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Local Expression of Mel_{1a} and Effect of Melatonin on Expression of PLP-A Gene in the Rat Placenta

Chang Sook Shin, Chae Kwan Lee[1], Han Seung Kang, Haekwon Kim[2], Yong Dal Yoon[3], Deog Hwan Moon[1] and Sung Goo Kang†

Department of Biology, College of Natural Sciences, Inje University, Kimhae, Gyeongnam 621-749, Korea
[1]Institute of Industrial Medicine, Inje University, Kimhae, Gyeongnam 621-749, Korea
[2] Department of Biotechnology, College of Natural Sciences, Seoul Women's University, Seoul 139-774, Korea
[3]Department of Life Science, College of Natural Sciences, Hanyang University, Seoul 133-791, Korea

흰쥐 태반에서의 Mel_{1a} 유전자 발현과 멜라토닌이 PLP-A 유전자 발현에 미치는 영향

신창숙 · 이채관[1] · 강한승 · 김해권[2] · 윤용달[3] · 문덕환[1] · 강성구†

인제대학교 자연과학대학 생물학과,
[1]인제대학교 산업의학연구소,
[2]서울여자대학교 자연과학대학 생명공학과,
[3]한양대학교 자연과학대학 생명과학과

ABSTRACT : Seasonal changes and circadian rhythm of plasma prolactin(PRL) concentration in mammals are mediated by melatonin. Pinealectomy or denervation of the pineal gland produces an increase in plasma PRL level. In the rat placenta, several members of the PRL family gene are expressed during the late pregnancy. However, the full spectrum of their expression mechanisms and regulatory factors are not elucidated yet. Present study aimed to investigate the local expression of the melatonin receptor 1a(Mel_{1a}) gene and the effect of melatonin on expression of prolactin-like protein A(PLP-A), a member of the PRL-family gene in the rat placenta. According to the RT-PCR, northern blot and *in situ* hybridization experiments, Mel_{1a} gene was locally expressed in the rat placenta. Mel_{1a} mRNA was localized mainly in the placental junctional and labyrinth zones. Interestingly, junctional zone of the placenta showed strong expression of Mel_{1a} at daytime(16:00) than at nighttime(22:00). Melatonin agonist, chloromelatonin decreased the PLP-A mRNA levels in the rat placenta. These results suggest that melatonin, coupled with Mel_{1a}, may act as a regulation factor that mediates the expression of the PLP-A gene in the rat placenta.

Key words : Melatonin, Mel_{1a}, PLP-A, Placenta.

요 약 : 포유동물의 혈중 프로락틴 농도는 일주기와 연주기의 변화를 나타내며 송과체에서 분비되는 멜라토닌이 조절인자로 관여한다. 인위적인 송과체의 기능 억제는 혈중 프로락틴 농도를 증가시킨다. 임신 후반기에 태반에서는 수종의 프로락틴군 호르몬들이 분비되어 태반기능 및 배아발생에 중요한 역할을 한다. 그러나 이들 호르몬 유전자들의 발현 조절 기작과 조절 인자들에 관한 연구 결과는 미비하다. 본 연구에서는 RT-PCR과, *in situ* hybridization 방법으로 흰쥐의 태반에서 Mel_{1a} 유전자의 발현을 확인하였다. 발현되는 주요 세포는 junctional zone과 labyrinth zone의 spongiotrophoblast 세포와 trophoblast giant 세포였다. 특이한 것은 junctional zone의 Mel_{1a} 유전자의 발현이 밤시간(22:00)에 비하여 낮시간(16:00)에 높게 조사되었다. 그리고 멜라토닌 수용체 agonist인 chloromelatonin은 PLP-A 유전자의 발현을 억제하였다. 이러한 결과들로 보아 흰쥐의 태반에서 Mel_{1a} 유전자가 발현되며, 멜라토닌에 의해 유도되는 Mel_{1a}의 활성화는 PLP-A 유전자의 발현에 중요한 조절인자로 작용할 것이다.

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†Correspondence: Department of Biology, College of Natural Sciences, Inje University. Obang-dong Kimhae Gyeongnam 621-749 Korea. Tel: 82-55-320-3213, Fax: 82-55-336-7706, E-mail: biosgkan@ijnc.inje.ac.kr)

INTRODUCTION

Most mammals show marked seasonal changes and circadian rhythm of plasma PRL concentration during pregnancy (McMillen et al., 1987; Basset et al., 1988). The environmental signal that drives these hormonal changes in mammals is photoperiod (Pelletier et al., 1973; Kennaway et al., 1983). The effect of photoperiod on PRL in mammals is mediated by melatonin (Basset et al., 1989). Pinealectomy(Kennaway et al., 1981; Yellon & Longo 1988; Mcmillen & Nowak 1989) or denervation of the pineal gland(Lincoln et al., 1982) produces an increase in plasma PRL level. Melatonin is produced rhythmically by the pineal gland and has two well-described biological effects (Reppert et al., 1994; Arendt, 1999; 2000). First, melatonin controls the annual timing of reproductive function in seasonally breeding mammals. Second, melatonin speeds up the adjustment of the circadian clock to changes in the light-dark cycle. Cloning studies in mammals have identified two melatonin receptor subtypes, Mel_{1a} and Mel_{1b}. Mel_{1a} is expressed in suprachiasmatic nuclei(SCN) and hypophyseal pars tuberalis(PT), presumed sites of the circadian and some of the reproductive effects of melatonin(Reppert et al., 1995; Roca, et al., 1996). Mel_{1b} receptor mRNA has been identified in human retina and brain(Reppert et al., 1995).

Several members of the PRL family gene are expressed in the rat placenta during the late pregnancy(Lytras et al., 1994; Soares et al., 1998). Well known members of this family are placental lactogen(PL) I, Iv, Ⅱ(Robertson et al., 1982; Duckworth et al., 1986A; Robertson et al., 1990; Deb et al., 1991A), PLP-A, B, C, Cv, D(Duckworth et al., 1986B; Croze et al., 1990; Deb et al., 1991B; Conliffe et al., 1995; Iwatsuki et al., 1996) and decidual/trophoblast prolactin related protein(d/t PRP)(Roby et al., 1993; Orwig et al., 1997). These genes are very similar with PRL in the structure and activities(Soares et al., 1998). The PLP-A mRNA is synthesized from approximately day 14 of pregnancy until term at day 21. *in situ* hybridization studies have localized the expression of PLP-A to the spongiotrophoblast and some giant cells of the junctional zone of the rat placenta, a region which has been reported to have direct access to the maternal blood supply(Duckworth et al., 1990)

Although biological actions and regulation mechanisms of PLP-A and other members of the PRL family gene are presently unknown, the structural relationship of PLP-A to PRL and other members of the PRL family gene suggests a possible role for PLP-A in coordination of metabolic adjustments between fetal and maternal compartments(Soares et al., 1991). In this study, we aimed to investigate the local expression of Mel_{1a} and the effects of melatonin on the expression of the PLP-A gene in the rat placenta.

MATERIALS AND METHODS

1. Animal and tissue preparation

Pregnancy of Sprague Dawley rats was checked with the presence of a copulatory plug or sperms in the vaginal smear and defined as pregnant day 0. For the tissue preparation of *in situ* hybridization histochemical studies, pregnant rats(day 19) were anesthetized with pentobarbital sodium(Sigma) and perfused intracardially with 25 mM phosphate-buffered saline(PBS) (pH 7.2) for 10 min, and then with 4% paraformaldehyde for 10 min. For the preparation of tissue culture and northern blot hybridization, placental junctional zone was removed from the labyrinth zone and then minced(>0.5 mm) using the micro forceps. Tissues were treated with lysis buffer(17 mM Tris-HCl, pH 7.3, 0.144 M NH4Cl) to eliminate the contamination of blood components such as lymphocytes in which Mel_{1a} gene is expressed(Pozo et al., 1997). Tissues were cultured in MEM with or without melatonin agonist, chloromelatonin(1.0 μM) for 12 h.

2. Cloning of Mel_{1a}

To clone the Mel_{1a} cDNA using the RT-PCR technique, a set of Mel_{1a} specific primer, an upstream primer(5'-CGTTACTGC-TACATTTGCCA-3') and a downstream primer(5'-ATGGTGAC-AAAGTTCCTGAA-3'), was synthesized based on the Mel_{1a} cDNA sequence reported previously in the rat hypothalamus (Reppert et al., 1994). Total RNAs(0.5 μg) were reverse transcribed by 200 units of moloney murine leukemia virus(MMLV) reverse transcriptase and amplified with 10 units of Taq DNA polymerase(Perkin-Elmer Cetus). Samples were amplified for 30 cycles with thermofile of denaturation at 95℃ for 1 min, annealing at 55℃ for 1 min and extension at 72℃ for 1 min, respectively. Mel_{1a} cDNA fragments were subcloned into the

pGEM-T vector(Promega) and sequenced using the fmol PCR sequencing system(Promega).

3. *In situ* hybridization

The specific activity of the probes was greater than 1×10^8 cpm/mg. Before *in situ* hybridization, the tissue sections(10 μm) were fixed in ice-cold 4% paraformaldehyde in phosphate-buffered saline, and washed in 2X sodium chloride-sodium citrate buffer(SSC; 0.5 M NaCl; 0.3 M sodium citrate, pH 7.0). Subsequently, the sections were covered with prehybridization buffer (50% formamide, 0.6 M NaCl, 10 mM Tris-HCl(pH 7.5), 0.02% Ficoll, 0.02% polyvinyl pryollidone, 0.1% bovine serum albumin, 1 mM EDTA(pH 8.0) and dextran sulfate) and incubated at 37℃ for 1 h. After removal of the prehybridization buffer, slides were covered with hybridization buffer. Hybridization with the antisense or sense probes were carried out in this same solution with the addition of 50 μg/ml yeast tRNA, 10 mM dithiothreitol, 10% dextran sulfate and 6×10^5cpm of RNA probe per μg of solution. The slides were cover-slipped and incubated at 60℃ for 24 h. Tissue slides were then posthybridized in a posthybridization buffer. Subsequently, following a wash in 2× SSC for 30 min, the sections were treated with RNase A(50 μg/ml), washed twice in warmed(50℃, high-stringency) 2X SSC buffer, transferred to a wash buffer containing 0.1X SSC at 65℃ for 15 min. Following dehydration, the slides were dipped in Kodak NTB 2 emulsion(1:1 dilution), exposed for two weeks at 4℃, developed in Kodak D19 developer(1:1 dilution, 15℃) and counter-stained with methyl-green. The slides were observed under a dark or a bright fields microscope, and then photographed.

4. Northern blot analysis

Total RNAs were extracted by the acid guanidium thiocyanate -phenol chloroform method using the tri-reagent(Sigma). For northern blot analysis, RNAs were fractionated on a 1% agarose /2.2 M formaldehyde gel at 100 V for 2 h, transferred to nytran membrane(0.45 μm; Schleicher and Schuell). The membranes were prehybridized with 10ml of hybridization buffer at 55℃ for 2 h. Hybridization buffer consisted of 50% deionized formamide, 5X SSC(1X SSC=0.15 M NaCl and 0.015 M sodium citrate), 5X Denhardt's solution(1X Denhardt's solution=0.01% polyvinyl-pyrrolidone, 0.01% Ficoll and 0.01% BSA), 0.1% SDS and 2 mg of heat-denatured salmon sperm DNA. Hybridization was carried out in a hybridization incubator(Stuart scientific) with hybridization buffer plus ^{32}P-labeled cDNA probe. Hybridization temperatures were adjusted at 55℃. After overnight hybridization, the membranes were washed at high stringency and exposed to X-ray film(Kodak) for 1~4 days. Cloned and sequenced Mel_{1a} and PLP-A cDNA fragments were labeled with ^{32}P dCTP by the oligolabelling kit(Pharmacia) for cDNA probes. Uncorporated ^{32}P dCTP was removed using the Nick column(Pharmacia). The specific activities of probes were about 1×10^9 cpm/ml.

5. Statistics

Statistical comparison between the groups was analyzed by either an unpaired student's t-test for two groups or a one-way analysis for variance for more than two groups. The level of statistical significance was set at $p<0.05$.

RESULTS

1. Local expression of Mel_{1a} gene in the rat placenta

In order to determine the local expression of Mel_{1a} gene in the rat placenta, we carried out RT-PCR, northern blot and *in situ* hybridization analyses. Fig. 1A shows the RT-PCR product and sequences of amplified Mel_{1a} cDNA fragment in the rat placenta. PCR product of Mel_{1a} mRNA in the rat placenta was exactly same with the expected size(347 bp) on the basis of hypothalamus Mel_{1a} cDNA. Sequences of cloned Mel_{1a} cDNA fragment in the rat placenta did not reveal any difference from corresponding region of hypothalamus Mel_{1a} cDNA sequence reported by others(Reppert et al., 1994). Northern blot analysis of Mel_{1a} in the rat placental cytoplasmic total RNA showed two transcripts of 6.0 kb and 1.8 kb and placental mRNA showed a distinctive single band about 1.8 kb in size(Fig. 1B).

2. Localization of Mel_{1a} mRNA in the rat placenta

In situ hybridization study using ^{35}S-UTP labeled Mel_{1a} cRNA probe was carried out to determine the localization of Mel_{1a} mRNA in frozen sections of the rat placental tissues. Fig. 2 shows brightfield(A, C) and darkfield(B, D) photographs of 19 -day-old rat placenta hybridized to antisense(A, B) and sense (C, D) Mel_{1a} cRNA probe. Signals were observed with the anti-sense Mel_{1a} cRNA probe but not with the sense cRNA probe, indi

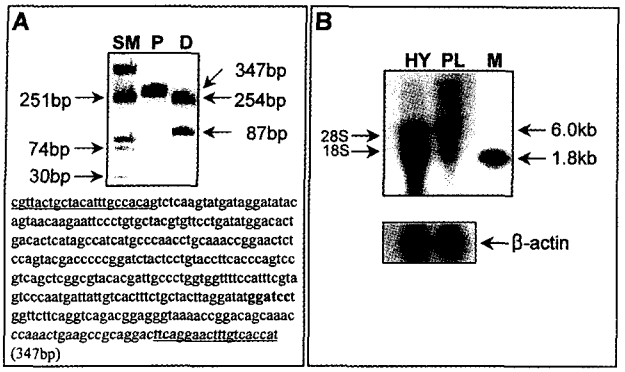

Fig. 1. Local expression of the melatonin receptor 1a(Mel_{1a}) gene in the rat placenta. Pregnant rats were perfused at gestational day 19(PM 10:00). Minced(> 0.5 mm) placental tissues were treated with lysis buffer. (A), RT-PCR of Mel_{1a} mRNA and sequence of amplified Mel_{1a} cDNA (347 bp). Underlines and bold letters indicate the primers for RT-PCR and digesting site of Bam HI restriction enzyme. P, RT -PCR product of Mel_{1a}; D, Mel_{1a} cDNA digested with BamHI; SM, size marker. (B), Northern blot analysis of Mel_{1a} in the rat hypothalamus and placenta. Total RNA(45 μg) and placental mRNA(1 μg) were fractionated on an 1% formaldehyde agarose gel, transferred to nytran membrane and hybridized with ^{32}P labeled Mel_{1a} or β-actin cDNA probe. HY, hypothalamus; PL, placenta; M, placental mRNA. 28S and 18S indicate the 28S and 18S ribosomal RNA.

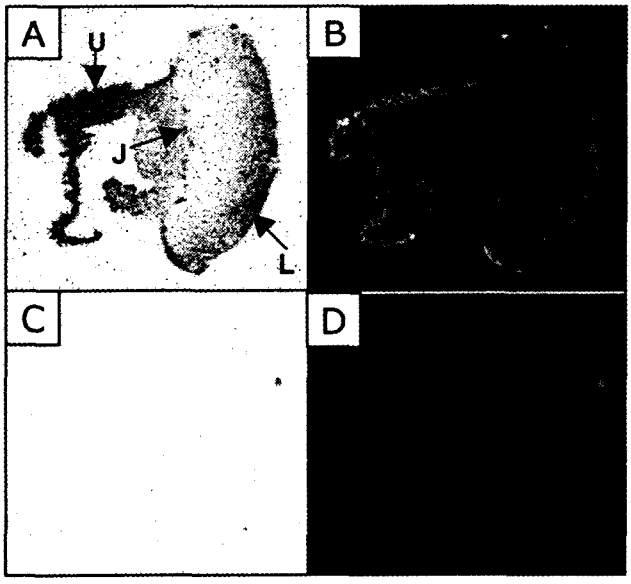

Fig. 2. *in situ* hybridization of melatonin receptor 1a(Mel_{1a}) mRNA in the rat placenta. Frozen sections(10 μm) were obtained at pregnant day 19(PM 10:00) and hybridized with ^{35}S-labeled Mel_{1a} anti -sense(A, B) and sense(C, D) RNA probes. A and C show the brightfield photographs, B and D corresponding the darkfield photographs(× 3, reproduced at 85%). U, uterus; J, Junctional zone; L, labyrinth zone.

cating the signals are specific to Mel_{1a} mRNA. Signals of Mel_{1a} mRNA were mainly localized in the trophoblast giant cells and spongiotrophoblast cells of placental junctional zone(Fig. 2, 3B).

3. Circadian rhythm of Mel_{1a} gene expression

To investigate the circadian rhythms of Mel_{1a} mRNA levels, pregnant rats were sacrificed at daytime(gestational day 19, 16:00) and nighttime(gestational day 19, 22:00). According to the *in situ* and northern blot hybridization date, there was clear circadian rhythmicity in the mRNA levels of Mel_{1a} in the rat placental junctional zone. Fig. 3 shows the *in situ* hybridization photographs of Mel_{1a} at daytime(A, B) and at nighttime(C, D). *in situ* hybridization signals for Mel_{1a} mRNA were stronger in the rat placental junctional zone sacrificed at daytime than nighttime. Fig. 4 shows the change of mRNA level in the rat placental junctional zone at daytime and nighttime analyzed by northern blot hybridization. Northern data of Mel_{1a} agree well with the *in situ* hybridization data(Fig. 4).

4. Effect of melatonin on expression of PLP-A gene

The rat placental junctional zone was isolated from the labyrinth zone and cultured in MEM with or without melatonin agonist, chloromelatonin(1.0 μM). Northern signal of PLP-A

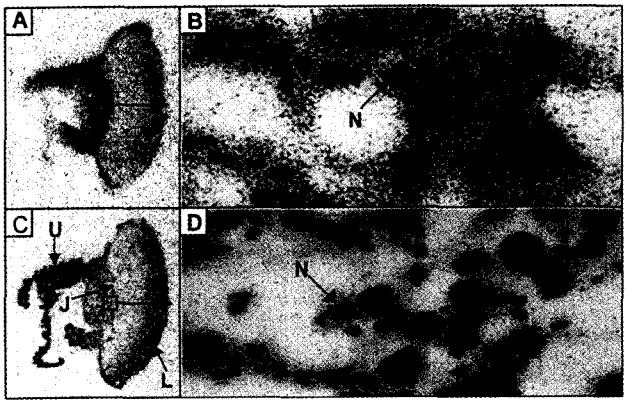

Fig. 3. Differential localization of melatonin receptor 1a(Mel_{1a}) in sections of rat placenta at daytime and at nighttime. Hybridization signals were identified as black dots in the brightfield photoradiographs. Sections were hybridized with ^{35}S-labeled Mel_{1a} anti-sense cRNA probe. A and C sections, the brightfield photographs of whole rat placenta, are seen at daytime(gestational day 19, 16:00) and nighttime(gestational day 19, 22:00)(× 2.5, reproduced at 85%) respectively. B and D, the brightfield microphotographs of placental junctional zone (× 400 reproduced at 70%). U, uterus; L, labyrinth zone; J, junctional zone; N, nucleus stained with methyl blue.

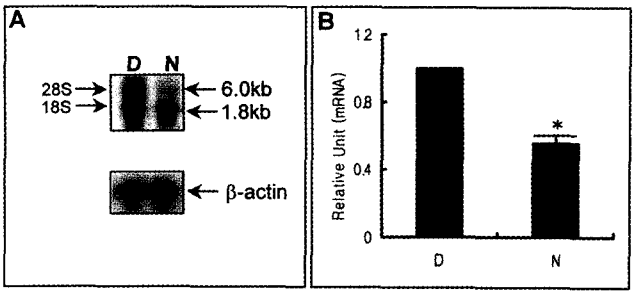

Fig. 4. Changes of melatonin receptor 1a(Mel_{1a}) mRNA level at daytime and at nighttime in the rat placental junctional zone. Placental tissues were obtained at 16:00(D) and 22:00(N) of gestational day 19 as described in Fig. 1. Northern blot analysis of Mel_{1a}(A). Total RNAs(45 μg) were fractionated on a formaldehyde agarose gel. Transferred to nytran membrane and hybridized with ^{32}P-labeled Mel_{1a} cDNA probe. β-actin was hybridized to certify the equal loading of total RNA. (B), Northern signals were normalized with β-actin mRNA levels and expressed as relative unit over D value of 1.0. Experiments were repeated for three times and individual values are expressed mean ± SEM(*, $p<0.05$).

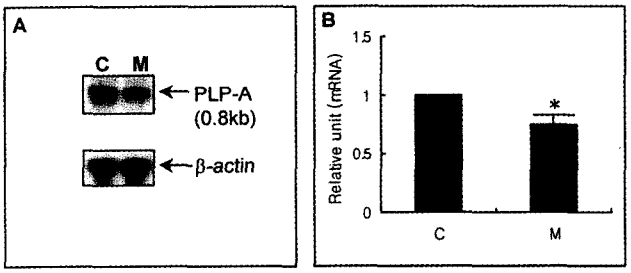

Fig. 5. Effect of melatonin on the expression of PLP-A mRNA level in the rat placenta. Placental junctional zone was minced(> 0.5 mm) and cultured in MEM with(M) or without(C) melatonin agonist, chloromelatonin(1.0 μM). (A), Northern blot analysis of PLP-A. Total RNA (10 μg) was fractionated on a formaldehyde gel. Transferred to nytran membrane and hybridized with ^{32}P labeled PLP-A cDNA probe. β-actin was hybridized to certify the equal loading of total RNA. (B), Northern signals were normalized with β-actin mRNA levels and expressed as relative unit over C value of 1.0. Experiments were repeated for three times and individual values are expressed mean ± SEM(*, $p<0.05$).

showed a distinctive single band(about 0.8 kb). Melatonin agonist, chloromelatonin, reduced the PLP-A mRNA levels in the cultured placental junctional zone(Fig. 5).

DISCUSSION

Seasonal changes and circadian rhythm of plasma PRL concentration are regulated by photoperiod(Pelletier et al., 1973; Kennaway et al., 1983). The effects of photoperiod on PRL in mammals are mediated by melatonin(Basset et al., 1989). Several members of the PRL gene family are expressed during the late pregnancy(Lytras et al., 1994; Soares et al., 1998). In this report, we confirmed the local expression of Mel_{1a} in the rat placenta. There was no blood contamination because pregnant rats were perfused with fixer and placental tissues were treated with lysis buffer to eliminate any contamination of blood cells in which Mel_{1a} gene was expressed(Pozo et al., 1997). PCR product of Mel_{1a} from the placental total RNA was exactly same with the expected size(347 bp) on the based of hypothalamus Mel_{1a} cDNA. And the sequence of cloned Mel_{1a} cDNA fragment showed not any difference from corresponding fragment of hypothalamus Mel_{1a} cDNA sequence by previous report(Reppert et al., 1994). Addition to the RT-PCR and northern blot hybridization, we have employed the *in situ* hybridization histochemistry to identify the temporal and spatial expression of Mel_{1a} in the rat placenta. Mel_{1a} mRNA was localized in the developing rat placental tissue. Hybridization signals for Mel_{1a} mRNA were localized mainly in the spongiotrophoblast and trophoblast giant cells in the rat placental junctional zone and labyrinth zone(Fig. 2, 3). This result was supported by the previous report that almost of PRL-family genes are expressed in the spongiotrophoblast and trophoblast giant cells(Soares et al., 1998). And interestingly, junctional zone of the placenta showed strong expression of Mel_{1a} at daytime(16:00) than at nighttimt(22:00) (Fig. 4).

By the studies of *in vitro* tissue culture, we suggested the strong evidence that melatonin, coupled with Mel_{1a}, acts as a regulatory factor that mediates expression of the rat PLP-A gene. Melatonin agonist, chloromelatonin reduced the PLP-A mRNA levels in the cultured placental junctional tissues(Fig. 5). Mel_{1a} is the G-protein coupled protein, affects to the cAMP level (Reppert et al., 1994; 1995) as the dopamine receptors(Vaillancourt et al., 1994). Recently, local expressions of dopamine receptor $1(D_1)$ and $2(D_2)$ genes were reported by our group in the rat placenta(Kim et al., 1997). And it has been reported that dopamine plays an inhibitor of PRL family gene expression in the human and rat placenta(Petit et al., 1993; Vaillancourt et al., 1994; 1997; Lee et al., 1998). In the rat placenta, the inhibitory controls of dopamine and melatonin for PRL family gene may

be mediated through the D_2 and Mel_{1a} binding and may be due to the decrease of cAMP accumulation just as dopamine inhibits the PRL gene expression in the pituitary lactotrophs(Elsholtz et al., 1991). However, the full spectrum of mechanisms that melatonin and dopamine regulate the expression of PLP-A gene is uncertain. In summary, we have reported the local expression of Mel_{1a} gene in the rat placenta for the first time. Melatonin decreased the PLP-A mRNA level in the rat placental tissues. Together with these data it is presumed that melatonin coupled with Mel_{1a} acts as one of the regulatory factors that control the expression of rat PLP-A gene in the rat placenta.

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