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Author(s)	Nio-Kobayashi, Junko; Iwanaga, Toshihiko
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Galectin-1 and galectin-3 in the corpus luteum of mice are differentially regulated by prolactin and prostaglandin $F_{2\alpha}$

Junko Nio-Kobayashi and Toshihiko Iwanaga

Laboratory of Histology and Cytology, Hokkaido University Graduate School of Medicine, Kita 15-Nishi 7, Kita-ku, Sapporo 060-8638, Japan

Correspondence should be addressed to J Nio-Kobayashi;
Laboratory of Histology and Cytology, Hokkaido University Graduate School of Medicine, Kita 15-Nishi 7, Kita-ku, Sapporo 060-8638, Japan. Tel. & Fax: +81-11-7067151 E-mail: niojun@med.hokudai.ac.jp

Short title: Regulation of galectin in the CL of mice

Long summary statement: The differential effect of prolactin (PRL) and prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) on the expression of galectin-1 and galectin-3 in the mouse corpus luteum was analyzed using pregnant, postpartum, and non-pregnant cyclic mice. Compulsorily weaning in postpartum mice and Bromocriptine administration to non-pregnant mice revealed a suppressive effect of PRL on the expression of galectin-1 but not that of galectin-3. $PGF_{2\alpha}$ is an excellent candidate which regulates galectin expression but its effect may be abolished by LH-R-mediated signal.

Abstract

Galectin-1 and galectin-3, *β*-galactoside-binding lectins, are specifically expressed in the regressing corpus luteum (CL) of mice, however, their function remains unclear. In this study, we examined the effects of prolactin (PRL) and prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), two main regulatory molecules of mouse CL function, on galectin expression. *In situ* hybridization analysis clearly demonstrated an initial increase of galectin-1 in the CL newly formed (CLN) after postpartum ovulation 48 h after compulsory weaning. This was accompanied by a decline in 3β-hydroxysteroid dehydrogenase (3β-HSD) and luteinizing hormone receptor (LH-R) expression, suggesting a withdrawal of PRL stimulation. At 72 h after the weaning, the expression of both galectins in CLN was remarkably increased, being associated with an intense expression of progesterone degradation enzyme (20α-HSD). Compulsory weaning did not significantly alter both galectin expression in the remaining CL of pregnancy (CLP), while $PGF_{2\alpha}$ strongly up-regulated both galectin expression only in the remaining CLP which lacked LH-R in postpartum mice. Administration of Bromocriptine, an antagonist for PRL secretion, to non-pregnant cyclic mice induced an accumulation of galectin-1 —but not galectin-3— in all CL of various generations, and additional PRL treatment reduced its accumulation, suggesting a direct suppressive effect of PRL on galectin-1 expression. Although the function and regulatory mechanism of galectin in the CL is not fully understood, $PGF_{2\alpha}$ is an excellent candidate which regulates galectin expression but its effect may be abolished by LH-R-mediated signal. PRL withdrawal seems to be necessary for an initiation of luteolysis and the following $PGF_{2\alpha}$-induced galectin expression.

Introduction

Galectin is a *β*-galactoside-binding animal lectin involved in various physiological and pathological events regulating cell proliferation, differentiation, migration, and apoptosis. Fifteen members of galectin family have been identified in mammalian tissues, and the predominant subtypes expressed in the mouse ovary are galectin-1 and galectin-3 (Nio and Iwanaga 2007). Recently, we demonstrated that both galectin subtypes in the corpus luteum (CL) of mice showed a stage- and cell-specific expression pattern during the estrous cycle, and their expression was especially intense in the regressing CL, which contains a progesterone degradation enzyme —20α-hydroxysteroid dehydrogenase (20α-HSD)— and abundant numbers of apoptotic cells (Nio-Kobayashi and Iwanaga 2010). Although the characteristic expression of galectins in the regressing CL confirms their contribution to luteolysis, the function and regulation of galectin in the ovary remains unclear.

In rodents, luteolysis is characterized by an initial decline in progesterone secretion that is commonly designated as *functional luteolysis*, which is distinct from *structural luteolysis* that signifies the subsequent structural change with its gradual involution to form a small scar composed of connective tissue (Bachelot and Binart 2005). Functional luteolysis is accompanied by the expression of 20α-HSD, whereas the structural luteolysis is characterized by apoptosis of luteal cells. CL function in rodents is regulated by two main factors: prolactin (PRL) from the pituitary gland and prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) from the uterus. PRL is a main luteotrophic hormone, and it induces gene expression essential for progesterone production, such as 3β-hydroxysteroid dehydrogenase (3β-HSD) and luteinizing hormone receptor (LH-R). PRL also possesses a luteolytic role when the CL cease to produce progesterone by

expressing 20α-HSD (Stocco et al. 2007). It is currently believed that the pre-ovulatory surge of PRL on the evening of proestrus causes apoptosis of luteal cells, and thus structural luteolysis, in the CL during estrous cycles of non-pregnant mice (Gaytán et al. 2001). On the other hand, $PGF_{2\alpha}$ plays a crucial role in the functional luteolysis by inducing 20α-HSD expression.

Galectin modulates transductions of various signals by recognizing *β*-galactoside of glycoconjugates on cell surface and extracellular matrix to regulate cell viability and motility. Because various factors regulating angiogenesis, immune cell function, and cancer progression and metastasis are involved in luteolysis, elucidating the role and regulation of galectin in luteolysis should provide the valuable information regarding the fundamental role of galectin and its carbohydrate ligands in mammals. Our recent report demonstrated a biphasic expression of galectin-1 in the mouse CL during the estrous cycle (Nio-Kobayashi and Iwanaga 2010). The first and temporal increase in galectin-1 expression occurred on the second day after ovulation preceding the expression of 20α-HSD, namely functional luteolysis. The mRNA expression of galectin-1 was again increased in the structurally regressing CL, which also intensely expressed galectin-3 and 20α-HSD and contained abundant numbers of apoptotic luteal cells. The dramatic change in the galectin expression during CL formation and regression suggests that ovarian galectin-1 and galectin-3 may be regulated by PRL and/or $PGF_{2\alpha}$ and differentially contribute to the control of luteolysis. The present study aimed to elucidate whether or not PRL and/or $PGF_{2\alpha}$ regulate the expression of galectin in the CL of mice by analyzing the effect of following treatments: 1) Compulsory weaning of postpartum mice to reduce plasma PRL concentration as serum PRL level is elevated by suckling in lactating animals after parturition (Taya et al. 1982).

2) $PGF_{2\alpha}$ administration to pregnant and postpartum mice. 3) Withdrawal of progesterone in pregnant mice by administration of a progesterone receptor antagonist (Mifepristone/RU486) to evaluate an indirect regulation of galectin expression by luteal progesterone which is altered by PRL and/or $PGF_{2\alpha}$. 4) Bromocriptine administration to suppress the secretion of PRL from the pituitary gland in non-pregnant cyclic mice to confirm the effect of PRL on galectin expression.

Results

Effect of compulsorily weaning on galectin expression in the ovary of postpartum mice

Since postpartum ovulation occurs within 24 h after parturition, newly formed CL (CLN) and the remaining CL of pregnancy (CLP) are intermingled in the postpartum ovary (Connor et al. 1980). Only the CLP expressed galectin-3 and 20α-HSD in lactating mice as we previously reported (Nio and Iwanaga 2007). To elucidate the effect of PRL on galectin expression in the CL, we examined changes in the expression of mRNA for galectins and related molecules in the ovaries of compulsorily weaned mice. No significant changes could be found in the ovary 24 h after the weaning in both CLN and CLP (Fig. 1A-E). Namely, galectin-3/20α-HSD-negative CLN (*arrows* in Fig. 1B, C) and galectin-3/20α-HSD-positive CLP (*asterisks* in Fig. 1B, C) intermingled as so do in the ovary of lactating mice. The expression of 3β-HSD was intense in all CL (Fig. 1D), whereas LH-R was expressed only in the CLN (*arrows* in Fig. 1E). The gene expression patterns indicated differential status of these CL: the CLN, corresponding to the CL of lactation which have been over-stimulated by PRL, are functional CL actively producing progesterone, while the remaining CLP is undergoing functional luteolysis. At 48 h after the weaning, a remarkable change in the gene expression was observed in the CLN. The expression of galectin-1 in the CLN increased in intensity associated with a significant expression of 20α-HSD, indicating the start of functional luteolysis (*arrows* in Fig. 1F, H). At this stage, the intensity of the expression of 3β-HSD and LH-R decreased in the CLN (*arrows* in Fig. 1I, J), suggesting a withdrawal of PRL stimulation, while 3β-HSD expression was still high in the CLP (*asterisks* in Fig 1I). Intense expression of both galectin-1 and galectin-3 in the CLN was observed at 72 h after the weaning associated with marked

expression of 20α-HSD and weak or no expression of 3β-HSD and LH-R (*arrows* in Fig. 1K-O). On the other hand, the expression of both galectins in the CLP was slightly reduced 48 h after the weaning (*asterisks* in Fig. 1F, G) and became intense at 72 h (*asterisks* in Fig. 1K, L), although the signals in the CLP were less intense than that in the CLN. The changes in the gene expression caused by compulsory weaning are summarized in Figure 1P and Q.

These observations indicate that an initial increase in galectin-1 expression in the CLN was accompanied by a decline in the expression of both 3β-HSD and LH-R, implying a withdrawal of PRL stimulation by compulsory weaning. The further increase in galectin-1 72 h after the weaning was associated with intense expressions of 20α-HSD and galectin-3. As the effect of compulsory weaning on galectin expression differed between in the CLN and CLP, and there was a time-lag in the expression of each galectin in the CLN of compulsorily weaned mice, the effect of PRL seems to differ with regards to galectin-1 and galectin-3 as well as to the status of the CL.

Effect of $PGF_{2\alpha}$ injection on galectin expression in the ovaries of pregnant and postpartum mice

It is well established that the synthesis of 20α-HSD in rodent CL is regulated by $PGF_{2\alpha}$ possibly released from the uterus. As galectin-3 and 20α-HSD are concomitantly expressed in the regressing CL (Nio and Iwanaga 2007), we hypothesized that galectin-3 would be also regulated by $PGF_{2\alpha}$. To investigate this, we examined the effect of $PGF_{2\alpha}$ treatment on galectin-3 expression in the late CL of pregnancy (pregnancy day 17.5), which lacks both galectins and 20α-HSD (Fig. 2A-C). Contrary to our expectations, $PGF_{2\alpha}$ administration did not induce galectin-3 expression in the

functional CLP of pregnant mice (Fig. 2B, F), although 20α-HSD expression was slightly increased by $PGF_{2\alpha}$ treatment in these CL (Fig. 2C, G). There were no significant effect of $PGF_{2\alpha}$ on the expression of both galectin-1 (Fig. 2A, E) and LH-R in the functional CLP (Fig. 2D, H). On the other hand, when $PGF_{2\alpha}$ was administrated to postpartum mice at day 3, the expression of galectin-3 remarkably increased only in the remaining CLP, which intensely expressed 20α-HSD but lacked LH-R (*asterisks* in Fig. 2J-L, N-P). Galectin-3 in the CLN with intense expression of LH-R was not affected by $PGF_{2\alpha}$ (*arrows* in Fig. 2J-L, N-P). Galectin-1 expression also increased in intensity in the remaining CLP by $PGF_{2\alpha}$ administration (*asterisks* in Fig. 2I, M) but again did not change in the CLN (*arrows* in Fig. 2I, M). These findings indicate that galectin-3, as well as galectin-1, is up-regulated by $PGF_{2\alpha}$ only when CL does not express LH-R.

Effect of Mifepristone (RU486) administration on galectin expression in the functional CL of pregnancy

To clarify whether progesterone could influence galectin expression we administrated a progesterone receptor antagonist to mice at day 17.5 of pregnancy. There were no significant change in the expression of either galectin, 3β-HSD, 20α-HSD, or LH-R 12 h after Mifepristone treatment (Fig. 3, control were shown in Fig. 2A-D), suggesting that PRL and/or $PGF_{2\alpha}$ may not indirectly regulate galectin expression via alternations in progesterone production.

Effect of Bromocriptine administration on the expression of galectins in the ovary of non-pregnant cyclic mice

As the increased expression of galectin-1 mRNA in weaned mice was accompanied by a reduction of 3β-HSD and LH-R, which are known to be up-regulated by PRL, it is anticipated that a decrease in a PRL signal would induce the expression of galectin-1 in the CL. We therefore examined the effect of continuous administration of Bromocriptine, which suppresses the secretion of PRL from the pituitary gland, and the effect of an additional PRL treatment on luteal galectin expression. Because PRL is known to induce structural luteolysis when the CL cease progesterone production, we first expected that Bromocriptine treatment must prevent the CL from undergoing structural luteolysis. However, it does not influence on the rate of ovulation. Thus, the ovaries of the Bromocriptine-treated mice contained several generations of cyclic CL: the functional CL formed at the latest estrous cycle, and old regressing CL under functional but not structural regressing stage formed during previous estrous cycles. The weight of the ovaries were significantly higher in the Bromocriptine-treated mice compared to both control (normal cyclic) and Bromocriptine plus PRL treated mice, corresponding to the previous report by Gaytán et al. (2001).

The continuous administration of Bromocriptine induced an accumulation of galectin-1 mRNA in all CL (Fig. 4A) while the loss of PRL signaling reduced the expressions of both 3β-HSD and LH-R (Fig. 4C, D). PRL administration to the Bromocriptine-treated mice resulted in a recovery of the expression of 3β-HSD —but not that of LH-R— and a simultaneous reduction in galectin-1 accumulation in the CL (Fig. 4E, G, H). There were no significant changes in the expression of galectin-3 in either Bromocriptine or Bromocriptine plus PRL-treated mice (Figure 4B, F). Immunohistochemical analysis revealed an accumulation of galectin-1 protein in both functional and old regressing CL in the ovaries of Bromocriptine-treated mice (Fig. 4I,

J), although the newly formed CL (*asterisks* in Fig. 4I–L) possessed less intense immunoreactions both for galectin-1 and galectin-3 than those in the old regressing CL. These findings suggest that galectin-1 is negatively regulated by PRL but galectin-3 expression is not affected by PRL.

Discussion

Although the regulation of luteolysis is complicated and its exact mechanism remains to be elucidated, PRL and $PGF_{2\alpha}$ are likely to be main regulatory molecules of the CL function in mice. A remarkable change in the expression of galectin during the estrous cycle associated with an intense expression in the regressing CL suggests an involvement of galectin in the PRL and $PGF_{2\alpha}$-regulated luteolysis. In this study, we found for the first time that $PGF_{2\alpha}$ was an excellent candidate for a molecular regulation of the expression both of galectin-1 and galectin-3 in the CL, although its effect was dependent on the status of the CL. When the CL intensely expressed LH-R, $PGF_{2\alpha}$ failed to induce the expression of either galectin. On the other hand, PRL seems to suppress the expression of galectin-1 but not that of galectin-3, suggesting differential regulatory mechanisms between galectin-1 and galectin-3 in the regressing CL.

As summarized in Fig. 5A, galectin-1 showed a biphasic expression pattern in the compulsorily weaned mice as previously reported by us in non-pregnant cyclic mice (Nio-Kobayashi and Iwanaga 2010). The first small increase in galectin-1 expression in the CLN of postpartum mice after compulsory weaning was accompanied by decreased expression of 3β-HSD and LH-R, which are known to be up-regulated by PRL. At this stage, the CLN started to weakly express 20α-HSD and it is likely due to the reduction in PRL stimulation as PRL suppresses luteal 20α-HSD expression (Stocco et al. 2001). Therefore, the first small increase in galectin-1 expression in the CLN of compulsorily weaned mice seems to be caused by a decline of PRL stimulation resulting from the absence of the suckling reflex. This is supported by the observation that Bromocriptine administration induced an accumulation of galectin-1 both in the functional and in the regressing CL of non-pregnant cyclic mice, and that this effect is

suppressed by a single injection of PRL. Although PRL can signal through multiple pathways it appears that the Jak 2 (Janus kinase 2)-Stat 5 (signal transducer and activator of transcription 5) pathway is the primary route by which PRL regulates gene transcription (Goffin et al. 2002). It has been reported that galectin-1 is significantly down-regulated in ERK 2 (extracellular signal-regulated kinase 2)-deficient mice where the Jak-Stat signaling pathway is up-regulated (Imamura et al. 2008). These findings support the conclusion that PRL may directly suppress the expression of galectin-1 both in the functional and the regressing CL, possibly through a Jak 2-Stat 5 signaling pathway.

The expression of galectin-1 in the compulsorily weaned mice increased again more intensely in the CLN 72 h after the weaning and this was associated with a strong expression of 20α-HSD (Fig. 5A), which is known to be up-regulated by $PGF_{2\alpha}$. As $PGF_{2\alpha}$ administration to the postpartum mice increased the expression of galectin-1 in the remaining CLP which lacked LH-R (Fig. 5B), this second intense increase in galectin-1 mRNA with the intense expression of 20α-HSD found in the CLN at 72 h after the weaning is likely to be caused by $PGF_{2\alpha}$ secreted from the uterus or the CL itself after a withdrawal of PRL stimulation.

Galectin-3 was also increased in the CLN of compulsorily weaned mice, however, its expression was observed later (at 72 h after the compulsory weaning) than an initial increase of galectin-1 in the CLN found at 48 h after the weaning (Fig. 5A). This suggests that unlike galectin-1 the PRL withdrawal may not directly influence on the galectin-3 expression in the CLN. This idea is supported by the finding that the expression of galectin-3 was unaffected by Bromocriptine and PRL injection to non-pregnant cyclic mice. On the other hand, an increase of galectin-3 in the CLN at

72 h after compulsorily weaning was accompanied with an intense expression of 20α-HSD, which is known to be induced by $PGF_{2\alpha}$. Because $PGF_{2\alpha}$ administration to postpartum mice induced the expression of galectin-3 in the remaining CLP, $PGF_{2\alpha}$ is likely an excellent candidate to control the expression of galectin-3 as well as galectin-1 (Fig. 5B). It is known that $PGF_{2\alpha}$ functions via activation of the calcium and phospholipid-dependent protein kinase, protein kinase C (PKC) in human luteal cells (Abayasekara et al. 1993). In addition, in macrophages, PKC activator (phorbol myristate acetate) administration directly increased galectin-3 expression (Kim et al. 2003). Thus, PKC pathway may be an important cascade to induce the expression of galectin-3 as well as galectin-1 in the CL.

Notably, the $PGF_{2\alpha}$-induced increase in galectin-3 as well as galectin-1 was only seen in the remaining CLP that lacked an expression of LH-R but not in the CLN with an intense expression of LH-R in the postpartum mice (Fig. 5B). This suggests that the stimulating effect of $PGF_{2\alpha}$, possibly via activation of PKC pathway, on the expression of galectins differs depending on the condition of the CL, particularly with regards to whether it is expressing LH-R or not. Similarly, $PGF_{2\alpha}$ administration to mice at pregnancy day 17.5 failed to increase the expression of either galectin-1 or galectin-3 in the functional CLP, which intensely expressed LH-R (Fig. 5B). Thus, the up-regulation of galectin-3 as well as galectin-1 by $PGF_{2\alpha}$ may be blocked by the signal mediated through LH-R in the functional CLP of the pregnant mice and in the CLN of lactating postpartum mice (Fig. 5B).

Since PRL and $PGF_{2\alpha}$ regulate luteal progesterone production through the regulation of the expression of 3β-HSD, LH-R, and 20α-HSD, it was considered that progesterone might be a candidate molecule involved in the regulation of galectin

expression. However, the administration of Mifepristone, a progesterone receptor antagonist, had no effect on the galectin expression in the functional CLP of pregnant mice, suggesting that progesterone is not a key molecule for the regulation of luteal galectin expression. Nevertheless, we cannot exclude the possibility that other molecules —which are produced from luteal steroidogenic cells or other cellular components of the CL under the control of PRL and/or $PGF_{2\alpha}$— affect the expression of galectin in a paracrine manner.

Although the function of galectin in the CL is still unclear, we previously demonstrated that luteal cells intensely expressing galectin-3 in the cytoplasm were resistant to apoptosis but gradually eliminated by a fragmentation of their cytoplasm (Nio-Kobayashi and Iwanaga 2010). In the compulsorily weaned mice of this study, the number of apoptotic cells were remarkably few in the remaining CLP which intensely expressed galectin-3 and 20α-HSD, whereas abundant apoptotic cells were observed in the CLN 96-120 h after the weaning, suggesting anti-apoptotic effect of galectin-3 in the CLP (our unpublished data). Takiguchi et al. (2004) demonstrated that a differential regulation of apoptosis between the CLP and CLN of rats after parturition under non-lactating condition. Galectin-3 may be involved in the regulation of viability and apoptosis in luteal cells, however, further investigation is required to elucidate the exact role of galectin as well as its carbohydrate ligands in the CL.

In conclusion, the expression of galectin in the mouse CL is differentially mediated by PRL and $PGF_{2\alpha}$ (Fig. 5). The expression of both galectin-1 and galectin-3 is induced by $PGF_{2\alpha}$ but the effect may be abolished if the CL is intensely expressing LH-R. On the other hand, PRL directly inhibits the expression of galectin-1 but not

that of galectin-3. As the expression of LH-R is up-regulated by PRL, a withdrawal of PRL stimulation is considered to be necessary for the $PGF_{2\alpha}$-induced expression of galectins, especially for galectin-3. Although the mechanisms involved in the regulation of luteal galectin expression as well as its function are still not fully understood, the data presented here enhances the understanding the regulation of galectin not only in the CL but also potentially in other tissues under both physiological and pathological conditions.

Materials and methods

Animals and tissue sampling

Female ddY mice were purchased from Japan SLC (Shizuoka, Japan), and maintained under controlled light (12 hours light and 12 hours dark, lights-on at 8:30 a.m.) and temperature (20–22 °C) with free access to chow and tap water. All experiments using animals were approved by the local ethical committee of Hokkaido University (Approval no. 08-0054). At least three animals were used for each group and the similar results were obtained from all animals examined. The most representative data are shown in the results.

After natural labor at days 18.5 or 19.5 of pregnancy, mice were left to lactate for 3 days. On the morning of postpartum day 3 the pups were removed and the mothers were sacrificed 24, 48, and 72 h after the weaning by cardiac exanimation under deep anesthesia with pentobarbital, and fresh ovaries were obtained. Other mice were treated subcutaneously with $PGF_{2\alpha}$ (25 μg in ethanol; Biomol International. L. P., PA) or Mifepristone (RU486; 25 mg/kg in ethanol; Sigma Chemical Company, MO) at day 17.5 of gestation, or at postpartum day 3, and sacrificed 12 h later for tissue collection.

In the experiments of Bromocriptine treatment, 8 week old females were subcutaneously administrated 5 IU of serum gonadotropin (Serotropin®; Aska Pharmaceutical Co. Ltd., Tokyo, Japan) followed 48 h later by an injection of 5 IU of human chorionic gonadotrophin (hCG; Gonatropin®; Aska Pharmaceutical Co. Ltd.) to synchronize the ovarian cycle. Commencing the following day (day 1), Bromocriptine (Parlodel®, 5 mg/kg in 0.5% carboxymethylcellulose; Novartis Pharmaceutical Co. Ltd., Tokyo, Japan) was orally administrated twice a day for 10 days. On day 9, some mice were subcutaneously injected with sheep pituitary PRL (50 μg in physiological saline;

Sigma Chemical Company). All mice treated with Bromocriptine or Bromocriptin plus PRL were sacrificed on day 10 for tissue collection. The ovaries obtained were immediately embedded in O.C.T. compound (Sakura Fintechnical Co., Ltd., Tokyo, Japan), snap frozen in liquid nitrogen, and stored at –30°C until use. For immunohistochemical analysis, the ovaries from Bromocriptine- and Bromocriptine plus PRL-treated mice were fixed with 4% paraformaldehyde at 4°C overnight.

In situ hybridization

Two non-overlapping 45-mer antisense oligonucleotide probes for galectin-1 (*Lgals1*), galectin-3 (*Lgals3*), 3β-HSD (*Hsd3b1*), 20α-HSD (*Akr1c18*), and LH-R (*Lhcgr*) were synthesized as reported previously (Nio and Iwanaga 2010). All probes were labeled with ^{33}P-dATP using terminal deoxynucleotidyl transferase (Invitrogen, Carlsbad, CA). The procedure for *in situ* hybridization has been described previously (Nio et al. 2005). In brief, fresh frozen sections, 10 μm-thick, were fixed with 4% paraformaldehyde in 0.1 M phosphate buffer for 15 min and acetylated with 0.25% acetic anhydride in 0.1 M triethanolamine-HCl (pH 8.0) for 10 min. Hybridization was performed at 42°C for 12 h by adding 10,000 cpm/μL ^{33}P-labeled oligonucleotide probes. Then sections were rinsed at room temperature for 30 min in 2× SSC (1× SSC: 150 mM sodium chloride, 15 mM sodium citrate) containing 0.1% sarkosyl, immersed twice at 55°C for 40 min in 0.1× SSC containing 0.1% sarkosyl, dehydrated through a graded series of ethanol, and air-dried. Sections were exposed onto BioMax MR films (Kodak, Rochester, NY) for 1 day (for 3β-HSD and 20α-HSD) or for 10 days (for galectins and LH-R). The hybridized sections used for the exposure on X-ray films were stained with Hematoxylin & Eosin and observed under a microscope to determine type of the CL.

The films were developed and scanned by a film scanner. The obtained images were cut into appropriate sizes by use of Photoshop Elements 8 software (Adobe Systems Inc., CA, USA). The color was reversed on this software, thus the positive signals representing each gene expression are shown in white color on each figure. The scanned images were also analyzed by Image J (Rasband, U.S. National Institutes of Health, Bethesda, Maryland, USA, http://rsb.info.nih.gov/ij/, 1997-2009) to compare the signal intensities for each gene in the individual CL. All statistical analyses were performed using Prism (GraphPad Software, La Jolla, USA). Differences between groups were analyzed using one-way ANOVA and differences were considered as significant when $P<0.05$.

In situ hybridization using two non-overlapping antisense probes exhibited consistent labeling in all tissues examined. Specificity of the hybridization was also confirmed by the disappearance of the signals with an excess dose of unlabeled antisense probes.

Immunohistochemistry

The paraformaldehyde-fixed ovaries were embedded in paraffin according to the conventional method. The sections were cut from these tissue blocks and the immunohistochemistry using specific antibodies for galectin-1 and galectin-3 was performed. The detailed procedure for immunohistochemistry was previously described (Nio-Kobayashi et al. 2010).

Declaration of interest

Authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Figure legends

Figure 1 Changes in the expression of galectins and related molecules in the ovaries of compulsorily weaned mice. Two types of CL intermingle in the postpartum ovary: CL newly formed at postpartum ovulation (CLN, *arrows*) and remaining CL of pregnancy under a functional regressing stage (CLP, *asterisks*). Like the lactating mouse ovary, galectin-3 (**G3**) and 20α-hydroxysteroid dehydrogenase (**20α-HSD**) mRNAs are expressed only in the CLP (*asterisks* in **B**, **C**), whereas signals for luteinizing hormone receptor (**LH-R**) are found only in the CLN (*arrows* in **E**) at 24 h after the weaning. At this stage, 3β-hydroxysteroid dehydrogenase (**3β-HSD**) is expressed in all CL (**D**) and there is a weak expression of galectin-1 (**G1**) (**A**). Galectin-1 and 20α-HSD mRNAs increase 48 h after the weaning in the CLN (*arrows* in **F, H**) when both the signals for 3β-HSD and LH-R are decreasing (*arrows* in **I**, **J**). After 72 h from the weaning, both galectin-1 and galectin-3 are intensely expressed in the CLN (*arrows* in **K**, **L**) as well as in the CLP (*asterisks* in **K**, **L**), although the expressions of both 3β-HSD and LH-R have almost disappeared from the CLN (*arrows* in **N**, **O**). The percentages of the positive signals per area for each gene in the CLN and CLP are summarized in **P** and **Q**. Asterisks in P and Q show the significant difference compared to the signals at 24 h after the weaning. $*P<0.05$, $**P<0.01$, $***P<0.001$, respectively. n.s.: not significant Bars = 0.5 mm

Figure 2 Effect of $PGF_{2\alpha}$ on galectin expression in the ovaries of pregnant and postpartum mice. $PGF_{2\alpha}$ administration to pregnant mice (day 17.5) has no effect on the expression of either galectin or LH-R (**A-H**) in all CL whereas the signals for 20α-HSD is slightly increased (**G**). On the other hand, both galectin-1 and galectin-3

are increased in the remaining CLP of postpartum mice (day 3) by $PGF_{2\alpha}$ treatment (*asterisks* in **I**, **J**, **M**, **N**). These CL are intensely express 20α-HSD (*asterisks* in **K**, **O**) but lack LH-R (*asterisks* in **L**, **P**). Bars = 1 mm

Figure 3 Effect of Mifepristone on galectin expression in pregnant mice (at day 17.5). Administration of Mifepristone does not alter the expression of galectin or 20α-HSD and has no influence on the expression of both 3β-HSD and LH-R in all CL of pregnancy (**A**-**E**). Bar = 1 mm

Figure 4 Changes in the expression of galectins, 3β-HSD, and LH-R in the Bromocriptine-treated mice. When Bromocriptine (**Bromo**) is administrated for 10 days, an accumulation of galectin-1 (**G1**) mRNA in the CL is observed (**A**) whereas signals for both 3β-HSD and LH-R are negligible in all CL (**C**, **D**). Prolactin (**PRL**) treatment in the Bromocriptine-administrated mice at day 9 inhibits the accumulation of galectin-1 and restores the expression of 3β-HSD but not that of LH-R (**E, G, H**). Galectin-3 (**G3**) is not significantly affected by either Bromocriptine or Bromocriptine plus PRL treatment (**B**, **F**). Immunohistochemical (**IHC**) analysis shows an accumulation of galectin-1 in all CL of Bromocriptine-treated mice (**I**, **J**), however, galectin-3 staining does not significantly alter by the treatment (**K**, **L**). Asterisks in I–L indicate the functional CL formed at the latest estrous cycle. Bars = 0.5 mm

Figure 5 A schema summarizing the effect of compulsory weaning (**A**) and $PGF_{2\alpha}$ administration (**B**). Arrows indicate an increase or a decrease in the galectin expression compared to the expression in the CL at 0/24 h after compulsory weaning or

in the CL without $PGF_{2\alpha}$ treatment. Genes up-regulated by PRL (3β-HSD and LH-R) are indicated in red letters, whereas 20α-HSD, which is induced by $PGF_{2\alpha}$, is indicated in blue. +++: extremely intense expression; ++: intense expression; +: moderate expression; +/–: weak expression; –: no expression. N: CL newly formed after the postpartum ovulation, P: CL of pregnancy.

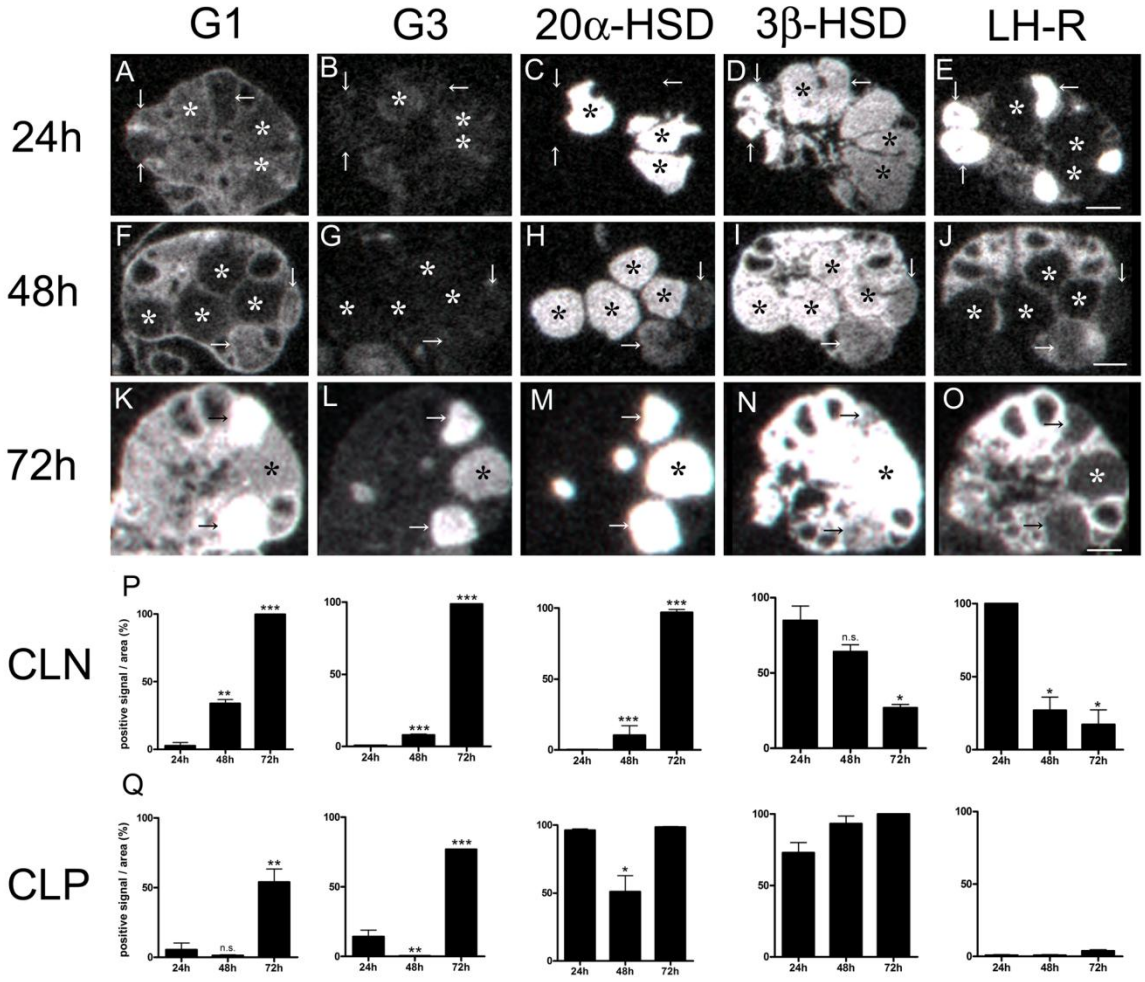

Figure 1 Nio-Kobayashi and Iwanaga

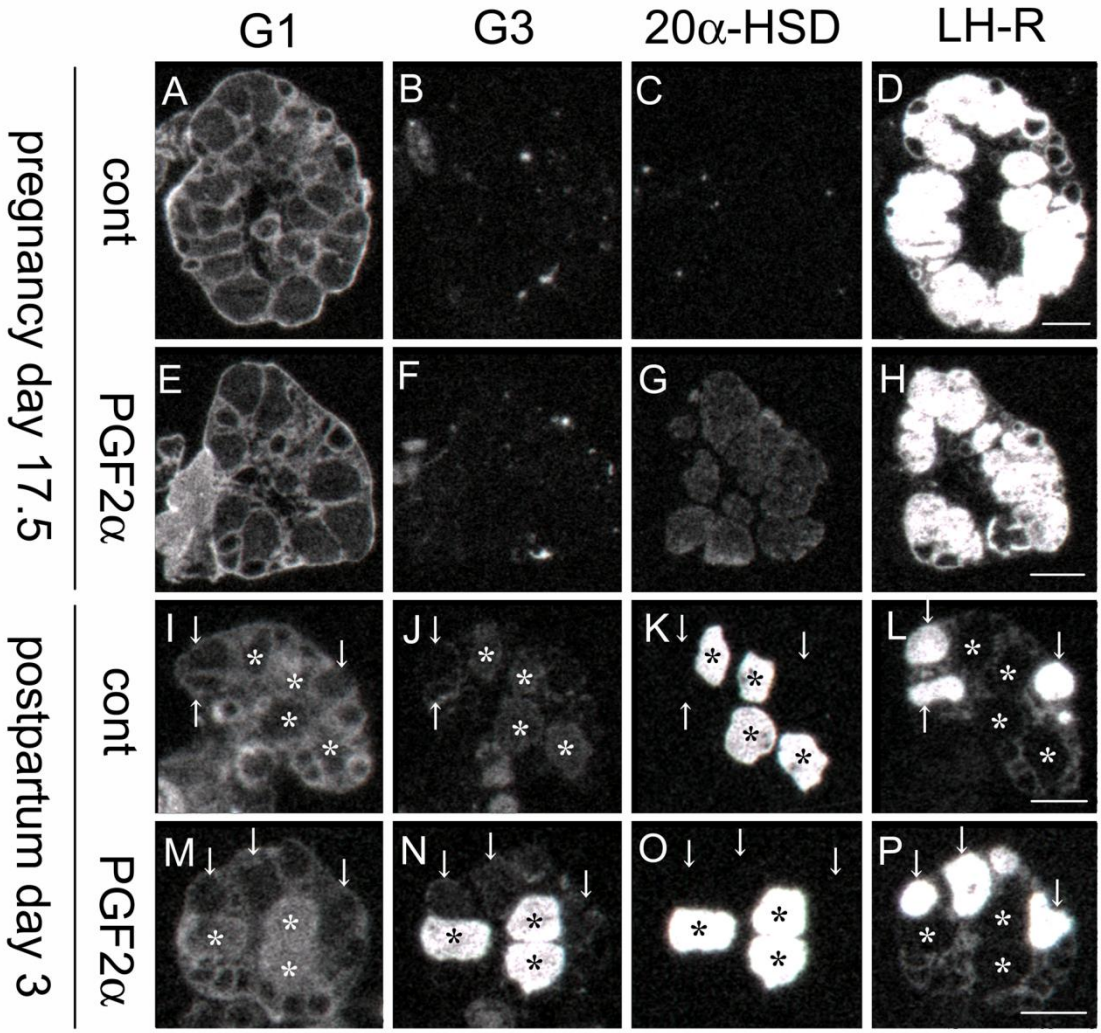

Figure 2 Nio-Kobayashi and Iwanaga

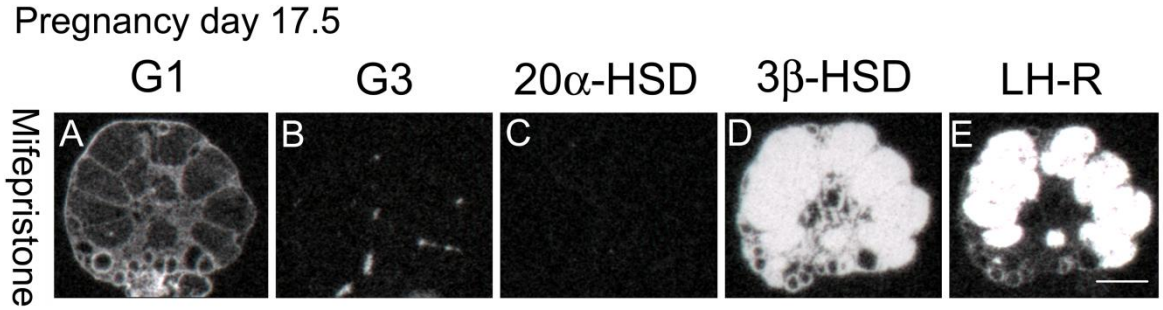

Figure 3 Nio-Kobayashi and Iwanaga

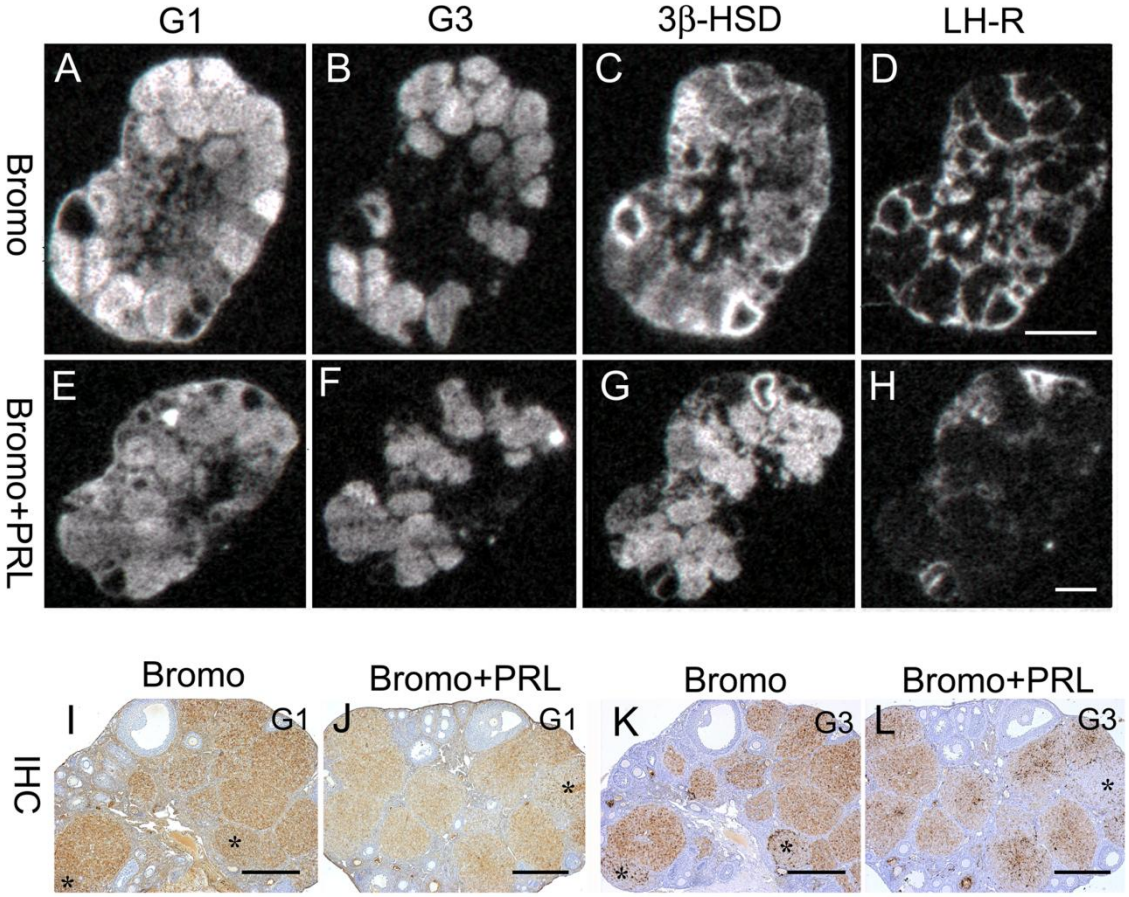

Figure 4 Nio-Kobayashi and Iwanaga

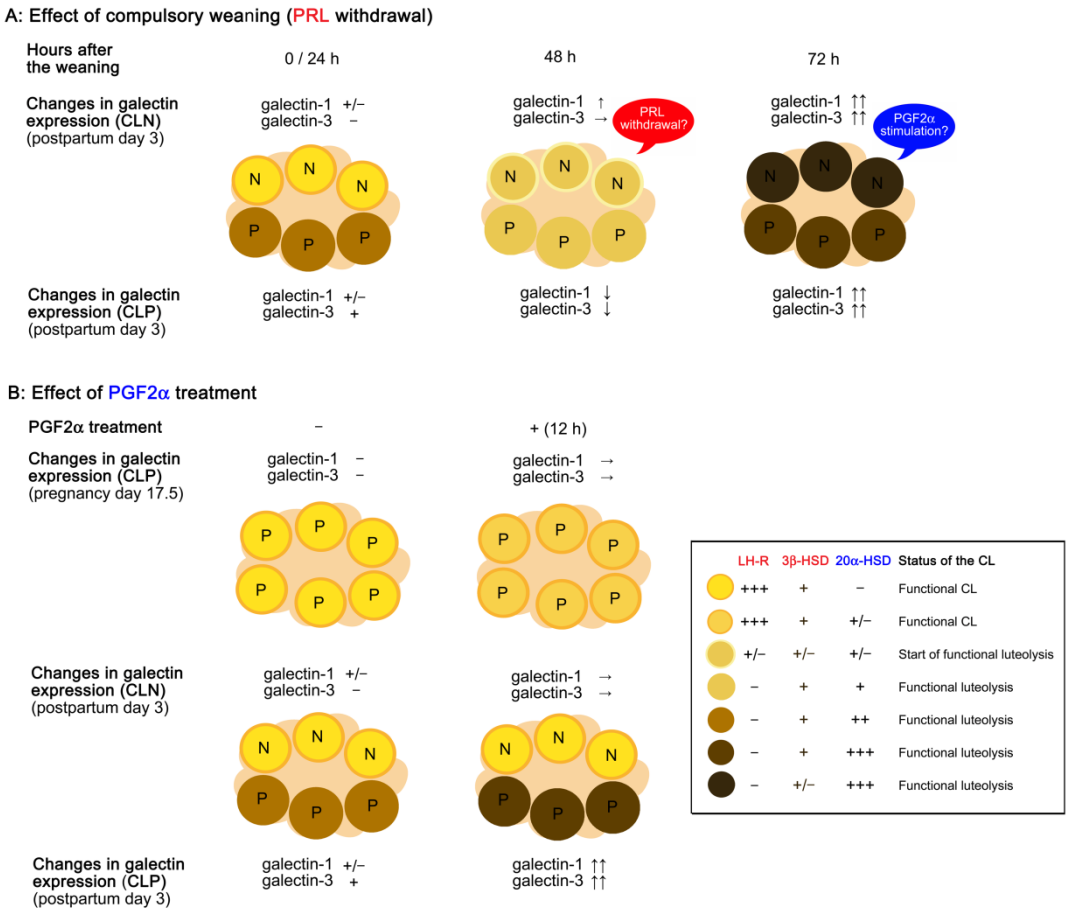

Figure 5 Nio-Kobayashi and Iwanaga