MT1-mOE female mice at 8 weeks of age following treatment with melatonin, E2 + progesterone, or melatonin + E2 + progesterone. In control mice, administration of exogenous E2 + progesterone increased ductal branching and TEB formation, which was repressed by melatonin administration, particularly with regards to TEB formation. In MT1-mOE transgenic mice, administration of exogenous E2 + progesterone enhanced ductal development and TEB formation compared to diluent controls, but less so than non-transgenic mice treated with E2 + progesterone. In both MT1-mOE and control mice, concomitant administration of melatonin with E2 + progesterone significantly slowed ductal development and TEB formation.

Studies by Brisken et al. [4] convincingly demonstrate the contribution of Wnt4 signaling in mediating the morphogenic response of the mammary epithelium to E2 + progesterone cues. As shown in Fig. 8c, during pregnancy and lactation when E2 and progesterone levels are elevated, Wnt4 expression levels are increased in control mice, but are dramatically suppressed in MT1-mOE transgenic dams.

Impaired lobulo-alveolar development in MT1-mOE mice suggests a possible role of melatonin and the MT $_1$ receptor in lactation. Therefore, we explored whether elevated MT $_1$ expression is accompanied by defective milk production. For this analysis, we examined the expression of two major milk proteins, β -casein and WAP, by immunofluorescence staining. The levels of the milk proteins β -casein (Fig. 8A) and WAP (Fig. 8B) in the mammary glands of MT1-mOE mice compared with those in the age- and stage-matched control mice were visibly reduced at day 18 of pregnancy and day 1 of lactation, respectively. Given that

the Akt1 and 2 regulate a host of cellular functions including growth, proliferation, survival, cell migration, and intermediary metabolism [26, 27], that ablation of Akt1 causes developmental defects in the mammary gland during pregnancy, and our report that melatonin can repress Akt expression in human breast cancer cells [17], we evaluated whether expression and activation of the MT₁ receptor can regulate Akt expression and/or phosphorylation in the mammary epithelium. Figure 9A shows a dramatic repression of Akt1 protein levels in the mammary epithelium during late puberty and pregnancy, with a less robust, but still significant decrease in lactating epithelium of MT1-mOE mice compared to non-transgenic controls. Immunohistochemical analysis of Akt1 expression (Fig. 9B) shows greatly enhanced expression of Akt1 in mammary epithelial cells of non-transgenic pregnant control mice as compared to MT1-mOE pregnant mice.

Recent work by Maroulakou et al. [28] demonstrates that ablation of Akt1 interferes with the expression and phosphorylation of Stat5. In Figure 9C, Western blot analyses show that the levels of total Stat5 in the mammary epithelium are diminished in MT1-mOE mice during pregnancy and lactation. The loss of Stat5 is much greater during pregnancy in MT1-mOE mice compared to non-transgenic mice, and the levels of phosphorylated Stat5 are greatly reduced during lactation in the mammary epithelium of MT1-mOE mice compared to controls. Furthermore, Stat5 is induced during pregnancy and lactation by E2 and progesterone and is involved in ductal differentiation and epithelial cell survival during pregnancy. As shown in Fig. 9C, we observed that during day 18 of pregnancy and day 1 of lactation Stat5 is highly expressed in the mammary epithelium of control mice, but is almost completely suppressed on day 18 of pregnancy in MT1-mOE mice, while rebounding to control values at day 1 of lactation.

DISCUSSION

Pubertal and pregnancy-induced mammary gland development is a complex and tightly controlled process. Though many positive regulators of mammary development have been described, few negative regulators have been identified [29]. To date, there have been several reports that melatonin can suppress or slow mammary gland development [22, 23, 30]. However, these studies did not demonstrate if the inhibitory actions of melatonin on the mammary gland were mediated via the MT₁ or MT₂ melatonin receptors or other mechanisms. We report, for the first time that elevated expression of the melatonin MT₁ GPCR in the mammary epithelium results in significant impairment of mammary gland development in mice in both phases of development. In this report, we demonstrate that the MMTV-promoter driven MT1-Flag tagged transgene is expressed at high levels, specifically in the mammary epithelial cells of the lobulo-alveolar ducts, TEBs, and acini in our MT1-mOE mice, and that expression of the MT₁-transgene was modest at the beginning of puberty, increased throughout puberty, and peaked during pregnancy and lactation.

Since both control and MT1-mOE mice were not ovariectomized, we were able to compare the effects of elevated MT₁ expression in the mammary gland in the presence of ovarian E2 and progesterone. MT1-mOE mice with elevated expression of the MT₁-transgene showed impaired ductal elongation and branching compared to mammary glands from control mice throughout puberty. As MT₁ receptor expression increased during pregnancy and lactation, so did the suppressive effect of MT₁ on mammary gland development, with increased repression of lobulo-alveolar and alveoli development. Although some laboratories consider that C57Bl/6J mice, from which the B6SJLF2 mice were derived, do not synthesize pineal melatonin, Conti et al. [31] demonstrated by HPLC that C57Bl/6J mice generate a short-term melatonin peak in the middle of the dark period, with a pattern that mirrors their observations of serum melatonin in this strain [32].

In these studies, it is possible that overexpression of MT₁ might cause the nonspecific, nonphysiological, heterodimer formation of this GPCR, leading to MT₁ autoactivation. However, the expressed recombinant MT₁ receptor is still activated by exogenous melatonin (Fig. 7) decreasing ductal development and TEB formation. Furthermore, administration of exogenous melatonin to vector control mice also impaired basal ductal branching and repressed E2 and progesterone-induced TEB development (Fig. 7), suggesting that endogenous MT₁ levels are sufficient to drive this inhibitory effect on mammary gland development.

The ability of melatonin, via its MT₁ receptor, to suppress mammary gland morphogenesis could result from either the suppression of cell proliferation, the blockade of key developmental pathways, or both. BrdU incorporation studies suggest that at a minimum, overexpression and/or activation of the MT₁ GPCR suppresses the proliferation of ductal and TEB epithelial cells. However, it is possible that melatonin, via the MT₁ receptor, may also regulate developmental processes.

It is well established that E2 and progesterone promote ductal growth and branching and TEB formation in the mammary gland [25, 33–35]. In fact, epithelial-specific ablation of exon 3 of the ER α gene in virgin mice severely impairs ductal elongation and side branching, decreasing the proliferation of alveolar progenitors [32]. Furthermore, we have demonstrated that melatonin via activation of its MT₁ receptor can repress ER α transcriptional activation [36] and ER α mRNA expression [11]. Thus, it is possible that elevated expression of MT₁ in the mammary gland, activated by endogenous melatonin, can repress the expression of ER α and suppress the expression of E2-regulated genes, including the milk proteins β -casein and WAP. As shown in Figure 6 B and C, elevated expression of the MT₁ melatonin receptor in the mammary epithelium of our MT1-mOE transgenic female mice induced a significant suppression of ER α protein expression by weeks 8 and 12, time frames that correlate well with the decline in ductal growth, ductal branching, and TEB formation in the mammary glands of MT1-mOE mice. Follow-up analysis of the expression milk proteins β -casein and WAP (Figs. 8A and B), which can be regulated by E2, clearly show a greater than 50% decrease in both β -casein and WAP by day 18 of pregnancy and day 1 of lactation in mammary glands from MT1-mOE mice compared to controls.

Given that E2 regulates the growth and development of the ductal epithelium, alveoli, and milk proteins, it is clear that the inhibitory effects of melatonin, via its MT₁ receptor, could be mediated through the repression or blockade of the E2/ER α signaling pathway in all stages of mammary gland development. However, the E2/ER α signaling pathway is not the only pathway by which melatonin, via its MT₁ receptor, can impact the mammary gland. We have demonstrated that other nuclear receptors, including the glucocorticoid receptor (GR), retinoic acid receptor (RAR), and retinoid X receptor (RXR), also play a role in differentiation and have enhanced transactivation in response to melatonin [12, 37]. The ability of melatonin to repress the transcriptional activation of the GR in MCF-7 human breast cancer cells [12] is particularly relevant since glucocorticoids, via the GR, cross talk with Stat5 to mediate mammary gland development and involution. During lactation, the successful differentiation of the secretory epithelium and milk production depend mainly on the action of prolactin and glucocorticoids [38]. Circulating prolactin, via activation of its receptor, phosphorylates Stat5 through Jak2. In the mammary gland, GR potentiates the lactogenic and survival activities of Stat5 [39]. Thus, it is clear that Stat5 plays a major role in mammary gland differentiation, epithelial cell survival, and milk production and that both glucocorticoids and prolactin are central to Stat5 activation. Given our report that melatonin represses glucocorticoid transactivation of the GR in MCF-7 breast cancer cells [12], and that melatonin inhibits prolactin-induced MCF-7 cell proliferation [40], it is not surprising

that elevated expression of the MT₁-melatonin receptor can repress Stat5 expression and/or activation to mediate melatonin inhibitory effects on mammary gland development.

As noted earlier, ablation of Akt1 induces developmental defects in the mammary gland during pregnancy [27], and ablation of Akt1 interferes with the expression and phosphorylation of Stat5 [28]. Our previous report that Akt expression was elevated during the daytime, when serum melatonin levels are the lowest and repressed at night-time when melatonin levels are elevated in tissue-isolated human breast tumor xenografts [17, 39], that melatonin and vitamin D3 can act to synergistically down-regulate AKT in breast cancer cells in culture [40], and that Akt1 can induce Stat5 phosphorylation/activation in the mammary gland [28], our data in Figure 9A showing that elevated expression of the MT₁ receptor can repress Akt1 protein levels in the mammary epithelium during late puberty and pregnancy and even lactation, and that elevated MT₁ expression can down-regulate Stat5 expression/phosphorylation (Fig. 8C), suggests another possible pathway by which melatonin, via its MT₁ receptor, can regulate Stat5 and mammary gland development and the expression of the milk proteins β -casein and WAP.

The inhibitory effects of melatonin and MT₁ on β -casein and WAP expression suggest a defect in terminal differentiation independent of the defect in proliferation, as WAP plays a role in terminal differentiation of the mammary gland and is a well-recognized marker of terminal differentiation [43, 44]. Furthermore, the ability of elevated MT₁ expression to suppress Wnt4, a known ovarian steroid-regulated mediator of epithelial-to-mesenchymal transition (EMT) and thus, ductal branching [4], further supports the role of melatonin in altering developmental paradigms in the breast. Finally, the ability of elevated MT₁ expression to suppress not only Akt1 and Stat5 expression but also Stat5 phosphorylation during pregnancy suggests that melatonin can also regulate terminal differentiation events and may play a role in mammary gland remodeling after ovarian cycles. Despite a wealth of knowledge regarding the role of Stat5 in mammary gland development and the transcriptional regulation of milk genes [45, 46], there is little information about Stat5 mediated gene expression regulating the proliferation and survival of mammary epithelial cells. Recent studies by Wanger et al. [46] indicate that activation of the Jak2/Stat5 signaling pathway regulates the expression of total and phosphorylated levels of Akt1. Akt1 is known to inhibit GSK3 β , a kinase that phosphorylates cyclin D1 on threonine 286 [47–49], and β -catenin, allowing their degradation and repressing both mammary epithelial cell proliferation by cyclin D1 and ductal branching by Wnt/ β -catenin. Thus, part of melatonin's mechanism of action in mammary gland development may be related to its repression of Akt's blockade of GSK3 β activity, leading to decreased cell proliferation and ductal branching, by increased ubiquitination of cyclin D1 and β -catenin, respectively.

Our studies in female mice demonstrate the potent inhibitory actions of melatonin, via its MT₁ GPCR, on mammary gland development. The peri-pubertal and pubertal periods of mammary gland development depend heavily on E2 and progesterone, while the post pubertal mammary gland depends on these as well as adrenal hormones, prolactin, and growth factors such as IGF-1 [50–52]. It is clear from previous studies that a blockade of E2 function by either suppression of ovarian estrogen or knockdown of ER α [53] can impede ductal morphogenesis and that pregnancy and that E2 implantation can rescue impeded ductal morphogenesis. As shown in Figure 10, our study demonstrates that enhanced expression of the melatonin MT₁ receptor suppresses ER α expression in ductal and TEB epithelial cells by weeks 8 and 12 to represses cell proliferation, and the expression of the milk proteins WAP and β -casein in mammary ductal and alveolar cells supporting the importance of ER α regulation as a mechanism for melatonin's actions on mammary gland development and proliferation. In pregnancy and lactation mediated mammary gland development, it appears that the inhibitory effects of elevated MT₁ expression are likely

regulated via multiple pathways including ER α , PR, GR, prolactin, and even Akt1 to suppress the expression of both Wnt4 and Stat5 impairing acini development and the expression of the milk proteins β -casein and WAP. Taken together, our data demonstrate that melatonin, via its MT₁ GPCR, is an important negative regulator of pubertal and post-pubertal mammary gland proliferation and development.

Acknowledgments

This work was supported by NIH/NCI grant CA-054152-12 and Army DOD grant DAMD-123201 to S.M.H.

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Fig. 1.

Expression of MT1-Flag transgene in mammary glands of MMTV-MT1 B6SJLF2 transgenic mice. **(A)** Diagram of the MT1 transgene construct, containing the MMTV-LTV promoter, the 5' untranslated region, the human MT1 coding sequence with the Flag tag (5' end) and the SV40 poly-A sequence. **(B)** Western blot analysis of MT1-Flag transgene expression. Total cellular protein was isolated from the number 4 inguinal mammary gland of developmentally staged female mice. Seventy-five micrograms of total protein from each sample was separated by 12% PAGE and subjected to Western blot analysis using an anti-flag specific antibody.

Fig. 2. Reduced weight of pups born to MT1 transgenic mothers. On postnatal day 10, 20, and 30 (10 days after weaning), the litters were weighed and the average neonatal body weights calculated. N= 15 pups per group, * $P<0.05$ vs. pups born to and nursed by control dams.

Fig. 3.

Elevated expression of MT1 in mammary glands inhibits ductal branching and TEB formation at puberty. (A) Mammary gland whole mount preparations. Control and MT1-mOE mice (MT1 transgenic) were sacrificed at 4, 8, and 12 weeks of age. The number 4 inguinal mammary gland of each mouse was excised and spread onto a glass microscope slide, fixed in acidic ethanol, processed using the whole-mount procedure, and stained with carmine. (B) Number of TEB in mammary gland at 4 weeks, (C) Branching in mammary gland at 8 weeks. The number of TEBs and ductal branchings were quantitated and compared between control mice and MT1-mOE mice. A total of 5 mammary glands from each group were evaluated. Data are expressed as numbers per mm² ± SEM, **P*<0.05 vs. control mice.

Fig. 4. Overexpression of the MT1 receptor in MT1-mOE female mice inhibits lobulo-alveolar development in mouse mammary gland. MT1-transgenic and control mice were sacrificed at days 14 and 18 of pregnancy and day 1 of lactation. The number 4 inguinal mammary gland was excised and spread onto a glass microscope slide, fixed in acidic ethanol, processed using the whole-mount procedure, and stained with carmine.

Fig. 5. Histological analysis of developmentally staged mammary glands from non-transgenic controls and MT1-mOE (MT1) transgenic mice. MT1-mOE transgenic mice exhibit decreased mammary epithelial cell proliferation. **(A)** A portion of the number 4 inguinal mammary gland at 8 weeks of age (puberty), 14 and 18 days of pregnancy, and 1 day of lactation was fixed in paraformaldehyde, embedded in paraffin, and stained with hematoxylin/eosin. **(B)** Immunohistochemical analysis of BrdU staining in mammary epithelial cells. BrdU incorporation was determined by immunohistochemistry of paraffin-embedded mammary glands from control and MT1-mOE (MT1 transgenic) mice at weeks 8 and 12 of age (puberty), days 14 and 18 of pregnancy, and day 1 of lactation. **(C)** Quantitation of BrdU staining in mammary glands of control and MT1 transgenic mice. N= 5 independent samples, * P 0.05 vs. controls, ** P 0.01 vs. controls.

Fig. 6.

Elevated expression of the MT1 transgene reduces ERα and PR_{A/B} expression in mouse mammary glands. **(A)** Western blot analysis of ERα expression. Total cellular protein was isolated from the number 4 inguinal mammary gland of developmentally staged female vector control (-) and MT1-mOE transgenic (+) mice. Seventy-five micrograms of total protein from each sample was separated by 12% PAGE and subjected to Western blot analysis using an antibody directed against the mouse ERα. **(B)** Quantification of ERα expression. N= 5 independent samples, * P 0.05 vs. controls, ** P 0.01 vs. controls. **(C)** Immunohistochemical analysis of ERα expression in ductal epithelium from control and MT1-mOE (MT1 transgenic) mice at 8 weeks of age. **(D)** Western blot analysis of PR expression. Total cellular protein was isolated from the number 4 inguinal mammary gland of pregnant (day 18) and lactating (day1) female mice. Seventy-five micrograms of total protein from each sample was separated by 12% PAGE and subjected to Western blot analysis using an antibody directed against the mouse PR (detects both A and B forms).

Fig. 7.

Whole mount analysis of mammary gland development induced by estrogen and progesterone and modulated by melatonin from non-transgenic controls and MT1-mOE transgenic mice. Four-week-old control and MT1-mOE mice were grouped and treated as: controls, melatonin (MLT) [25 μ g/day i.p.], 17 β -estradiol (E) [0.1 mg/pellet] + progesterone (P) [10 mg pellet], and E + P + melatonin. After 4 weeks of treatment (8 weeks of age), mice were sacrificed and the entire number 4 inguinal mammary gland was prepared as whole mount and stained with carmine.

Fig. 8.

Elevated expression of the MT1 transgene alters the expression of the milk proteins β -casein and Whey acidic protein (WAP) in mouse mammary glands. **(A–B)** Immunofluorescence staining of milk proteins in mammary gland tissue section from day 18 of pregnancy and day 1 of lactation in control and MT1-mOE mice. **(A)** β -casein expression (green), **(B)** WAP expression (green). **(C)** Elevated expression of the MT1 transgene reduces Wnt4 expression in mouse mammary glands. Total cellular protein was isolated from the number 4 inguinal mammary gland of developmentally staged female mice. Seventy-five micrograms of total protein from each sample was separated by 10% PAGE and subjected to Western blot analysis using antibodies directed against the mouse Wnt4 and GAPDH.

Fig. 9.

Elevated expression of the MT1 transgene alters the expression of Akt1 and total and phosphorylated forms of Stat5 in mouse mammary glands. **(A)** Western blot analysis of Akt1 expression in mouse mammary glands from week 12 of development, pregnancy day 18, and lactation day 1. **(B)** Immunohistochemical analysis of Akt1 in mammary gland tissue section from day 18 of pregnancy and day 1 of lactation in control and MT1-mOE mice. **(C)** Elevated expression of the MT1 transgene reduces expression of both total and phosphorylated forms of Stat5 in mouse mammary glands. Total cellular protein was isolated from the number 4 inguinal mammary gland of developmentally staged female mice. Seventy-five micrograms of total protein from each sample was separated by 10%

PAGE and subjected to Western blot analysis using an antibodies directed against the mouse Akt1, total and phosphorylated Stat5, and GAPDH.

Fig. 10.
Pathways by which melatonin via its MT₁ GPCR suppresses mouse mammary gland development.