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A 914-bp promoter is sufficient to reproduce the endogenous prolyl oligopeptidase gene localization in the mouse placenta if not subject to position effect

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Abstract

Prolyl oligopeptidase (POP) is a widely distributed multifunctional protein which has an endopeptidase activity to cleave a -Pro-X- peptide bond. In spite of numerous studies about POP, the mechanism by which its transcription is controlled has not been well investigated. Here we generated transgenic mice bearing a transgene which contained a 914-bp POP gene promoter linked to the enhanced green fluorescent protein (EGFP) gene to assess the *in vivo* promoter activity. We established six transgenic lines with different copy numbers, but no EGFP signal was observed in four lines due to a high level of DNA methylation, which suggested that the transgene was subject to position effect. However, in the other two lines, we detected the EGFP expression in many tissues, and its placental localization showed a similar change to POP. A strong EGFP signal was observed in the junctional and labyrinthine zones of E10.5-E12.5 placentas and in the junctional zone and the maternal decidua after that. This placental gene activation might be attributed to AP-2 γ because we detected its binding to the POP promoter. In contrast, we did not obtain any evidence that EGFP was expressed in a similar pattern compared with POP in the ovary. The current data demonstrated that the 914-bp promoter had sufficient activity to reproduce the POP localization in the placenta if it was not subject to position effect and suggest that the regulatory mechanism of the POP gene expression differs between tissues.

Key words: prolyl oligopeptidase, transgenic mouse, promoter, position effect, placenta, AP-2 γ

Abbreviations: ANOVA, analysis of variance; E, embryonic day; EGFP, enhanced green fluorescent protein; LCR, locus control region; PCR, polymerase chain reaction; POP, prolyl oligopeptidase; qPCR, quantitative polymerase chain reaction; qRT-PCR, quantitative reverse transcription-polymerase chain reaction; SD, standard deviation; TGC, trophoblast giant cell

1. Introduction

Prolyl oligopeptidase (POP, E.C.3.4.21.26, prolyl endopeptidase, post proline cleaving enzyme; gene symbol *Prep*) is a serine endopeptidase which hydrolyzes a short peptide at the carboxyl side of an internal proline residue (Wilk, 1983). POP is known to be a multifunctional protein and has been reported to play some roles in the degradation of hormones and neuropeptides (Mentlein, 1988; Wilk, 1983), learning and memory (Miura et al., 1997; Shishido et al., 1998; Toide et al., 1995; Yoshimoto et al., 1987), cell signaling (Williams et al., 1999), cell differentiation (Hannula et al., 2011; Ohtsuki et al., 1994), sperm motility (Kimura et al., 2002; Yoshida et al., 1999), obesity (Perroud et al., 2009; Warden et al., 2009), and so on. POP has also attracted attention as a therapeutic target because it is possibly involved in several diseases such as bipolar disorder, Alzheimer's disease, and cancer (García-Horsman et al., 2007; Myöhänen et al., 2009; Williams, 2004). In the patients of such diseases, the expression level of POP aberrantly increased or decreased (Mantle et al., 1996; Breen et al., 2004; Goossens et al., 1996). Therefore, it is of great importance to understand the mechanism by which the POP expression is regulated, but only a few studies have investigated it.

POP is expressed in a wide variety of tissues, but its expression level varies greatly between tissues. For example, POP mRNA was detected at a high level in the porcine heart and muscle (Rennex et al., 1991), the human skeletal muscle (Goossens et al., 1997; Shirasawa et al., 1994), the rat brain and thymus (Kimura and Takahashi, 2000), and the mouse ovary and placenta (Matsubara et al., 2010, 2011), compared to other tissues. Moreover, we have shown that the mouse POP mRNA was localized to granulosa cells in the ovary (Matsubara et al., 2010) and to spermatids in the testis (Kimura et al., 2002). In the placenta, POP mRNA changed its localization at embryonic day 12.5 (E12.5) from the junctional and labyrinthine zones to the junctional zone alone (Matsubara et al., 2011). These indicate that the POP gene transcription is

highly regulated in a tissue-specific manner. Considering that few endogenous POP inhibitors are present (Salers, 1994; Yoshimoto et al., 1982; Yamakawa et al., 1994) and POP is not translated as a precursor or a zymogen (Szeltner and Polgár, 2008), the transcription is likely to be a main step for regulating the POP gene expression.

The transcriptional regulation is one of the most important steps for appropriate expression of many genes. To analyze the regulatory mechanism of transcription, a reporter gene assay is often used as a powerful technique, and it can uncover the *in vitro* activity of a regulatory sequence. However, to assess the *in vivo* activity, transgenic mice have to be generated and analyzed, and the result could be different from that of the reporter gene assay (Shewchuk et al., 2002). This is because a transgene is integrated into the mouse genome and influenced by the adjacent chromosomal environment in transgenic mice. In other words, a transgene can be subject to position effect in transgenic mice. Therefore, the elements identified by the *in vitro* assay should also be analyzed *in vivo*.

A promoter is the most important element for transcriptional activation. It is defined as the sequence to which RNA polymerase binds to begin transcription and is usually identified as an immediately upstream sequence of the transcriptional start site. Previous reports have shown that some promoters had sufficient activity to drive the appropriate gene expression in transgenic mice. For example, promoters of the *Pem/Rhox5* gene (Rao et al., 2003), the *CYP19* aromatase gene (Kamat et al., 1999), the *tyrosinase* gene (Klüppel et al., 1991), and the human *Ig β* gene (Yoo et al., 2006) reproduced the tissue-specific expression patterns of the corresponding genes in transgenic mice. Several testis-specific promoters have also been reported to be sufficient for the appropriate gene activation in transgenic mice (Reddi et al., 2007). However, the full gene activation by its promoter alone seems to be a rare case in mammals, and most genes require other regulatory elements such as an enhancer and an insulator that are often identified in intronic or intergenic regions (Bagheri-Fam et al., 2006;

Grosveld et al., 1987; Jeong et al., 2006; Jones et al., 1995; Kim et al., 2007; Nobrega et al., 2003; Shima et al., 2005, 2008). The enhancer is the sequence to which activator proteins bind to increase the rate of transcription (Bulger and Groudine, 2011), and the insulator is a sequence to protect a transgene from position effect (Gaszner and Felsenfeld, 2006; Yang and Corces, 2011). In case of the POP gene, we determined a minimum promoter to drive its transcription *in vitro* (Kimura et al., 1999) and identified a CpG island in the genebody to be an enhancer candidate in the ovarian granulosa cell (Matsubara et al., 2010), but mechanisms for its transcriptional regulation are largely unknown.

In this study, as an initial step in understanding the regulation of POP gene expression, we evaluated whether the POP promoter is sufficient to establish the endogenous POP expression patterns *in vivo*. To this end, we generated transgenic mice harboring the enhanced green fluorescent protein (EGFP) gene driven by the POP promoter. The EGFP gene was not expressed in any tissues of four established lines possibly due to position effect. However, in the other two lines, the EGFP gene was expressed in many tissues and its localization in the placenta was similar to POP. The current data demonstrated that the POP promoter was sufficient for the appropriate localization in the placenta if it was not subject to position effect.

2. Materials and methods

2.1. Animals

C57/BL6 and BDF1 (C57/BL6 $\times$ DBA2 F1) mice were obtained from CLEA Japan Inc. (Tokyo, Japan) and maintained under the constant temperature (25 °C), with enough food and water, and a photoperiod of 14-h light/10-h dark cycle. Transgenic mice were kept in the same

condition. The experimental procedures used in this study were approved by the Institutional Animal Use and Care Committee at Hokkaido University.

2.2. *Generation, maintenance, and genotyping of transgenic mice*

A 914-bp mouse POP promoter (an *EcoRI*-*Bam*HI fragment) was already subcloned into the upstream of the EGFP gene of a pEGFP-1 vector (Clontech Laboratories Inc, Palo Alto, CA, USA) (Kimura et al., 1999). By digesting it with *Xho*I and *Afl*III restriction enzymes, the transgene composed of the POP promoter, the EGFP gene, and the SV40 poly A signal was isolated from the vector sequence. The transgene was separated by agarose gel electrophoresis and purified using GENECLAN kit III (MP Miomedicals, Solon, OH, USA) according to the manufacturers' protocol. Transgenic mice were generated at Yamagata University, School of Medicine (Yamagata, Japan) by microinjecting the transgene into the male pronuclei of fertilized BDF1 oocytes. Founder mice were identified by a dot blot analysis using the tail DNA with a 772-bp EGFP probe which was prepared by digesting the pEGFP-1 vector with *Eco*RI and *Not*I restriction enzymes. Positive founders were mated with BDF1 mice to generate heterogenous F1 offsprings and we established the lines.

After the establishment of transgenic lines, we used polymerase chain reaction (PCR) for routinely genotyping their offsprings using the tail DNA with primers, 5'-ACGTAAACGGCCACAAGTTC-3' and 5'-TTGAAGTTCACCTTGATGCC-3', under the following conditions: 94 °C for 3 min, and 32 cycles of 94 °C for 30 s, 65 °C for 30 s, and 72 °C for 1 min. Transgenic mice were identified by the amplified 436-bp EGFP fragment within the transgene. The offspring of each line was maintained at heterogenous genetic background.

2.3. *Copy number determination*

To assess the copy number of the transgene in the established transgenic line, we adopted quantitative PCR (qPCR) method. We first digested 500 ng of the tail DNA with *Pst*I at 37 °C for overnight. After the purification by phenol/chloroform-isoamylalcohol extraction and ethanol precipitation, qPCR was performed using 1.5 ng of the DNA with the 7300 real-time PCR system and Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA) in a total volume of 10 µl per well. The PCR conditions were 50 °C for 2 min, 95 °C for 10 min, and 40 cycles of 95 °C for 15 sec and 60 °C for 1 min. Dissociation curves were obtained to confirm the specificity of the amplified DNA. The primer sequences were 5'-AGCAAAGACCCCAACGAGAA-3' and 5'-GGCGGCGGTCACGAA-3' for EGFP (Harraghy et al., 2011), and 5'-CCATAGGCTTCACACCTTCCTG-3' and 5'-GCACTAACACTACCTTCCTCAACCG-3' for *β-actin* as an endogenous control of two copies (Brandt et al., 2008). A standard curve for each gene was generated using serial dilutions of genomic DNA and we confirmed that amplification efficiencies of both primer pairs were similar (data not shown). The copy number was calculated relative to *β-actin* per copy and expressed as mean ± SE.

2.4. Northern blot analysis

Northern blot was performed as previously described (Matsubara et al., 2010). Briefly, total RNAs from 17 mouse tissues of each F2-F4 generation mouse (lines #542 and #675) were purified using ISOGEN (Nippon gene, Tokyo, Japan) according to the manufacturers' instructions. Twenty micrograms of the RNA were electrophoresed on a formaldehyde/agarose gel, blotted to a Hybond-N⁺ membrane (GE Healthcare, Piscataway, NJ, USA), and hybridized with a ³²P-labeled EGFP cDNA probe. The membrane was washed and exposed to Kodak

Biomax film (Kodak, Rochester, NY, USA), and signals were visualized by autoradiography.

We used the same EGFP probe as the dot blot analysis described above.

2.5. *Detection of the EGFP fluorescence*

Wild type or transgenic adult mice (F7-F9 generation, lines #542 and #675) were sacrificed and tissues were collected. Placentas were isolated from wild type and transgenic pregnant mice as previously described (Matsubara et al., 2010, 2011). We washed the tissues with phosphate buffered saline, and the EGFP fluorescence was analyzed using a Leica MZ FLIII Fluorescence Stereo Microscope (Leica Microsystems, Tokyo, Japan) equipped with a cooled charged-coupled device camera (KEYENCE VB-7010, KEYENCE, Osaka, Japan). For analyzing the EGFP localization in the placenta at each developmental stage, a 3-5 mm of cross-section was prepared. For visualizing the primary granulosa cell, an Olympus IX70 inverted phase-contrast microscope (Olympus, Tokyo, Japan) was used with the same camera. An exposure time for all the tissues and cells was set at 5 sec.

2.6. *Bisulfite sequencing analysis*

Bisulfite sequencing analysis was performed using two sets of placentas at E15.5 (#542) and at E17.5 (#528) as previously described (Matsubara et al., 2010). In order to specifically amplify the DNA fragment derived from the transgene, we used a forward primer at the POP promoter (5'-GGTGT TTTTGTAGAGGGAGTTG-3', Matsubara et al., 2010) and a reverse primer at the EGFP gene (5'-ACCAAAATAAACACCACCCC-3').

2.7. *Primary granulosa cell culture*

Granulosa cells were isolated from superovulated female mice (3-4 weeks old) and cultured as previously described (Matsubara et al., 2010). After the culture for 3 days, the EGFP fluorescence was observed and total RNA was isolated at day 5.

2.8. *Quantitative reverse transcription-polymerase chain reaction (qRT-PCR)*

qRT-PCR was performed as reported before (Matsubara et al., 2010) with slight modifications. Total RNAs from tissues or cultured cells were purified as described above. After the treatment with TURBO DNase (Ambion, Austin, TX, USA), the first-strand cDNA was synthesized using a Superscript III reverse transcriptase (Invitrogen, Carlsbad, CA, USA). The primers used for PCR were 5'-GGAATCGATGCTGCTGATTA-3' and 5'-CCATCCAGCTTTATGCCTTT-3' for POP (Matsubara et al., 2010), 5'-AGCAAAGACCCCAACGAGAA-3' and 5'-GGCGGCGGTCACGAA-3' for EGFP (Harraghy et al., 2011), and 5'-GAGGACGGGATCCAAAAGC-3' and 5'-CTGTGCAGCGTCCGAAAGT-3' for *arylhydrocarbon receptor-interacting protein (Aip)*, Frericks and Esser, 2008; Matsubara et al., 2010). All the data were normalized to *Aip*.

2.9. *Chromatin immunoprecipitation (ChIP) assay*

ChIP was conducted with E13.5 transgenic placentas (#675) as previously described (Matsubara et al., 2010). Three micrograms of the anti-AP-2 γ antibody (sc-8977, Santa Cruz Biotechnology, Santa Cruz, CA, USA) or normal rabbit IgG were used. A primer pair of 5'-GACAGCGTCTTCCTGCTTG-3' and 5'-GGGTACTGGAAGGACAGCTA-3' was used to amplify the candidate AP-2 γ binding site at the endogenous POP promoter. Sequences for the

other primers were previously described (Matsubara et al., 2010).

2.10. Statistical analysis

Results were expressed as the average $\pm$ standard deviation (SD) of at least three independent experiments and analyzed by Student's *t* test or two-way analysis of variance (ANOVA) followed by Tukey's *post hoc* test using JSTAT statistical software (version 12.6 for Windows, Masato Sato, Japan). Differences were considered statistically significant at $P < 0.05$.

3. Results

3.1. Establishment of transgenic lines and determination of their copy numbers

To evaluate the *in vivo* activity of the mouse POP gene promoter, we generated transgenic mice harboring the EGFP gene driven by a 914-bp POP promoter. This promoter has been already proved to have sufficient activity to drive the transcription of reporter genes *in vitro* (Kimura et al., 1999; Matsubara et al., 2010). By injecting the transgene into fertilized eggs, we obtained nine transgenic founders. We mated them with wild type mice, screened their offsprings by PCR, and established six transgenic lines that were assigned as #528, #531, #542, #566, #675, and #676.

We first estimated the copy number of the transgene in each established line. We purified the genome DNA from the tail of each line of mouse and performed qPCR using primers to amplify the EGFP gene. We also amplified the endogenous *β -actin* gene as a control of two copies (Brandt et al., 2008) and the copy number of the transgene was calculated as a ratio to the

control per copy. Ratios of EGFP to β -actin per copy were 2.96 ± 0.80 for #528, 2.60 ± 0.56 for #531, 0.87 ± 0.30 for #542, 1.37 ± 0.55 for #566, 1.16 ± 0.39 for #675, and 1.38 ± 0.27 for #676 (Table1). Since the copy number cannot be decimal in theory, we represented it in an integral number. Consequently, two lines (#528 and #531) had 3 copies of the transgene and the remaining four lines (#542, #566, #675, and #676) had 1 copy (Table 1).

3.2. *Tissue distribution of the EGFP gene in the transgenic lines*

We investigated the EGFP mRNA expression by Northern blot analysis using 17 tissues of each line. Four lines (#528, #531, #566, and #676) had no EGFP mRNA expression in any of the 17 tissues (data not shown, Table 1), and we could not detect any tissues with the green fluorescence in these lines by microscopic observations (data not shown). Therefore, we concluded that the EGFP gene was silenced in these four lines, probably due to position effect. In contrast, the other two lines (#542 and #675) had the EGFP signals in all the tissues examined (Fig. 1). The expression level of EGFP mRNA was higher in the epididymis, heart, kidney, ovary, and small intestine of the #542 line and in the kidney of #675 than in the other tissues (Fig. 1). These expression patterns of EGFP mRNA were different from that of the endogenous POP mRNA which was expressed at higher levels in the ovary and placenta (Fig. 1, Matsubara et al., 2010, 2011). This indicated that the 914-bp promoter was insufficient to recapitulate the tissue distribution of POP mRNA.

We also confirmed the EGFP expression in the tissues from the #542 and #675 lines by detecting the green fluorescence. Consistent with the Northern blot analysis (Fig. 1), we observed the EGFP fluorescence in all the tissues examined of both lines including the tissues indicated in figure 2. The intensities of the EGFP fluorescence, however, seemed to be different from those in the Northern results. For example, the kidney showed intense EGFP bands in both

lines by Northern blot (Fig.1), but its EGFP fluorescence looked weaker than the brain, epididymis, and testis (Fig.2). This could be due to the presence of blood inside the tissue which might disturb excitation or emission light of the EGFP protein. Indeed, when we perfused the liver of the #675 line, we observed more intense fluorescence (data not shown). In any case, the intensity of the EGFP fluorescence was not the highest in the ovary and placenta of these lines, which indicated that the EGFP gene was expressed in different patterns from POP.

3.3. *DNA methylation in the transgenic placenta*

To reveal the mechanism by which the transgene was silenced in the four lines, we investigated the DNA methylation status of the transgene by bisulfite sequencing. The placenta was chosen for this assay because the endogenous POP promoter was known to be completely demethylated in this tissue (Matsubara et al., 2010). We used placentas from the #528 and #675 lines as the tissue which did not and did express EGFP, respectively. To specifically amplify the sequence of the transgene, we used an antisense primer designed within the EGFP gene (Fig. 3). As a result of sequencing 12 subclones from two placentas for each line, we found that the exogenous POP promoter was completely demethylated in the #675 placenta whereas it was highly methylated in the #528 line (Fig. 3). The overall methylation rate of the #528 placenta was 77.4 %. This indicated that the transgene was inactivated due to epigenetic silencing of the transgene in the #528 line. This is the first indication that the DNA methylation status is directly correlated to the POP promoter activity.

3.4. *Localization of EGFP in the transgenic ovary*

Although the 914-bp promoter did not reproduce the tissue distribution of POP (Figs. 1

and 2), it is possible that it could drive appropriate localization inside the tissues. Therefore, we investigated the localization of the EGFP signal in the ovary and placenta. These two tissues were chosen because they expressed the highest levels of POP mRNA and the localization of POP has been already determined in these tissues (Matsubara et al., 2010, 2011). We dissected the ovary from the #542 and #675 transgenic lines and prepared paraffin sections and cryosections. However, we could not detect any EGFP signals on these ovarian sections from either line (data not shown) in spite of the strong fluorescence in the whole ovary (Fig. 2). Then, we isolated and cultured the granulosa cell which specifically expressed POP mRNA in the mouse ovary (Matsubara et al., 2010). The EGFP fluorescence was barely observed in the cells from both lines (Fig. 4A), which suggested that EGFP was expressed at a low level in the granulosa cell. To see if the EGFP signal was present specifically in the granulosa cell, we performed the qRT-PCR analysis by using the whole ovary and the isolated granulosa cell of the #542 and #675 transgenic lines. In both lines, the EGFP mRNA level in the granulosa cell was lower than that in the whole ovary, while POP mRNA was detected at a significantly higher level in the granulosa cell than in the whole ovary (Fig. 4B). This result strongly suggested that EGFP was not specifically expressed in the granulosa cell of the transgenic ovaries. Therefore, the 914-bp POP promoter was probably insufficient for the appropriate POP localization in the ovary.

3.5. *Localization of EGFP in the transgenic placenta*

In the placenta, POP mRNA was mainly localized in the junctional and labyrinthine zones until E11.5 and thereafter in the junctional zone alone (Matsubara et al., 2011). Because the green fluorescence was not detected on 5-10 μ m sections of the placenta, we prepared 3-5 mm sections to roughly determine the EGFP localization. For the #542 line, the fluorescence was

observed in the junctional and labyrinthine zones of the E10.5 placenta and in the junctional zone alone at E13.5, while the signal appeared in the maternal decidua as well as the junctional zone in the E18.5 placenta (Fig. 5A). For the #675 line, we obtained similar results. The fluorescence was mainly restricted to the junctional and labyrinthine zones at E10.5 and E12.5 and to the junctional zone and the maternal decidua at E17.5 (Fig. 5A). The fluorescence was also observed around the region connected to the umbilical cord of E17.5 and E18.5 placentas (Fig. 5A, arrowheads). This is partly consistent with the POP localization because some results of *in situ* hybridization also had the POP signal in a similar region (Matsubara et al., 2010, 2011). Taken together, the results indicated that the 914-bp POP promoter contained most sets of elements to establish the appropriate POP localization in the placenta.

We also compared the expression levels between EGFP and the endogenous POP in the transgenic placentas. Using placentas at different embryonic days from the #542 and #675 lines, we performed qRT-PCR to determine the mRNA levels. In both lines, the EGFP mRNA level per copy was similar to POP mRNA at E10.5 and significantly increased at later stages (Fig. 5B). The level of the placental EGFP in the #542 and #675 lines was 1.6-fold and 3.2-fold higher, respectively, than that of POP at E18.5 and E17.5. Both of the differences were statistically significant. These suggested that the 914-bp promoter was sufficient to an appropriate level of the POP mRNA expression at E10.5, but not necessarily at E17.5 and E18.5.

3.6. *The binding of AP-2 γ to the POP promoter*

We finally assessed what controlled the localization change of the placental POP. A placenta-specific transcription factor, AP-2 γ , could be a candidate because it was expressed in the same manner as POP (Sapin et al., 2000; Shi and Kellems, 1998) and the POP promoter had several AP-2 binding sites (Kimura et al., 1999). This time, we searched for the candidate AP-2

binding sites again by using the TRANSFAC software

(<http://www.gene-regulation.com/pub/databases.html>) and found nine sites in the 914-bp POP promoter, one of which was the sequence for AP-2 γ (Fig. 6A). Therefore, we investigated whether the transcription factor is binding to the POP promoter in the placenta.

We performed the ChIP analysis using the #675 transgenic placentas at E13.5. Although we used transgenic placentas, we amplified the promoter sequence from the endogenous POP locus alone but not from the transgene, because the antisense primer was designed outside the 914-bp promoter (Fig. 6A). Normal IgG was used instead of the antibody to determine the background level. As a result, we detected a peak of the AP-2 γ binding signal only in the promoter region (Fig. 6B), which indicated that AP-2 γ did actually bind to the POP promoter in the placenta. This suggested that AP-2 γ might be involved in controlling the placental POP localization.

4. Discussion

A promoter is the most important element for every gene to be actively transcribed, but its activity is different from one gene to another. Some promoters have an almost complete set of elements to fully activate the gene (Kamat et al., 1999; Klüppel et al., 1991; Rao et al., 2003; Reddi et al., 2007; Yoo et al., 2006), but others have little activity (Grosveld et al., 1987; Heckert et al., 2000; Jones et al., 1995). Therefore, it is very important to evaluate the promoter activity of each gene, and we analyzed the *in vivo* activity of the POP promoter for the first time by using transgenic mice.

One problem of the study with transgenic mice is that the expression pattern of a transgene could be different between transgenic lines (Boyer et al., 1997; Grosveld et al., 1987; Jones et al., 1995; Frazar et al., 2003). This is usually because of position effect, which makes it difficult

to interpret the results and to understand the genuine promoter activity. A locus control region (LCR) was discovered as a set of *cis*-elements that could overcome position effect (Grosveld et al., 1987), and an insulator was key for the LCR activity (Higgs, 1998; Li et al., 2002a). If the transgene contains an insulator activity, it shows site of integration independent and copy-number dependent expression (Kellum and Schedl, 1991; Li et al., 2002b). Alternatively, when the transgene contained a strong enhancer activity, it could be expressed in a site of integration independent manner (Shewchuk et al., 2002). In case of the POP promoter, we could not detect any EGFP expression in four lines out of six (Table 1), which indicated that the transgene expression was dependent on its integration site. Moreover, the expression was not copy-number dependent because the lines bearing three copies of transgene (#528 and #531) did not express EGFP but two single-copy lines (#542 and #675) did (Table 1). Therefore, we concluded that the 914-bp POP promoter could not overcome position effect. In other words, the promoter did not contain an insulator activity and the promoter activity was not strong enough to drive the transgene expression independent of its integration site.

How was the transgene silenced in the four lines? We expected that the 914-bp promoter in the transgene could be highly methylated, as is the case for other transgenes (Herbst et al., 2012; Mehta et al., 2009; Pedram et al., 2006). Indeed, we detected a high level of DNA methylation in one line whose transgene was silenced (Fig. 3). This suggests that the transgene is inactivated by DNA methylation in the four lines. Generally, the DNA methylation status of a promoter affects its transcription activity (Illingworth and Bird, 2009), and we showed that the active POP promoter is hypomethylated (Matsubara et al., 2010). However, because most tissues expressed the POP gene, it was unclear whether DNA methylation of the promoter was correlated to gene silencing. In the current study, for the first time, we showed that the inactive POP promoter was highly methylated. This highlights the importance of the epigenetic regulation in controlling the POP gene.

In the #542 and #675 lines, whose transgenes were probably integrated in active chromatin regions, the tissue distribution of EGFP was different from that of POP (Fig. 1). While the endogenous POP was expressed at higher levels in the ovary and placenta, the EGFP expression levels in several transgenic tissues were comparable to or even higher than those in the ovary and placenta. This provided two possibilities: one was that the POP promoter might become aberrantly active in several transgenic tissues and the other was that the promoter activity might decrease in the ovary and placenta of the transgenic mice. To see which hypothesis is correct, we compared the expression level of EGFP with that of POP in the #542 and #675 placentas. The result demonstrated that the placental EGFP level was similar to the endogenous POP in both lines (Fig. 5B), which supported the first possibility that the activity of the POP promoter became stronger in several transgenic tissues. Higher signal intensities of EGFP than POP in many tissues by the Northern analysis were consistent with this hypothesis (Fig. 1). In an active chromatin region, many *cis*- and *trans*-elements are likely to perform their functions for gene activation, and they could be effective on an integrated transgene. This could result in the aberrant enhancement of the EGFP expression. In contrast, the transgene was probably not much affected by the surrounding sequences in the placenta, which could result in the appropriate level of EGFP expression. These suggest that several elements other than the promoter are involved in the POP gene regulation in many tissues.

In the ovary, POP mRNA is specifically expressed in the granulosa cell (Matsubara et al., 2010), but our qRT-PCR analysis with the whole ovary and the isolated granulosa cell strongly suggested that the EGFP signal was not only localized to the granulosa cell but also to other ovarian tissues in the #542 and #675 transgenic lines (Fig. 4B). This means that more elements are required to enhance the POP transcription in the granulosa cell and/or to suppress the POP gene transcription in other ovarian tissues. Consistent with this, we identified a CpG island in the POP gene body as an enhancer candidate for its expression in the ovary (Matsubara et al.,

2010). However, we have also shown in the same study that the CpG island appeared to be insufficient to fully activate the POP transcription in the granulosa cell (Matsubara et al., 2010). Thus, the regulatory mechanism of the POP gene activation in the ovarian granulosa cell is probably complicated, and identification and characterization of other regulatory elements will be necessary.

In the placenta, POP showed the interesting localization change at E12.5 (Matsubara et al., 2011), which was largely reproduced in the #542 and #675 lines (Fig. 5A). However, the following three points were different between the endogenous POP and the exogenous EGFP. First, the EGFP signal was observed in the junctional and labyrinthine zones of the E12.5 placenta, while POP mRNA was localized in the junctional zone alone at E12.5 (Matsubara et al., 2011). This could be because the translated EGFP protein was retained in the labyrinth at E12.5 when its transcript had been already degraded. Second, the EGFP fluorescence of the maternal decidua seemed to be as strong as that of the junctional zone in the transgenic mice at E17.5 and E18.5 (Fig. 5A), whereas the endogenous POP was expressed at a higher level in the junctional zone (Matsubara et al., 2011). This suggested that the POP promoter in the transgene was more active in the maternal decidua than the endogenous promoter at E17.5-E18.5. Third, EGFP was also expressed at a higher level around the region connected to the umbilical cord than POP at E17.5 and E18.5 (Fig. 5A, arrowheads). In our previous *in situ* hybridization analyses, the POP signal was detected in similar regions of some sections, but its intensity was not as strong as that in the junctional zone (Matsubara et al., 2010, 2011). Presumably, the transgene was ectopically activated by surrounding *cis*-elements in the maternal decidua and in the region connected to the umbilical cord, which might be a cause of significant increases of the EGFP mRNA levels in the E17.5 and E18.5 placentas (Fig. 5B). Nevertheless, the current results strongly suggest that the 914-bp POP promoter contains most elements to control the localization change of the placental POP.

To identify the transcription factor involved in the placental POP activation, we focused on AP-2 γ because this protein has a critical role in the placenta (Auman et al., 2002; Werling et al., 2002) and the POP promoter contains a consensus sequence for its binding (Fig. 6A). As expected, our ChIP data demonstrated that AP-2 γ did bind to the promoter (Fig. 6B). Although we used whole placentas for this analysis, AP-2 γ probably bound to the POP promoter in the cells that expressed POP, because the two proteins were expected to be co-localized in the placenta (Matsubara et al., 2011; Sapin et al., 2000; Shi and Kellems, 1998). This suggests that AP-2 γ may play some role in the POP gene activation. However, the mechanism does not seem to be simple because we observed a decrease of the POP promoter activity by overexpression of AP-2 γ in non-placental cells in our preliminary analysis (data not shown). AP-2 γ may activate the POP promoter by collaborating with other transcription factors in the placenta, and indeed, many co-factors of AP-2 have been reported (Eckert et al., 2005; Tan et al., 2011). Alternatively, the POP gene might be activated by different transcription factors. Further studies will be required to reveal how the POP gene is activated in the placenta, but our current results indicate that the promoter plays a main role in controlling the placental POP localization.

5. Conclusions

In the present study, we have assessed the *in vivo* activity of the POP promoter for the first time. The transgene was silenced in the four lines due to a high level of DNA methylation, which indicated that the promoter was insufficient to overcome position effect. However, in the other two lines, the reporter EGFP gene was expressed in all the tissues examined, and its localization in the placenta showed a similar change to POP. A transcription factor, AP-2 γ , might be involved in the placental POP activation. These suggest that the promoter contains sufficient

activity to drive the appropriate POP localization in the placenta.

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References

- Auman, H.J., Nottoli, T., Lakiza, O., Winger, Q., Donaldson, S., Williams, T., 2002. Transcription factor AP-2gamma is essential in the extra-embryonic lineages for early postimplantation development. *Development* 129, 2733-2747.
- Bagheri-Fam, S., Barrionuevo, F., Dohrmann, U., Günther, T., Schule, R., Kemler, R., Mallo, M., Kanzler, B., Scherer, G., 2006. Long-range upstream and downstream enhancers control distinct subsets of the complex spatiotemporal Sox9 expression pattern. *Dev. Biol.* 291, 382-397.
- Boyer, O., Zhao, J.C., Cohen, J.L., Depetris, D., Yagello, M., Lejeune, L., Bruel, S., Mattéi, M.G., Klatzmann, D., 1997. Position-dependent variegation of a CD4 minigene with targeted expression to mature CD4+ T cells. *J. Immunol.* 159, 3383-3390.
- Brandt, W., Khandekar, M., Suzuki, N., Yamamoto, M., Lim, K.C., Engel, J.D., 2008. Defining

- the functional boundaries of the Gata2 locus by rescue with a linked bacterial artificial chromosome transgene. *J. Biol. Chem.* 283, 8976-8983.
- Breen, G., Harwood, A.J., Gregory, K., Sinclair, M., Collier, D., St Clair, D., Williams, R.S., 2004. Two peptidase activities decrease in treated bipolar disorder not schizophrenic patients. *Bipolar Disord.* 6, 156-161.
- Bulger, M., Groudine, M., 2011. Functional and mechanistic diversity of distal transcription enhancers. *Cell* 144, 327-339.
- Eckert, D., Buhl, S., Weber, S., Jäger, R., Schorle, H., 2005. The AP-2 family of transcription factors. *Genome Biol.* 6, 246.
- Frazar, T.F., Weisbein, J.L., Anderson, S.M., Cline, A.P., Garrett, L.J., Felsenfeld, G., Gallagher, P.G., Bodine, D.M., 2003. Variegated expression from the murine band 3 (AE1) promoter in transgenic mice is associated with mRNA transcript initiation at upstream start sites and can be suppressed by the addition of the chicken beta-globin 5' HS4 insulator element. *Mol. Cell. Biol.* 23, 4753-4763.
- Frericks, M., Esser, C., 2008. A toolbox of novel murine house-keeping genes identified by meta-analysis of large scale gene expression profiles. *Biochim. Biophys. Acta* 1779, 830-837.
- García-Horsman, J.A., Männistö, P.T., Venäläinen, J.I., 2007. On the role of prolyl oligopeptidase in health and disease. *Neuropeptides* 41, 1-24.
- Gaszner, M., Felsenfeld, G., 2006. Insulators: exploiting transcriptional and epigenetic mechanisms. *Nat. Rev. Genet.* 7, 703-713.
- Goossens, F., De Meester, I., Vanhoof, G., Scharpé, S., 1996. Distribution of prolyl oligopeptidase in human peripheral tissues and body fluids. *Eur. J. Clin. Chem. Clin. Biochem.* 34, 17-22.
- Goossens, F., Vanhoof, G., De Meester, I., Augustyns, K., Borloo, M., Tourwe, D., Haemers, A.,

- Scharpé, S., 1997. Development and evaluation of peptide-based prolyl oligopeptidase inhibitors--introduction of N-benzyloxycarbonyl-prolyl-3-fluoropyrrolidine as a lead in inhibitor design. *Eur. J. Biochem.* 250, 177-183.
- Grosveld, F., van Assendelft, G.B., Greaves, D.R., Kollias, G., 1987. Position-independent, high-level expression of the human beta-globin gene in transgenic mice. *Cell* 51, 975-985.
- Hannula, M.J., Männistö, P.T., Myöhänen, T.T., 2011. Sequential expression, activity and nuclear localization of prolyl oligopeptidase protein in the developing rat brain. *Dev. Neurosci.* 33, 38-47.
- Harraghy, N., Regamey, A., Girod, P.A., Mermoud, N., 2011. Identification of a potent MAR element from the mouse genome and assessment of its activity in stable and transient transfections. *J. Biotechnol.* 154, 11-20.
- Heckert, L.L., Sawadogo, M., Daggett, M.A., Chen, J.K., 2000. The USF proteins regulate transcription of the follicle-stimulating hormone receptor but are insufficient for cell-specific expression. *Mol. Endocrinol.* 14, 1836-1848.
- Herbst, F., Ball, C.R., Tuorto, F., Nowrouzi, A., Wang, W., Zavidij, O., Dieter, S.M., Fessler, S., van der Hoeven, F., Kloz, U., Lyko, F., Schmidt, M., von Kalle, C., Glimm, H., 2012. Extensive methylation of promoter sequences silences lentiviral transgene expression during stem cell differentiation *in vivo*. *Mol. Ther.* 20, 1014-1021.
- Higgs, D.R., 1998. Do LCRs open chromatin domains? *Cell* 95, 299-302.
- Illingworth, R.S., Bird, A.P., 2009. CpG islands--'a rough guide'. *FEBS Lett.* 583, 1713-1720.
- Jeong, Y., El-Jaick, K., Roessler, E., Muenke, M., Epstein, D.J., 2006. A functional screen for sonic hedgehog regulatory elements across a 1 Mb interval identifies long-range ventral forebrain enhancers. *Development* 133, 761-772.
- Jones, B.K., Monks, B.R., Liebhaber, S.A., Cooke, N.E., 1995. The human growth hormone gene is regulated by a multicomponent locus control region. *Mol. Cell. Biol.* 15, 7010-7021.

- Kamat, A., Graves, K.H., Smith, M.E., Richardson, J.A., Mendelson, C.R., 1999. A 500-bp region, approximately 40 kb upstream of the human CYP19 (aromatase) gene, mediates placenta-specific expression in transgenic mice. *Proc. Natl. Acad. Sci. U. S. A.* 96, 4575-4580.
- Kellum, R., Schedl, P., 1991. A position-effect assay for boundaries of higher order chromosomal domains. *Cell* 64, 941-950.
- Kim, T.H., Abdullaev, Z.K., Smith, A.D., Ching, K.A., Loukinov, D.I., Green, R.D., Zhang, M.Q., Lobanenko, V.V., Ren, B., 2007. Analysis of the vertebrate insulator protein CTCF-binding sites in the human genome. *Cell* 128, 1231-1245.
- Kimura, A., Yoshida, I., Takagi, N., Takahashi, T., 1999. Structure and localization of the mouse prolyl oligopeptidase gene. *J. Biol. Chem.* 274, 24047-24053.
- Kimura, A., Takahashi, T., 2000. cDNA cloning of rat prolyl oligopeptidase and its expression in the ovary during the estrous cycle. *J. Exp. Zool.* 286, 656-665.
- Kimura, A., Matsui, H., Takahashi, T., 2002. Expression and localization of prolyl oligopeptidase in mouse testis and its possible involvement in sperm motility. *Zoolog. Sci.* 19, 93-102.
- Klüppel, M., Beermann, F., Ruppert, S., Schmid, E., Hummler, E., Schütz, G., 1991. The mouse tyrosinase promoter is sufficient for expression in melanocytes and in the pigmented epithelium of the retina. *Proc. Natl. Acad. Sci. U. S. A.* 88, 3777-3781.
- Li, Q., Peterson, K.R., Fang, X., Stamatoyannopoulos, G., 2002a. Locus control regions. *Blood* 100, 3077-3086.
- Li, Q., Zhang, M., Han, H., Rohde, A., Stamatoyannopoulos, G., 2002b. Evidence that DNase I hypersensitive site 5 of the human beta-globin locus control region functions as a chromosomal insulator in transgenic mice. *Nucleic Acids Res.* 30, 2484-2491.
- Mantle, D., Falkous, G., Ishiura, S., Blanchard, P.J., Perry, E.K., 1996. Comparison of proline

- endopeptidase activity in brain tissue from normal cases and cases with Alzheimer's disease, Lewy body dementia, Parkinson's disease and Huntington's disease. Clin. Chim. Acta 249, 129-139.
- Matsubara, S., Takahashi, T., Kimura, A.P., 2010. Epigenetic patterns at the mouse prolyl oligopeptidase gene locus suggest the CpG island in the gene body to be a novel regulator for gene expression. Gene 465, 17-29.
- Matsubara, S., Takahashi, T., Kimura, A.P., 2011. Localization and subcellular distribution of prolyl oligopeptidase in the mouse placenta. J. Mol. Histol. 42, 251-264.
- Mehta, A.K., Majumdar, S.S., Alam, P., Gulati, N., Brahmachari, V., 2009. Epigenetic regulation of cytomegalovirus major immediate-early promoter activity in transgenic mice. Gene 428, 20-24.
- Mentlein, R., 1988. Proline residues in the maturation and degradation of peptide hormones and neuropeptides. FEBS Lett. 234, 251-256.
- Miura, N., Shibata, S., Watanabe, S., 1997. Z-321, a prolyl endopeptidase inhibitor, augments the potentiation of synaptic transmission in rat hippocampal slices. Behav. Brain. Res. 83, 213-216.
- Myöhänen, T.T., García-Horsman, J.A., Tenorio-Laranga, J., Männistö, P.T., 2009. Issues about the physiological functions of prolyl oligopeptidase based on its discordant spatial association with substrates and inconsistencies among mRNA, protein levels, and enzymatic activity. J. Histochem. Cytochem. 57, 831-848.
- Nobrega, M.A., Ovcharenko, I., Afzal, V., Rubin, E.M., 2003. Scanning human gene deserts for long-range enhancers. Science 302, 413.
- Ohtsuki, S., Homma, K., Kurata, S., Komano, H., Natori, S., 1994. A prolyl endopeptidase of *Sarcophaga peregrina* (flesh fly): its purification and suggestion for its participation in the differentiation of the imaginal discs. J. Biochem. 115, 449-453.

- Pedram, M., Sprung, C.N., Gao, Q., Lo, A.W., Reynolds, G.E., Murnane, J.P., 2006. Telomere position effect and silencing of transgenes near telomeres in the mouse. *Mol. Cell. Biol.* 26, 1865-1878.
- Perroud, B., Alvarado, R.J., Espinal, G.M., Morado, A.R., Phinney, B.S., Warden, C.H., 2009. *In vivo* multiplex quantitative analysis of 3 forms of alpha melanocyte stimulating hormone in pituitary of prollyl endopeptidase deficient mice. *Mol. Brain* 2, 14.
- Rao, M.K., Wayne, C.M., Meistrich, M.L., Wilkinson, M.F., 2003. Pem homeobox gene promoter sequences that direct transcription in a Sertoli cell-specific, stage-specific, and androgen-dependent manner in the testis *in vivo*. *Mol. Endocrinol.* 17, 223-233.
- Reddi, P.P., Urekar, C.J., Abhyankar, M.M., Ranpura, S.A., 2007. Role of an insulator in testis-specific gene transcription. *Ann. N. Y. Acad. Sci.* 1120, 95-103.
- Rennex, D., Hemmings, B.A., Hofsteenge, J., Stone, S.R., 1991. cDNA cloning of porcine brain prollyl endopeptidase and identification of the active-site seryl residue. *Biochemistry* 30, 2195-2203.
- Salers, P., 1994. Evidence for the presence of prollyl oligopeptidase and its endogenous inhibitor in neonatal rat pancreatic beta-cells. *Regul. Pept.* 50, 235-245.
- Sapin, V., Bouillet, P., Oulad-Abdelghani, M., Dastugue, B., Chambon, P., Dollé, P., 2000. Differential expression of retinoic acid-inducible (Stra) genes during mouse placentation. *Mech. Dev.* 92, 295-299.
- Shewchuk, B.M., Liebhaber, S.A., Cooke, N.E., 2002. Specification of unique Pit-1 activity in the hGH locus control region. *Proc. Natl. Acad. Sci. U. S. A.* 99, 11784-11789.
- Shi, D., Kellems, R.E., 1998. Transcription factor AP-2gamma regulates murine adenosine deaminase gene expression during placental development. *J. Biol. Chem.* 273, 27331-27338.
- Shima, Y., Zubair, M., Ishihara, S., Shinohara, Y., Oka, S., Kimura, S., Okamoto, S., Minokoshi, Y., Suita, S., Morohashi, K., 2005. Ventromedial hypothalamic nucleus-specific enhancer of

- Ad4BP/SF-1 gene. *Mol. Endocrinol.* 19, 2812-2823.
- Shima, Y., Zubair, M., Komatsu, T., Oka, S., Yokoyama, C., Tachibana, T., Hjalt, T.A., Drouin, J., Morohashi, K., 2008. Pituitary homeobox 2 regulates adrenol4 binding protein/steroidogenic factor-1 gene transcription in the pituitary gonadotrope through interaction with the intronic enhancer. *Mol. Endocrinol.* 22, 1633-1646.
- Shirasawa, Y., Osawa, T., Hirashima, A., 1994. Molecular cloning and characterization of prolyl endopeptidase from human T cells. *J. Biochem.* 115, 724-729.
- Shishido, Y., Furushiro, M., Tanabe, S., Taniguchi, A., Hashimoto, S., Yokokura, T., Shibata, S., Yamamoto, T., Watanabe, S., 1998. Effect of ZTTA, a prolyl endopeptidase inhibitor, on memory impairment in a passive avoidance test of rats with basal forebrain lesions. *Pharm. Res.* 15, 1907-1910.
- Szeltner, Z., Polgár, L., 2008. Structure, function and biological relevance of prolyl oligopeptidase. *Curr. Protein. Pept. Sci.* 9, 96-107.
- Tan, S.K., Lin, Z.H., Chang, C.W., Varang, V., Chng, K.R., Pan, Y.F., Young, E.L., Sung, W.K., Cheung, E., 2011. AP-2 γ regulates oestrogen receptor-mediated long-range chromatin interaction and gene transcription. *EMBO J.* 30, 2569-2581.
- Toide, K., Okamiya, K., Iwamoto, Y., Kato, T., 1995. Effect of a novel prolyl endopeptidase inhibitor, JTP-4819, on prolyl endopeptidase activity and substance P- and arginine-vasopressin-like immunoreactivity in the brains of aged rats. *J. Neurochem.* 65, 234-240.
- Warden, C.H., Fisler, J.S., Espinal, G., Graham, J., Havel, P.J., Perroud, B., 2009. Maternal influence of prolyl endopeptidase on fat mass of adult progeny. *Int. J. Obes.* 33, 1013-1022.
- Werling, U., Schorle, H., 2002. Transcription factor gene AP-2 γ essential for early murine development. *Mol. Cell. Biol.* 22, 3149-3156.
- Wilk, S., 1983. Prolyl endopeptidase. *Life. Sci.* 33, 2149-2157.

- Williams, R.S., Eames, M., Ryves, W.J., Viggars, J., Harwood, A.J., 1999. Loss of a prolyl oligopeptidase confers resistance to lithium by elevation of inositol (1,4,5) trisphosphate. *EMBO J.* 18, 2734-2745.
- Williams, R.S.B., 2004. Prolyl oligopeptidase and bipolar disorder. *Clin. Neurosci. Res.* 4, 233-242.
- Yamakawa, N., Shimeno, H., Soeda, S., Nagamatsu, A., 1994. Regulation of prolyl oligopeptidase activity in regenerating rat liver. *Biochim. Biophys. Acta* 1199, 279-284.
- Yang, J., Corces, V.G., 2011. Chromatin insulators: a role in nuclear organization and gene expression. *Adv. Cancer Res.* 110, 43-76.
- Yoo, E.J., Cajiao, I., Kim, J.S., Kimura, A.P., Zhang, A., Cooke, N.E., Liebhaber, S.A., 2006. Tissue-specific chromatin modifications at a multigene locus generate asymmetric transcriptional interactions. *Mol. Cell. Biol.* 26, 5569-5579.
- Yoshida, K., Inaba, K., Ohtake, H., Morisawa, M., 1999. Purification and characterization of prolyl endopeptidase from the Pacific herring, *Clupea pallasii*, and its role in the activation of sperm motility. *Dev. Growth Differ.* 41, 217-225.
- Yoshimoto, T., Tsukumo, K., Takatsuka, N., Tsuru, D., 1982. An inhibitor for post-proline cleaving enzyme; distribution and partial purification from porcine pancreas. *J. Pharmacobiodyn.* 5, 734-740.
- Yoshimoto, T., Kado, K., Matsubara, F., Koriyama, N., Kaneto, H., Tsuru, D., 1987. Specific inhibitors for prolyl endopeptidase and their anti-amnesic effect. *J. Pharmacobiodyn.* 10, 730-735.

Figure Legends

Fig. 1. The EGFP mRNA expression in transgenic tissues. Total RNA was prepared from the indicated seventeen tissues of two transgenic mouse lines, #542 (top) and #675 (bottom), and 20 µg of the RNA were electrophoresed and blotted to a nylon membrane. The blot was hybridized with ³²P-labeled cDNA probes for EGFP and POP, and the signals were detected by autoradiography. The electrophoretic image of 28S and 18S ribosomal RNAs showed that a similar amount of total RNA was applied to each lane. In both lines, EGFP mRNA was expressed in all the tissues, but its expression pattern was different from that of POP.

Fig. 2. The EGFP fluorescence in transgenic tissues. Indicated thirteen tissues were collected from wild type and transgenic mice (#542 and #675) and observed with a fluorescence microscope. The tissues from #542 are shown in upper panels and those from #675 in lower panels. Bright (top) and dark images (bottom) are indicated for each tissue. In all images, the tissues from wild type (WT) and transgenic mice (TG) were observed together. Placentas were obtained by sacrificing pregnant mice at indicated embryonic days. The EGFP fluorescence was detected in all the tissues examined.

Fig. 3. DNA methylation patterns of the POP promoter in the transgenic placentas. Structure of the transgene is drawn at the top, and the amplified region and the primer positions are depicted with a grey bar and arrows. Middle and lower drawings indicate a detailed map and the methylation status of the proximal promoter region. The positions of the CpG dinucleotides are shown with vertical lines. The proximal POP promoter includes 38 CpG dinucleotides and all of which were analyzed. The methylation status of individual CpGs was determined by bisulfite sequencing. Genomic DNA was purified from two sets of transgenic placentas (#528 and #675) and treated with sodium bisulfite. A part of the transgene (grey bar) was amplified by PCR, and six subclones of the amplified product for each placenta were analyzed by DNA sequencing.

Note that we specifically amplified the transgene because the antisense primer was positioned within the EGFP gene (arrow). Open and filled circles represent unmethylated and methylated cytosines, respectively. The overall methylation rate for each tissue is indicated at the left.

Fig. 4. The EGFP localization and expression in the transgenic ovary. (A) The EGFP fluorescence in cultured primary granulosa cells. The granulosa cells were collected from superovulated wild-type or transgenic mice (#542 and #675), cultured for 3 days, and observed with the fluorescence microscope. The wild type granulosa cell was used as a negative control, and the Hepa1-6 cell transfected with the EGFP gene driven by a cytomegalovirus promoter was as a positive control. Images of bright and dark fields are shown at the top and bottom, respectively. Exposure time for granulosa cells was 5 sec while Hepa1-6 cells were exposed for 1 sec. Weak EGFP signals were observed in the transgenic granulosa cells. The scale bar represents 100 μ m. (B) Comparison of POP and EGFP mRNA levels in the whole ovary and the isolated granulosa cell. Total RNA was prepared from the whole ovary and the granulosa cell of transgenic mice (#542 and #675) and treated with TURBO DNase. cDNA was generated by reverse transcription with 500 ng of the RNA, and the expression of POP and EGFP mRNA was analyzed by real time PCR using the ABI Prism 7300 real-time PCR system. The mRNA levels were normalized to *Aip* and relative expression is shown as -fold difference calculated relative to the POP expression in the whole ovary, which was arbitrarily set at 1. The data are presented as mean $\pm$ SD. $n=3-4$. For both lines, the POP mRNA level was significantly higher in the granulosa cell than in the whole ovary, while the EGFP level was not. * $P<0.05$. ** $P<0.01$.

Fig. 5. The EGFP localization and expression in transgenic placentas. (A) Wild type (right) and transgenic placentas (left) from the #542 and #675 lines at indicated stages were harvested from pregnant mice and 3-5 mm cross-sections were prepared. The EGFP fluorescence was observed

with a fluorescence microscope. Bright and dark fields are shown for each tissue. Three layers of transgenic placentas in the bright images were outlined by dashed line. In both lines, the EGFP fluorescence was mainly localized to the junctional and labyrinthine zones at E10.5-12.5 and to the junctional zone and the maternal decidua at E17.5-E18.5. Arrowheads indicate the EGFP signal around the region connected to the umbilical cord. The scale bar represents 1 mm. Md, maternal decidua; Jz, junctional zone; La, labyrinth (B) Comparison of the POP and EGFP mRNA levels in the transgenic placenta. The expression levels of POP and EGFP mRNA were analyzed as in Fig.3B, and the mRNA levels were normalized to *Aip* and to copy number. The EGFP mRNA level was comparable to POP in both lines, although it was significantly higher than POP at E17.5 and E18.5. The data are presented as mean $\pm$ SD and analyzed by Student's *t* test. *n*=4-6. **P*<0.05, ***P*<0.01 compared to POP.

Fig. 6. A transcription factor, AP-2 γ , binds to the mouse POP gene promoter in the placenta. (A) A genomic structure of the mouse POP gene locus is illustrated. Exons are indicated by vertical lines or open boxes with the exon number. Enlarged structure of the region around exon 1 and a position of the 914-bp promoter (grey bar) are shown below. Candidate AP-2 and AP-2 γ binding sites were searched with TRANSFAC software (<http://www.gene-regulation.com/pub/databases.html>) and indicated by open and filled ovals, respectively. Positions of the primers to amplify the promoter for ChIP are shown by black arrows. Note that the antisense primer was outside of the 914-bp promoter which resulted in the specific amplification of the endogenous POP locus but not the transgene. (B) The AP-2 γ binds to the POP promoter. ChIP was conducted with chromatin isolated from the E13.5 transgenic placenta (#675). The sheared chromatin was immunoprecipitated with a polyclonal antibody against AP-2 γ or normal rabbit IgG, and DNA purified from the precipitated (bound) fraction was subjected to real time PCR amplification. The amplification efficiency was normalized by

calculating the ratio of the signal in the bound chromatin to that in the input fraction which was collected before immunoprecipitation. The value was further normalized by the bound to input ratio of normal rabbit IgG set as 1.0. ChIP was performed five times with two placentas. The graph was shown as the average $\pm$ SD. The white bar represents the AP-2 γ binding levels in the placenta and the black bar indicates the background level. Data were analyzed using two-way ANOVA and Tukey's *post hoc* test. Statistically significant AP-2 γ binding at the promoter (compared with rabbit IgG at the promoter or with the AP-2 γ signal at other regions) was observed ($n=5$; $**P<0.01$).

Table 1

Transgene expression and copy number of each line

line	EGFP expression	EGFP/ β -actin/copy*	copy number
#528	-	2.96 ± 0.80	3
#531	-	2.60 ± 0.56	3
#542	+	0.87 ± 0.30	1
#566	-	1.37 ± 0.55	1
#675	+	1.16 ± 0.39	1
#676	-	1.38 ± 0.27	1

* This was determined by qPCR with genome DNA from transgenic mice. The data are presented as mean $\pm$ SD. $n = 4$.

Fig. 1

#542

#675

Fig. 2

Fig. 3

A

B

Fig. 4

A

B

Fig. 5

A

B

Fig. 6