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ORIGINAL ARTICLE

The Effect of Light Regimen and Melatonin on the Development of Spontaneous Mammary Tumors in HER-2/neu Transgenic Mice is Related to a Downregulation of HER-2/neu Gene Expression

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Abstract

OBJECTIVES AND DESIGN: The effect and the mechanism of light regimen and melatonin on the development of mammary tumors in HER2/neu transgenic mice were investigated. Female HER-2/neu mice starting from the age of 2 months were kept under standard light/dark regimen (LD) or constant light illumination (LL) and a part of each group was given melatonin (20 mg/l) during the night time.

RESULTS: The exposure to LL failed to change the incidence of spontaneous mammary adenocarcinoma development, the size of mammary tumors, as well as the incidence and size of lung metastases. However, the number of tumors per mouse was significantly increased in the LL group as compared to the LD group. The number of mice bearing 4 and more tumors was higher in the LL group than in the LD group, whereas the number of mice bearing 1 to 3 tumors was lower in the LL group in comparison with the LD group. Melatonin decreased the incidence and size of mammary adenocarcinomas, and the incidence of lung metastases in the LD group but not in the LL group. The mean number of tumors per mouse was not changed by melatonin treatment in both light regimens. The number of mice bearing 4 and more tumors was reduced by melatonin more significantly in the LL group than in LD group. Melatonin treatment resulted in a 2.5-fold reduction in the expression of HER-2/neu mRNA in mammary tumors from HER-2 /neu transgenic mice.

CONCLUSION: The data demonstrate the influence of the LD light regiment and melatonin treatment in the development of spontaneous mammary tumors in HER-2/neu mice suggesting a melatonin-dependent modulation of HER-2/neu gene expression in mammary adenocarcinoma.

Abbreviations:

LD : standard light/dark regimen
LL : constant light illumination
MLT : Melatonin

1. Introduction

Breast cancer is one of the most common cancers and is a leading cause of mortality in women [1, 2]. The HER-2/neu, oncogene encodes a 185 kDa (p 185) receptor protein belonging to the epidermal growth factor receptor family involved in organogenesis and epithelial differentiation [3]. Amplification and mutation of HER-2/neu plays a pathogenetic role in several malignancies, including carcinoma of the breast, ovary and uterus, [4, 5]. Overexpression of ErbB-2/HER-2/neu occur in 15-40% of human breast cancers [6]. Its appearance correlated with poor prognosis and it is, therefore, an important target for physiologic investigation and therapeutic intervention [5]. Numerous experimental studies and clinical observations have shown the influence of biorhythms on development of tumors. The factor defining circadian rhythms is the light regimen, which, in turn, influences the function of the pineal gland, mainly through the production of its indole hormone – melatonin [7, 8]. Light entrains melatonin rhythm by suppressing its synthesis during the day [7]. Exposure to light-at-night inhibits the synthesis and secretion of melatonin [9, 10] and stimulates the development of transplantable, spontaneous and 7,12-dimethylbenz(a)anthracene (DMBA) or N-nitrosomethylurea (NMU) induced mammary adenocarcinomas [9–14], as well as N-nitrosodiethylamine induced hepatocarcinogenesis [15] and N-nitrosoethylurea induced transplacental carcinogenesis in rats [16]. At the same time, the treatment with melatonin inhibits the development of carcinogen-induced or transplantable tumors in mammary gland, uterine cervix, and colon in various animal experimental models [17–20]. The clinical observations suggest that light at night has the possibility to influence cancer development in humans. The well known "melatonin hypothesis", postulates that the lighting, especially in night time, suppresses the peak of melatonin secretion, and increases the risk of breast cancer [10]. At present, several mechanisms for the effect of melatonin on mammary cancer tumorigenesis have been proposed: an endocrine hypothesis [19], based on a possible role of melatonin on some of the pituitary or gonadal hormones which control mammary gland development; a direct action on tumor cells through melatonin-mediated antiproliferative, antioxidant or immunoenhancing effects [20–23]. In spite of these suggestions, no direct evidence has been reported until now on the molecular mechanism involved in melatonin protection in mammary cancer tumorigenesis.

The purpose of this study was to evaluate the influence of a the light regimen and melatonin on the spontaneous development of mammary tumors occurring in transgenic mice carrying the mammary cancer gene HER-2/neu. We hypothesized that exposure to constant light would exert a promoting effect on mammary carcinogenesis driven by HER-2/neu overexpression in mice, whereas melatonin supplementation should exert a protective effect. The mechanism of the pineal hormone action was investigated studying the HER-2/neu mRNA expression in tumors from melatonin treated animals.

2. Material and methods

2.1. Animals

Homozygous HER- 2/neu transgenic mice originally obtained from Charles River (Hollister, CA) by the Italian National Research Center for Aging were housed and breed in the Laboratory of Carcinogenesis and Aging. The mice were kept 5–7 in polypropylene cages (30 x 21 x 10 cm) at 22 ± 2°C and received standard laboratory chow and tap water *ad libitum*.

2.2. Experimental design

One hundred four female FVB/N HER-2/neu mice at the age of 2 months were randomly divided into four groups. Mice of groups 1 and 2 were kept under standard light/dark regimen (12 hours light :12 hours darkness) (LD) whereas the mice of groups 3 and 4 were exposed to constant light illumination for 24 hours a day (LL). Mice from groups 2 and 4 were given melatonin (Sigma, USA) dissolved in tap water (20 mg/l) during the night time (from 18.00 to 09.00 hours). Melatonin solution was prepared 3 times a week. It has been proved to be stable in water solution at least for 6 months. Melatonin was used at a dose which has been proved as effective for life span extension and anti-carcinogenic effect [17, 20, 24]. Once a week all mice were palpated for the detection of mammary tumors appearance. The localization and the size of tumors were registered on the special charts. Once a month all mice were weighted and, simultaneously, the amount of consumed food was measured, and the rate of the consumed food mass (g) per mouse and per body weight unit were calculated. Once in every 3 month, daily for 2 weeks vaginal smears of the animals were cytologically examined to estimate the estrus function. The time of appearance of mammary tumors was evaluated by palpation and the neoplastic masses were measured with calipers in the two perpendicular diameters. Progressively growing masses of >3 mm in mean diameter were regarded as tumors.

Because some treated mice did not display carcinomas in all mammary glands, the mean number of palpable mammary carcinomas/mouse was calculated as the cumulative number of incident tumors/number of tumor-bearing mice.

2.3. Pathomorphological examination

All the animals were autopsied. Site, number and size of mammary tumors and their metastases in lungs were checked. All the tumors, as well as the tissues and organs with suspected tumor development, were excised and fixed in 10% neutral formalin. After fixation the number of metastasis in each lobe of lungs as well as the size of metastases were estimated as recommended by the International Agency for Research on Cancer [25]. After the routine histological processing the tissues were embedded into paraffin. 5–7 μm thin histological sections were stained with haematoxylin and eosin and were microscopically examined. Tumors were classified according to the International Agency for Research on Cancer recommendations [25] and Annapolis Consensus Report [26].

2.4. RNA extraction and RT-PCR

The expression of mRNA for HER-2/neu was evaluated in mammary tumors from HER-2/neu transgenic mice by RT-PCR. After homogenization of tissue sample, RNA was extracted using TRI-REAGENT™ according to the manufacturer's instructions (Sigma Chemical Co., USA). RNA concentrations were determined using the spectrophotometer (scientific instruments UV1601 Shimadzu, Columbia, MD, USA). cDNA was synthesized from 0.1 µg RNA incubating RNA with dNTP(0.5mM), Oligo dT (12.5ng/µl), First Strand Buffer (1X), M-MLV Reverse Transcriptase (10 U/µl), Rnase Inhibitor (1U/µl) and DTT (0.01M) all from Gibco BRL, in a final volume of 20µl. The samples were incubated at 37°C for 1h and 95°C for 10 min., subsequently cDNA was frozen at –20°C until use. PCR was performed incubating 5µl cDNA with a reaction mixture containing: PCR Buffer (1X), $MgCl_2$(1.5mM), dNTP (200µM), specific forward and reverse primers (0.8µM of each), Taq DNA Polymerase (1U/µl) in a total volume of 50µl (all from Roche Diagnostics GmbH, Germany). The samples were incubated in a GeneAmp PCR System 9700 (Perkin Elmer) for a total of 35 cycles for HER-2/neu, and 30cycles for β-actin. Each cycle consisted of: 1min. at 94°C, 1min. at 65°C, 1min. at 72°C for HER-2/neu; 1min. at 94°C, 2min. at 63°C, 1min. at 72 °C for β-actin.

The primers for HER-2/neu and β-actin were purchased from Roche Diagnostics (GmbH, Germany) using DNA published cDNA sequences. The HER-2/neu fragment of 239 bp was defined by the forward primer:
5'-GATCGAATTCCTGGAGGACGTGCCGCTTGTA and the reverse primer:
5'-GATCAAGCTTATAGCTCCACACATCACTCTG. β-actin fragment of 349 bp by the forward primer:
5'-TGGAATCCTGTGGCATCCATGAAAC and the reverse primer:
5'-TAAAACGCAGCTCAGTAACAGTCCG. The PCR products and a molecular weight standard (DNA molecular weight marker VIII, Roche Diagnostics) were visualized after electrophoresis in a 1.5% agarose gel containing 1µg/µl ethidium bromide(EtBr). Densitometric analysis was performed using the GelDoc 2000 (BioRad Laboratories, Italy).

2.5. Statistics

Experimental results were statistically processed by the methods of variation statistics with the use of STATGRAPH statistic program kit. The significance of the discrepancies was defined according to the Student *t*-criterion, Fischer's exact method, c^2, non-parametric Wilcoxon-Mann-Whitney and Friedman RM Anova on Ranks. Student-Newman-Keuls Method was used for all pairwise multiple comparisons.

3. Results

3.1. Effects of the exposure to constant light illumination

As shown in Table I, the body weight gain as well as the food consumption were similar in the LD and LL groups. There were no significant differences in the length or regularity of the estrus cycles between these groups (data are not shown).

The exposure to constant light illumination failed to change the incidence of mammary adenocarcinoma development, the maximum size of mammary tumors as well as the incidence of lung metastases and their size (Table II and Fig. 1). However, the number of tumors per mouse was significantly increased in the LL group in comparison with the LD group (Table II). The mean latent period of mammary tumors was one month longer in the LL group as compared to the LD group ($p<0.05$, Table II). It is noteworthy that the number of mice bearing 4 and more tumors in the LL group was increased as compared to the LD group (60% and 33%, respectively,) $p<0.05$, and the number of mice bearing 1 to 3 tumors was 2.15 times less in the LL group in comparison to with the LD group (20% and 43%, Fig. 3).

At the microscopic examination all tumors of a mammary gland were classified as adenocarcinomas type B [21] and revealed a solid, lobular and cribrosis structure with the multiple hemorrhagic cysts. There was no difference in the morphological structure of mammary carcinomas between these groups.

Table I. Effect of exposure to constant light and melatonin on body weight gain and food consumption in HER-2/neu transgenic female mice

Group	Age (months) 2	3	4	6	7	8	9
LD	22.2±0.23* (5.1 ± 0.28)	24.0±0.28 (5.5 ±0.19)	25.1 ± 0.38 (5.5 ± 0.19)	26.8 ± 0.67 (4.5 ±0.51)	28.4 ± 0.60 (5.4 ± 0.91)	34.2 ± 1.26 (5.0 ± 0.23)	35.2 ± 2.02
LD ± MLT	21.6±0.23 (5.6 ± 0.10)-	22.3±0.35[b] (6.8 ±0.50)	24.4 ± 0.31 (4.7 ± 0.72)	25.0± 0.44[a] (4.4 ±0.33)	27.8 ± 0.65 (4.7 ± 0.90)	27.3 ± 0.69[b] (5.0 ± 0.35)	
LL	22.1±0.39 (4.4 ± 0.15)	23.9 ±0.38 (4.8 ±0.67)	25.1 ± 0.45 (3.9 ± 0.09)	28.0 ± 0.52 (4.0 ±0.39)	28.8 ± 0.66 (4.3 ± 0.37)	32.4 ± 1.04 (4.4 ± 0.38)	33.3 ± 1.14
LL ± MLT	21.7±0.28 (4.2 ± 0.29)	23.6 ±0.32 (4.9 ±0.56)	24.2 ± 0.31[a] (4.3 ± 0.39)	26.0 ± 0.46 (4.3 ±0.39)	27.2 ± 0.60 (5.5 ± 0.43)	30.3 ± 1.06 [a] (3.6 ± 0.4[a])	31.42 ± 1.89

Difference with the LD group is significant: [a] - $p < 0.05$; [b] - $p < 0.01$.
*Date represent body weight gain (food consumption) expressed as mean ± S.D.

Table II. Effect of exposure to constant light and melatonin on mammary tumorogenesis in HER-2/neu transgenic female mice.

Group	N° of mice	N° of tumor bearing mice	Mean latent period of tumors	Maximum size of tumors	N° of tumors per mouse
		(%)	*(days)*	*(cm)*	
LD	30	23 (76.7%)	236±5.9	1.88±0.15	3.3±0.4
LD +MLT	22	13 (59.1%)	230±6.7	1.35±0.17*	3.5±0.4
LL	25	19 (76.0%)	268±4.7*	1.82±0.15	5.0±0.5**
LL +MLT	27	18 (66.7%)	266±5.6	1.52±0.14	4.1±0.4

Difference with LD mice is significant, *-p<0.05; **-p<0.02

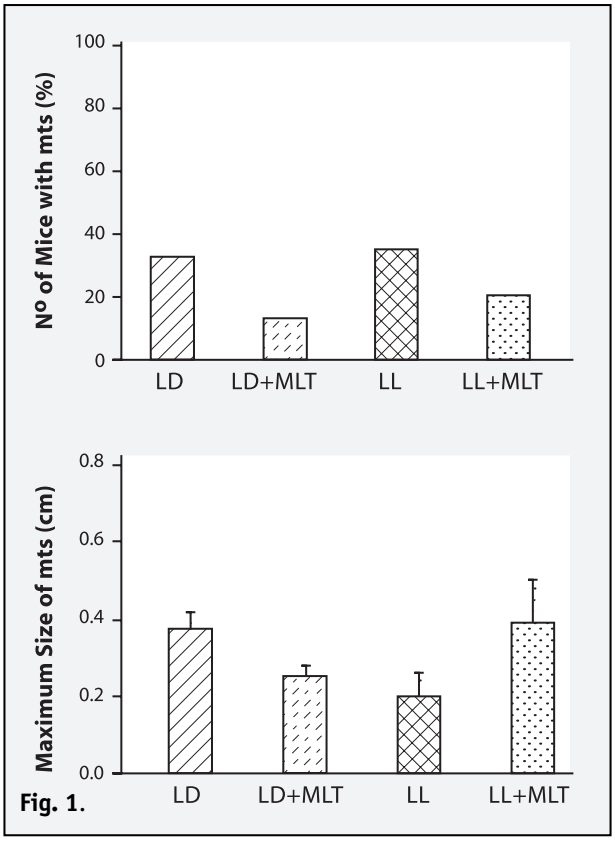

Fig. 1.

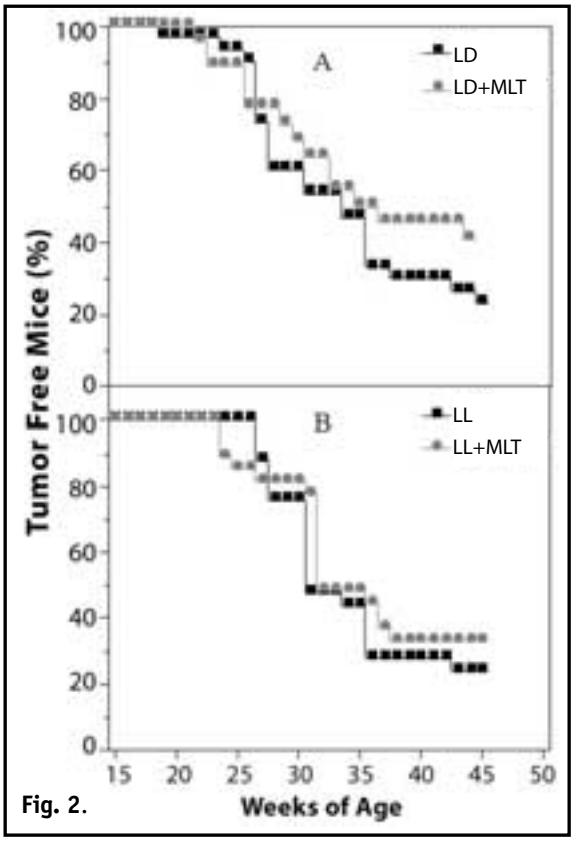

Fig. 2.

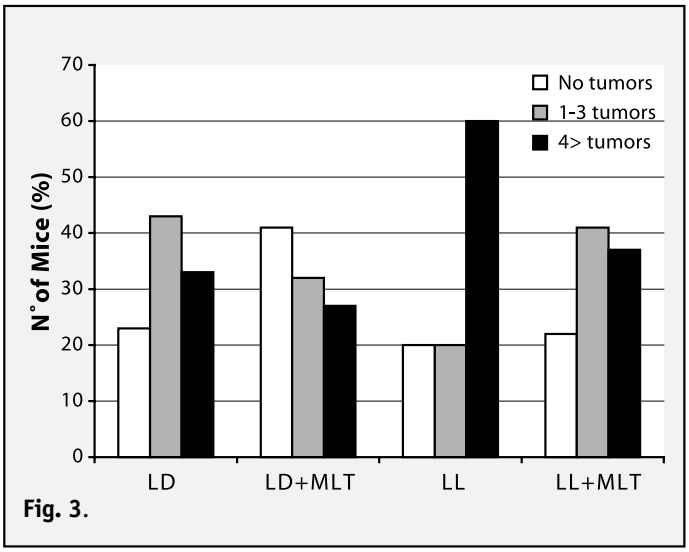

Fig. 3.

Fig. 1: Incidence and size of lung metastases in HER-2/neu transgenic mice kept at various light regiments and supplemented or not with melatonin. The percentage of mice with lung metastases (upper panel) and the maximum size of metastases (low panel) are shown. Melatonin administration significantly decreased the incidence of lung metastasis at the LD regimen ($p<0.07$) but not at the LL regimen. The number of mice for each treatment was 30 (LD), 22 (LD + MLT), 25 (LL), and 27 (LL + MLT).

Fig. 2: Progression of mammary carcinogenesis in HER-2/neu transgenic mice kept at various light regiments and supplemented or not with melatonin. The percentage of tumor free mice kept at LD or LD + MLT treatments (A) and LL or LL + MLT treatments (B) are shown. The number of mice for each treatment was 30 (LD), 22 (LD + MLT), 25 (LL), and 27 (LL + MLT). Statistical analysis shows that both LD + MLT and LL +MLT curves are significantly different in comparison with the respective LD or LL curve ($P< 0.0003$, Friedman RM Anova on Ranks test)

Fig. 3: Number of mammary carcinomas in HER-2/neu transgenic mice kept at various light regiments and supplemented or not with melatonin. The percentage of mice having 1-3 tumors or > 4 tumors is shown. LL treatment increased the number of mice bearing 4 or more tumors ($p<0.05$); melatonin administration significantly reduced the number of mice with 4 or more tumors at the LL regimen ($p<0.05$). The number of mice for each treatment was 30 (LD), 22 (LD + MLT), 25 (LL), and 27 (LL + MLT).

3.2. Effects of the exposure to melatonin

The treatment with melatonin slightly decreaesed the body weight gain at the age of 3, 6 and 8 months in the LD+MLT group as compared with the LD group. No body weight difference was observed in mice kept at the LL+MLT or LL regimen (Table I). Food consumption was not modified by melatonin at both light regimens (Table I). There were not significant differences in the length or the regularity of the estrus cycles between the groups exposed or not exposed to melatonin in both light regimens (data not shown). The administration of melatonin during the night time significantly decreased the incidence of mammary adenocarcinomas in the LD group and, in a lower manner, in the LL group ($p<0.0003$, Fig. 2). The mean number of tumors per tumor-bearing mouse was not changed by the melatonin treatment in both light regimens. However, at the LL regimen the number of mice bearing 4 and more tumors was reduced by melatonin more significantly than that in the mice kept at LD regimen ($p<0.05$, Fig. 3). Melatonin administration decreased the size of mammary adenocarcinomas ($p<0.05$, Table II) and the incidence of lung metastases ($p<0.07$, Fig. 1) at the LD regimen, but not at the LL regimen as compared with the LD and LL groups, respectively. There was not effect of melatonin treatment on the morphology of mammary adenocarcinomas in mice kept at either LD nor LL regimens.

3.3 Effect of Melatonin treatment on HER-2/neu expression

In order to evaluate whether the decreased incidence of mammary tumors observed in mice kept in standard light/dark regimen was due to an effect of melatonin on mammary gland, we performed RT-PCR analysis for HER-2/neu gene expression in the mammary tumors from mice of the LD group. As shown in Fig. 4, mRNA for HER-2/neu gene was greatly expressed in saline treated mice whereas it was significantly decreased in animals chronically treated with melatonin. The mean relative expression of the HER-2/neu gene as determined by densitometric analysis was 0.81 ± 0.06 and 0.33 ± 0.15 for control and melatonin-treated cells, respectively.

4. Discussion

There is a considerable amount of evidence to show that the light regimen and the pineal gland, via its hormone melatonin, exert a protective effect on tumor development both in animals and humans [17–20]. The results reported in this paper clearly demonstrate that: a) the exposure to constant light illumination promotes mammary carcinogenesis in transgenic HER-2/neu mice; b) the administration of melatonin decreases the incidence and the size of mammary carcinomas and the incidence of lung metastasis in transgenic HER-2/neu mice; c) *in vivo* melatonin decreases the expression of HER-2/neu mRNA in mammary tumors from HER-2 /neu mice.

The observation of a promoting effect of constant light exposure to mammary carcinogenesis is in

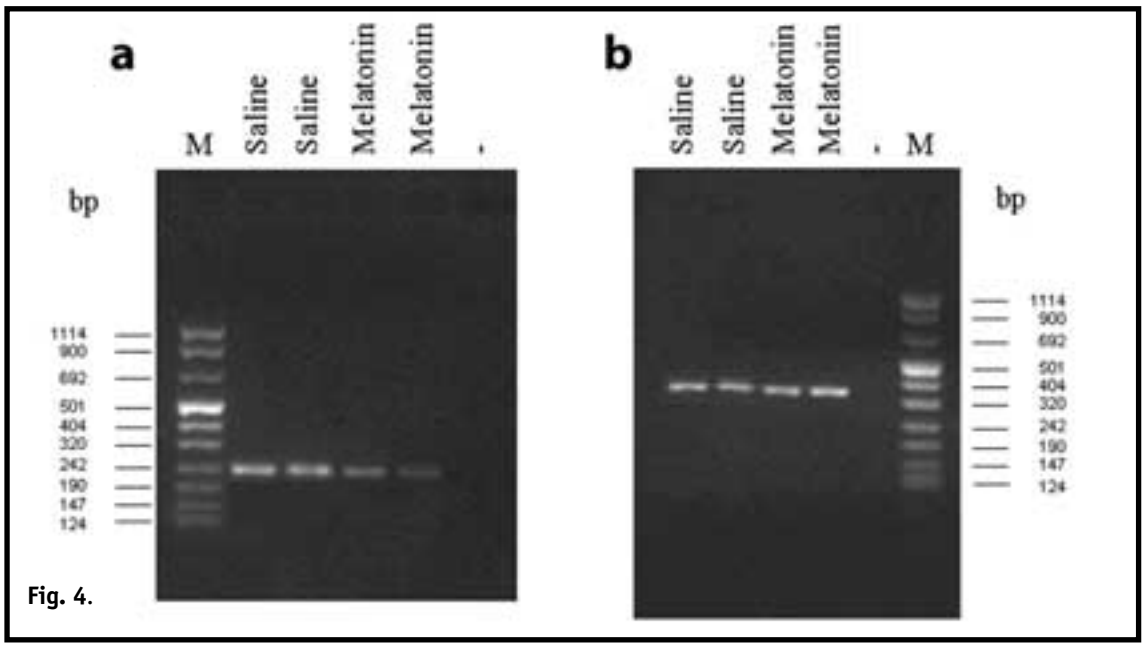

Fig. 4.

Fig. 4 : Expression of rat HER-2/neu mRNA (panel a) and β actin (panel b) in mammary tumors of FVB/N female mice transgenic for the activated rat HER-2/neu after chronic melatonin supplementation by RT-PCR. The RNA reverse transcribed and amplified was normalized for β actin expression in individual samples. The relative expression of HER-2/neu gene, as determined by densitometric analysis, was 0.81 and 0.33 for saline and melatonin-treated cells respectively. Data relative to two saline and two melatonin-treated mice are presented.

agreement with other studies which revealed a stimulating effect of light-at-night on the development of spontaneous and chemical carcinogen induced mammary adenocarcinomas in mice and rats [9, 11–13, 27, 28]. The treatment with melatonin attenuates the effect of constant light illumination, reducing the multiplicity of tumors and the incidence of lung metastases. Mediavilla *et al*. [29] observed an inhibitory effect of melatonin on the development of hyperplastic alveolar nodules and mammary carcinomas in thransgenic mice carrying the N-ras proto-oncogene under the control of the MMTV-LTR. Melatonin (50 mg/kg, orally for 30 weeks) delayed appearance of palpable mammary tumors and their growth in TG.NK transgenic mice expressing the c-neu oncogene under the control of a MMTV promoter [30].

Our observation is in agreement with other reports on the inhibitory effect of melatonin on mammary tumor development obtained in various models [19, 20, 28, 31–33]. With regards to the mechanisms involved in the inhibitory effect of melatonin on mammary carcinogenesis, either anti-proliferative and anti-estrogen capacities of melatonin, or inhibiton of prolactin level, or antioxidative potential and immunostimulating activity of the pineal hormone [20–23], have been reported. In this paper, we describe for the first time a new mechanism by which melatonin may bring about its anticancer action preventing the development of spontaneous tumors in HER-2/neu transgenic mice. This mechanism is based on the down-regulation of the HER-2/neu gene transcription. In fact, as shown in the RT-PCR results (Fig.4), the mRNA for HER-2/neu was expressed at lower levels in melatonin treated mice in comparison with control mice. Whether melatonin may directly act on tumor cells inhibiting HER-2/neu expression or its effect is indirectly due to the *in vivo* modulation of other factors [23, 34] remains to be demonstrated. Zinc, for example, may represent one of these factors, since melatonin modulates its production and circadian rytmicity [23, 34] and it has been shown to influence HER-2/neu transcription (Provinciali, personal communication). In conclusion, the data reported in this paper demonstrate that light regimen and melatonin may have an important role in mammary tumor development, and show that melatonin may modulate the expression of the HER-2/neu oncogene.

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