The loss of luteal progesterone production in women is associated with a galectin switch via α2,6-sialylation of glycoconjugates

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Abbreviated title: Galectins in the corpus luteum of women

Key terms: galectin, corpus luteum, hCG, PGE_2, α2,6-sialic acids, ST6GAL1

Word count: 3595 words

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Grants: This study was supported by the Cunningham Trust to W.C.D., and the Postdoctoral Fellowship for Research Abroad and Grant-in-Aid for Scientific Research (C) from Japan Society for the Promotion of Science, and the Suntory Foundation for Life Sciences to J. N.-K.

Disclosure Summary: The authors state that there are no conflicts of interest related to this manuscript.

Clinical trial registration number: N/A

Abstract

Context: Luteal progesterone is fundamental for reproduction but the molecular regulation of the corpus luteum (CL) in women remains unclear. Galectin-1 and galectin-3 bind to the sugar chains on cells to control key biological processes including cell function and fate.

Methods: The expression and localization of *LGALS1* and *LGALS3* was analyzed by quantitative PCR and histochemical analysis, with special reference to α2,6-sialylation of glycoconjugates, in carefully-dated human CL collected across the menstrual cycle and after exposure to hCG *in vivo*. The effects of hCG and prostaglandin E_2 (PGE_2) on the expression of galectins and an α2,6-sialyltransferase, ST6GAL1, in granulosa lutein cells were analyzed *in vitro*.

Results: Galectin-1 was predominantly localized to healthy granulosa lutein cells and galectin-3 was localized to macrophages and regressing granulosa lutein cells. Acute exposure to luteotrophic hormones (hCG and PGE_2) up-regulated *LGALS1* expression ($P<0.001$). *ST6GAL1*, which catalyzes α2,6-sialylation to block galectin-1 binding, increased during luteolysis ($P<0.05$) as did *LGALS3* ($P<0.05$). Luteotrophic hormones reduced *ST6GAL1* and *LGALS3 in vivo* ($P<0.05$) and *in vitro* ($P<0.001$). There was an inverse correlation between the expression of *ST6GAL1* and *HSD3B1* ($P<0.01$), and a distinct cellular relationship among α2,6-sialylation, 3β-HSD, and galectin expression.

Conclusions: Galectin-1 is a luteotrophic factor whose binding is inhibited by α2,6-sialylation in the human CL during luteolysis. ST6GAL1 and galectin-3 expression is increased during luteolysis and associated with a loss of progesterone synthesis. Luteotrophic hormones differentially regulate galectin-1 and galectin-3/α2,6-sialylation in granulosa lutein cells, suggesting a novel galectin switch regulated by luteotrophic stimuli during luteolysis and luteal rescue.

Introduction

The corpus luteum (CL) is a temporal endocrine gland producing large amount of progesterone that is essential for the establishment and maintenance of pregnancy. In the absence of pregnancy, the CL degenerates, and the associated fall in progesterone secretion is responsible for the induction of menstruation (1). In a conception cycle, the CL is maintained by human chorionic gonadotrophin (hCG) and continues to produce progesterone during early pregnancy (1). Low luteal progesterone concentrations and inadequate luteal function are associated with subfertility and miscarriage (2, 3). Although the CL plays an essential role in the menstrual cycle, fertility, and the endocrine maintenance of pregnancy, the molecular mechanisms governing human luteal function are not fully understood. One key barrier to our understanding of human luteal pathophysiology is that the CLs of disparate species are regulated differently to that of women, and there are no suitable non-primate pre-clinical animal models.

Although the CL of non-primate species has a different endocrine regulation, it is likely that cellular insights from other animals can be informative in the investigation of the human CL. We previously reported in the mouse ovary that galectin-1 and galectin-3 were expressed in the regressing CL (4, 5). Galectins are animal lectins that recognize β-galactoside of glycoconjugates and are involved in various biological functions including cell differentiation, apoptosis, and signal transduction. Fifteen galectin subtypes have been identified in mammals and are distributed throughout the body with subtype-specific localization (6, 7). All subtypes possess high affinity for *N*-acetyllactosamine (LacNAc), which consists of galactose β-linked to *N*-acetylglucosamine, however, modifications of LacNAc structure influence the affinity for each galectin subtypes. For example, α2,6-sialylation of terminal galactose interferes with the binding of galectin-1 (8-12).

The expression of galectin-1 and galectin-3 in the regressing mouse CL was not

synchronized, suggesting a differential contribution to the regulation of luteolysis. In addition there was evidence of endocrine regulation as both galectins were increased by luteolytic $PGF_{2\alpha}$ only when the CL did not intensely express luteinizing hormone (LH) receptors (13), suggesting an involvement of LH receptor-mediated signals in the regulation of luteal galectin expression. Interestingly, other than in mice, an elevated expression of galectin-3 has been suggested in the regressing CL of the cow (14, 15).

We hypothesized that regulated expression of galectins had a key role in the molecular regulation of the CL, as it was conserved in disparate species, and that regulated galectin expression would have an important role in the molecular regulation of the CL of women. In this study, we aimed to elucidate 1) the expression and localization of galectin-1 and galectin-3 in carefully-dated human CL, 2) the regulation of the galectin expression by luteotrophic molecules in the human CL *in vivo* and in human luteal cells *in vitro*, and 3) the functional interactions of galectins by investigating changes in the carbohydrates ligands, focused on α2,6-sialylation of glycoconjugates, which critically regulates the subtype-specific carbohydrate recognition of galectins.

Materials and Methods

Ethics

The administration of hCG to women and the collection of the CL at the time of hysterectomy was approved by the Lothian Medical Research Ethics Committee. Lothian Medical Research Ethics Committee separately approved the study of luteinized granulosa cells (LGC) collected during assisted conception. All women gave informed consent.

Reagents

Reagents were purchased from Sigma-Aldrich Corporation (St. Louis, MO) unless otherwise stated. Insulin-Transferrin-Selenium (ITS)-X was obtained from Life Technologies, Inc. (Carlsbad, CA). Matrigel is a product of BD Biosciences (San Jose, CA).

Collection of the human CL

The human CLs were collected at the time of surgery from women undergoing hysterectomy for benign conditions. The stages of the CLs were determined by endometrial morphology and by the concentration of LH in daily urine samples obtained prior to surgery as reported previously (16). The collected CLs were radially sectioned upon collection; half of the tissue was snap-frozen in liquid nitrogen and stored at −80°C, and the other half was fixed with formalin and processed into paraffin embedding. Only fixed tissue was available for some of these women. Some women were given daily injections of hCG at 125 IU (Profasi®, Serono Laboratories, Welwyn Garden City, UK), starting from 7 days after the LH surge for 5–8 days until surgery. This regimen has been shown to rescue the CL and mimic the hormonal changes of early pregnancy (16). Only frozen tissue was available from these women.

Human LGC culture

After removal of oocytes from the follicular aspirates obtained from the patients undergoing assisted conception, the remaining fluid was centrifuged at 1,500 rpm for 10 min, and cells were re-suspended in culture medium (DMEM/Ham's F-12 containing 2 mM L-glutamine, 10 unit/mL penicillin, 0.1 mg/mL streptomycin, 2.5 μg/mL amphotericin B, and 1× concentration of ITS-X). The cell suspension was layered over 45% Percoll/culture medium mixture and centrifuged at 1,200 rpm for 30 min to pellet blood cells. LGCs, visible in the interface, were collected by a pipette and washed with Dulbecco's phosphate buffered saline with $MgCl_2$ and $CaCl_2$ three times. One hundred-thousand viable cells were seeded on Matrigel-coated 24 well plates and cultured with 1 mL of culture medium at 37°C in 5% CO_2 in air. The culture medium was changed every two days, and cells were treated on day 6 with either 100 ng/mL hCG or 10 ng/mL PGE_2 for 24 h. For long-term treatment with hCG, cells were treated with 100 ng/mL hCG for 4 days from day 6 and collected daily from day 7 until day 11. For hCG withdrawal experiment, LGCs were treated with 1 ng/mL hCG from the next day of plating for continuous 6 days, changed to the media without hCG on day 7, and collected daily until day 11. Each experiment was repeated at least three times.

RNA extraction and reverse transcription (RT)

The CLs used for a quantitative gene expression analysis were classified as early-luteal (n=7), mid-luteal (n=8), late-luteal (n=7), menstrual phases (n=3), and rescued by hCG injection (n=4). Total RNA was purified from the frozen tissues of human CL or cultured LGCs using RNeasy Mini Kit (Qiagen Ltd., Crawley, UK) following the manufacturer's protocol. RNA (200 ng) was used to prepare cDNA using the TaqMan Reverse Transcription regents (Applied Biosystems, Foster City, CA).

Quantitative PCR (qPCR)

151 The sequences of the primer sets used for this study are listed in Table 1. Primers were
152 pre-validated by standard PCR and generating standard curves in qPCR. Each reaction buffer
153 contained 5.0 μL 2×PowerSYBR® Green PCR Master Mix (Applied Biosystems), 0.5 μL primer
154 pair (5 μM), 3.5 μL of nuclease free H_2O, and 1.0 μL cDNA, and each reaction was conducted
155 in duplicate. The qPCR cycling program consisted of a denaturing step (95°C for 10 min),
156 annealing and extension step (95°C for 15 sec and 60°C for 1 min repeated for 40 cycles), and a
157 dissociation step (95°C, 60°C, and 95°C for 15 sec each). The reactions were carried out using
158 a 7900 Sequence Detection System (Applied Biosystems). The relative expression levels of
159 each target gene to glucose-6-phosphate dehydrogenase (G6PD) as a house keeping gene, as
160 previously validated using geNorm analysis (Primerdesign Ltd, Southampton, UK), were
161 quantified using the $_{\Delta}$Ct or $_{\Delta\Delta}$Ct methods. All statistical analyses were performed using
162 un-paired *t*-tests or one-way ANOVA, after testing for normality, using Graphpad prism 6
163 software (Graphpad Software Inc., San Diego, CA, USA) and $P<0.05$ was regarded as
164 significant.
165
166 **Immunohistochemistry & lectin histochemistry**
167 Fixed human CL tissues at early-luteal (n=20), mid-luteal (n=34), late-luteal (n=22), and
168 menstrual phases (n=5) were used for histochemical analysis. The tissues were dehydrated
169 through ethanol and embedded into paraffin according to the conventional method. The
170 sections, at 5 μm thickness, were de-waxed and washed in phosphate-buffered saline (PBS).
171 Subsequently the sections were incubated with 3% hydrogen peroxide for 20 min and
172 Avidin/Biotin blocking solution (Vector Laboratories Inc., Burlingame, CA) for 15 min for each
173 reagent. Then the sections were incubated with normal rabbit or goat serum for 60 min at
174 room temperature. They were incubated with goat anti-human galectin-1 antibody (1:1000;
175 AF1152, R&D systems Inc., Minneapolis, MN), rabbit anti-human galectin-3 antibody (1:200;

sc-20157, Santa Cruz Biotechnology Inc., Dallas, TX), rabbit anti-3β-hydroxysteroid dehydrogenase (3β-HSD) antibody (1:1,000; kind gift from Prof. Ian Mason, The University of Edinburgh) in rabbit or goat serum at 4°C overnight. Control sections were incubated with non-immune serum.

After washing twice in PBS, the sections were incubated with biotinylated anti-goat or anti-rabbit IgG (1:500; Vector laboratories Inc.) for 60 min at room temperature. The reaction sites were visualized using Vectastain ABC Elite kit (Vector Laboratories Inc.) for 60 min followed by ImmPACT™ DAB Peroxidase Substrate Kit (Vector Laboratories Inc.) for 5 min. The sections were counterstained with haemotoxylin and observed under a light microscope (BX51; Olympus corporation, Tokyo, Japan).

Other sections were blocked using Carbo-free blocking solution (Vector Laboratories Inc.) for 60 min at room temperature following an incubation with Avidin/Biotin blocking solution. The sections were incubated with the biotinylated *Sambucus sieboldiana* (SNA) that specifically recognizes α2,6-sialic acids of glycoconjugates (Seikagaku corporation, Tokyo, Japan) diluted in 1:250 at 4°C overnight. The visualization of the lectin-binding sites was performed with Vectastain ABC Elite and ImmPACT™ DAB Peroxidase Substrate Kit as described above.

Results

Expression and regulation of galectin-1 in the human CL

We first assessed whether galectin-1 was expressed in the human CL. *LGALS1* was expressed in the CL at all luteal phases (Figure 1A). It could be localized by immunohistochemistry to the cytoplasm of granulosa lutein cells throughout the luteal phase (Figure 1B-E). It was also expressed in non-steroidogenic fibroblast-like cells distributed in the central clot (*arrowheads* in Figure 1B).

We next examined the effect of simulated early pregnancy using incremental hCG injections for 5 to 8 days *in vivo*. Luteal rescue with hCG did not alter the expression of *LGALS1* in the CL (Figure 1F). However, luteotrophic hormones could acutely increase *LGALS1* expression in steroidogenic cells: both hCG (Figure 1G; $P<0.001$) and PGE_2 (Figure 1H; $P<0.001$) increased *LGALS1* expression in human LGCs. In addition, the ability of hCG to augment *LGALS1* decreased with duration of exposure *in vitro* (Figure 1I), paralleling what is seen *in vivo* (Figure 1F). Overall galectin-1 is expressed in luteal steroidogenic cells and is acutely increased by molecules that promote normal luteal function.

Expression and regulation of galectin-3 in the human CL

We next addressed whether galectin-3 was expressed in the human CL. In contrast to *LGALS1*, *LGALS3* was low in the early- and mid-luteal CL (Figure 2A). Its expression during the functional luteal phase was maximal in the late-luteal phase (Figure 2A; $P<0.05$). However there was marked *LGALS3* expression in the non-functional CL collected during menstruation (Figure 2A; $P<0.001$). Galectin-3 immunoreactivity was mainly found in stellate or spindle-shaped cells scattered in the CL parenchyma (*arrows* in Figure 2B). These were identified as infiltrating macrophages and were more marked in the CL at late-luteal phase (Figure 2C). Thus the increasing *LGALS3* as the functional CL ages may reflect an increased

number of galectin-3-positive macrophages.

While galectin-3 immunoreactivity in granulosa lutein cells was faint in the functional CL (Figure 2B and 2C), this was not the case in a regressing CL from the previous cycle. Unlike the healthy CL at early-luteal phase which contained abundant 3β-HSD but does not express galectin-3 ("new" in Figure 2D and 2E), the regressing CL with minimal 3β-HSD displayed marked galectin-3 immunostaining ("old" in Figure 2D and 2E). Degenerating granulosa lutein cells in the old CL possessed large cytoplasmic lipid droplets and clear galectin-3 immunoreactivity (Figure 2F).

In contrast to *LGALS1*, PGE_2 reduced *LGALS3* expression in human LGCs (Figure 2G; $P<0.001$). While hCG had no acute effect (Figure 2H), *LGALS3* expression in LGCs increased with time (Figure 2I; $P<0.001$) and the inhibitory effect of hCG *in vitro* became more marked (Figure 2I; $P<0.001$). This was mirrored by the effect of exogenous hCG on the CL *in vivo* (Figure 2J; $P<0.05$). Overall galectin-3 is expressed in infiltrating macrophages and degenerating steroidogenic cells and is reduced by molecules that promote normal luteal function.

Luteotrophic stimuli regulate a galectin switch via α2,6-sialylation of glycoconjugates

Thus as the human CL undergoes luteolysis there seems to be a switch of the major galectin subtype in steroidogenic cells from galectin-1 to galectin-3. As the ligands for galectins are glycoconjugates, we next examined the effect of luteotrophic molecules on glycoconjugates on lutein cells. Galectins possess a high affinity for LacNAc structure, and α2,6-sialylation of its terminal galactose, catalyzed by ST6GAL1 sialyltransferase, inhibits galectin-1 action but does not influence that of galectin-3 (Figure 3A). We therefore examined the expression and regulation of ST6GAL1.

ST6GAL1 expression in the human CL during the functional luteal phase was increased in

the late-luteal CL (Figure 3B; $P<0.05$). Like *LGALS3*, its expression was marked in the regressing CL collected during menstruation (Figure 3B; $P<0.001$). There was a contrasting relationship between the expression of *ST6GAL1* and a key enzyme for progesterone synthesis, *HSD3B1* (Figure 3C) with an inverse correlation in level of expression ($r = -0.5428$, $P<0.01$). *ST6GAL1* expression was inhibited by luteotrophic molecules: both PGE_2 (Figure 3D; $P<0.001$) and hCG (Figure 3E; $P<0.001$) inhibited *ST6GAL1* in LGCs *in vitro*, and hCG reduced luteal *ST6GAL1* expression *in vivo* (Figure 3F; $P<0.05$). In addition withdrawal of luteotrophic signals increased the expression of *ST6GAL1* in LCGs (Figure 3G; $P<0.001$). Overall luteotrophic signals suppress *ST6GAL1* to promote galectin-1 binding while loss of luteotrophic signals increase *ST6GAL1* and promote galectin-3 binding to glycoconjugates. The increase of *ST6GAL1* in the human CL as it ages parallels the reduction in *HSD3B1*.

Intimate relationship of α2,6-sialylation and loss of progesterone production in luteal cells

We next examined the temporal association between increased ST6GAL1 activity and the loss of 3β-HSD in the human CL. Serial sections of human CL were stained with an α2,6-sialic acid-recognizing plant lectin (SNA) and for 3β-HSD. Intense SNA reaction was found in endothelial cells (*arrows* in Figure 3H and 3J) while most numbers of granulosa lutein cells were negative for the SNA reaction (Figure 3H and 3J). However, some cells contained α2,6-sialic acids in the cytoplasm or plasma membrane and these cells showed faint or negative immunoreactivity for 3β-HSD (*asterisks* in Figure 3H-K).

Cellular relationship between α2,6-sialylation and localization of galectins

A CL, collected as menstruation commenced, illustrated a distinct relationship between α2,6-sialylation, loss of 3β-HSD and galectin expression in serial sections (Figure 4A-D). Granulosa lutein cells with residual 3β-HSD ("1" in Figure 4A) did not express α2,6-sialic acids

("1" in Figure 4B) while they expressed galectin-1 ("1" in Figure 4C) and did not express galectin-3 ("1" in Figure 4C). Neighboring lutein cells not expressing 3β-HSD ("2" in Figure 4A) contained α2,6-sialic acids ("2" in Figure 4B) and less galectin-1 ("2" in Figure 4C) and more galectin-3 ("2" in Figure 4D). Certain areas were occupied with pyknotic cells with moderate 3β-HSD ("3" in Figure 4A) but increased α2,6-sialic acids ("3" in Figure 4B) and without galectins ("3" in Figure 4C and 4D). This suggests a co-ordinated relationship between galectins, α2,6-sialyation, and loss of 3β-HSD during luteolysis (Figure 4E). Differential staining patterns for galectins and α2,6-sialic acids were tightly correlated to the functional and structural status of granulosa lutein cells during luteolysis.

Discussion

This study revealed for the first time the differential expression and localization of galectin-1 and galectin-3 in the human CL throughout menstrual cycle. Galectin-1 was predominantly expressed in the human CL and its expression in luteal steroidogenic cells *in vitro* was up-regulated by luteotrophic molecules (hCG and PGE_2), suggesting an important role of galectin-1 in luteotrophic process. On the other hand, galectin-3 was dramatically increased in the regressing CL and its expression was suppressed by hCG and PGE_2 in luteal cells. Galectin-3 expression was intense in luteal cells without 3β-HSD immunoreactivity in the regressing human CL, and we previously showed the concomitant expression of galectin-3 and a progesterone degradation enzyme in the mouse CL (4). Thus, there could be an intimate relation between galectin-3 expression and the cessation of progesterone synthesis in the CL during luteolysis.

We also revealed for the first time that hCG and PGE_2 significantly down-regulate ST6GAL1 which, like galectin-3, is intensely expressed in the regressing CL at late-luteal and menstrual phases. This enzyme catalyzes α2,6-sialylation of the terminal galactose on glycoconjugates to interfere the binding of galectin-1, therefore we propose here an existence of galectin switch regulated by luteotrophic hCG and PGE_2 via α2,6-sialylation of glycoconjugates. This highlights the importance of the interaction between galectin-1 and glycoconjugates in luteal cells in the mechanism of luteal rescue regulated by hCG, and a contribution of galectin-3 and α2,6-sialic acids to the regulation of luteolysis.

Although luteal cells in the functional CL of mice do not express any galectins, human luteal cells in the functional CL are characterized by rich existence of galectin-1. As mentioned above, galectin-1 in human LGCs was significantly up-regulated by hCG and PGE_2, suggesting its luteotrophic contribution in the human CL. As galectins bind to glycoconjugates, the interaction between galectin-1 and sugar chains on cell surface molecules

may play an important role in the luteotrophic function of hCG. The ligand for galectin-1 could be *N*-gycans on hCG or LH/choriogonadotropin receptor (LHCGR), which possesses two or six *N*-glycosylation sites though the entire structure of sugar chains has not been characterized. Other glycoconjugates such as glycolipids represented by ganglioside GM1, a known ligand for galectin-1 (17), also could be a target. To elucidate the role of galectin-1 on luteal rescue, further experiments including identification of the ligand glycoconjugates for galectin-1 are underway in our laboratory.

On the other hand, intense expression of galectin-3 was found in the regressing CL of human, suggesting its involvement in luteolysis. In cultured human LGCs, galectin-3 was significantly down-regulated by PGE_2 but not influenced by hCG. Therefore, PGE_2 released from luteal cells by hCG stimulation seems to be an important negative regulator of galectin-3 expression in human luteal cells. In addition, histochemical analysis revealed a specific localization of galectin-3 in degenerating granulosa lutein cells without 3β-HSD immunoreactivity. These data suggest a correlation between galectin-3 expression and loss of ability for progesterone synthesis in luteal cells.

Cellular localization of galectins other than luteal cells in the human CL was similar to that in the mouse CL (5): galectin-1 was localized in fibroblasts and galectin-3 was contained in infiltrating macrophages, although fibroblasts were less intensely immunoreactive for galectin-3 in the human CL. An increased number of galectin-3-positive infiltrating macrophages in the regressing CL was observed both in the human and mouse CL (5), suggesting an involvement of galectin-3-positive infiltrating macrophages in the regulation of luteolysis. We have shown in this study that hCG down-regulates the expression of galectin-3 in the human CL *in vivo*. Because hCG suppresses macrophage infiltration into the human CL (18), the decreased number of galectin-3-positive macrophages may contributes to the suppressive effect of hCG on the expression of *LGALS3 in vivo*.

Interestingly, hCG and PGE_2 significantly down-regulated the expression of *ST6GAL1*, which catalyzes α2,6-sialic acids on the terminal galactose of glycoconjugates. Because α2,6-sialylation interferes the binding of galectin-1 to sugar chains (9, 12), luteotrophic stimuli may enhance an interaction between galectin-1 and glycoconjugates to support luteal function by inhibiting α2,6-sialylation. Histochemical analysis of α2,6-sialic acid using SNA, confirmed a luteolytic role of α2,6-sialylation by a specific localization of α2,6-sialic acids in pyknotic or degenerating granulosa lutein cells in the regressing CL at menstrual phase, but not in healthy granulosa lutein cells intensely expressing 3β-HSD and galectin-1. Although proteins carrying α2,6-sialic acids remain to be identified, α2,6-sialylation would be an important modification of glycoconjugates during luteolysis, and it is definitely inhibited by hCG and PGE_2.

Histochemical analysis of galectins and α2,6-sialic acids in the regressing CL at menstrual phase revealed their differential localization depending on the status of each granulosa lutein cell. According to the staining patterns of galectins, 3β-HSD, and α2,6-sialic acids, granulosa lutein cells in the regressing CL can be classified based on their functional status. Both intact and pyknotic cells contained intense immunoreactivity for 3β-HSD, thus they can produce progesterone. The difference between them is the staining pattern of α2,6-sialic acids: only pyknotic cells contained abundant α2,6-sialic acids, suggesting that loss of galectin-1 binding on glycoconjugates may lead to apoptosis of luteal cells. On the other hand, degenerating cells contained abundant α2,6-sialic acids and galectin-3 but lacked 3β-HSD. Since the expression both of *LGALS3* and *ST6GAL1* is down-regulated by hCG and PGE_2, a functional withdrawal of luteotrophic signals may result in the accumulation of α2,6-sialic acids and galectin-3.

Cytoplasmic galectin-3 possesses anti-apoptotic activity (19, 20) and recent reports showed that α2,6-sialylation of the Fas death receptor and the receptor for TNFα (TNFR1) by ST6GAL1 provides protection against Fas-/TNFα-mediated apoptosis in carcinoma cells and

macrophages (21, 22). Thus, it appears that galectin-3/α2,6-sialic acids-containing granulosa lutein cells may increase in number under low luteotrophic stimuli, and escape from apoptosis and degenerate slowly. In support of this idea, the old 3β-HSD-negative CL consisted with galectin-3-positive granulosa lutein cells remained in the ovary over the menstrual cycle as shown in Figure 2D and 2E.

In conclusion, we propose here a possible contribution of an interaction between galectin-1 and glycoconjugates in the regulation of luteal function, and a possible role of galectin-3 and α2,6-sialic acids in luteolysis. Accumulation of α2,6-sialic acids and galectin-3 in luteal cells may inhibit galectin-1 binding to its ligands and contribute to luteolysis, resulting in degeneration of luteal cells. The parallels with the rodent CL highlight the utility of selective knock-out models to further assess the molecular roles of galectins in luteal function. Although detailed experiments using knock-out or knock-down models will to reveal the exact function of galectins in the CL, it is likely that galectin and sugar chain interactions have fundamental roles in the regulation of luteal function.

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Acknowledgments

We are grateful to Prof. Hamish Fraser, The University of Edinburgh, and Prof. Tariq Sethi, King's College London, for giving us the chance to corroborate to carry out this study. We also thank Dr. Fiona Connolly and Ms. Eva Gay, The University of Edinburgh, for their excellent technical support.

501 **Figure legends**

502 **Figure 1.** Expression and localization of galectin-1 in the human CL and cultured human
503 LGCs. Human CL predominantly expresses *LGALS1* mRNA throughout the menstrual cycle
504 (**A**). Immunohistochemical analysis demonstrates an intense immunoreactivity for galectin-1
505 in the cytoplasm of glanulosa lutein cells at mid-luteal (ML; **B**) and late-luteal phases (LL; **C**).
506 Fibroblasts distributed in central clot are also immunostained for galectin-1 (*arrowheads* in **B**),
507 while endothelial cells are negative in the reaction (*arrows* in **D**). The expression of *LGALS1*
508 is not altered by hCG *in vivo* (**F**) while acutely increased by hCG and PGE_2 *in vitro* (**G**, **H**).
509 *LGALS1* expression does not alter with longer hCG exposure *in vitro* (**I**). **E**: control section in
510 which the antibody was omitted. EtOH: ethanol used as a vehicle of PGE_2, EL: early-luteal
511 phase, Men: menstrual phase, n.s.: not significant. The values on graphs represent mean ±
512 SEM. ***$P<0.001$.

513

514 **Figure 2.** Expression and localization of galectin-3 in the human CL and cultured human
515 LGCs. The expression of *LGALS3* mRNA is low in EL and ML phases but elevates at LL and
516 menstrual phases as revealed by qPCR (**A**). Galectin-3 immunoreactivity is observed in
517 scattered cells, being identifiable as infiltrating macrophages (*arrows* in **B**) in ML phase.
518 Fibroblasts in the central clot also show weak immunoreactivity for galectin-3 (*arrowheads* in
519 **B**). Galectin-3-positive infiltrating macrophages with vacuolated cytoplasm are significantly
520 increased in number at LL phase (*arrows* in **C**). In **D** and **E**, two CLs formed at different
521 menstrual cycles are shown in serial sections at EL phase. New CL formed after the latest
522 ovulation contains intense 3β-HSD immunoreactivity (**D**) but not galectin-3 (**E**). Old CL
523 formed at previous menstrual cycle abundantly contains galectin-3 (**E**) but minimal 3β-HSD (**D**).
524 Degenerating granulosa lutein cells in the old CL with large lipid droplets show clear galectin-3
525 immunoreactivity in the cytoplasm (**F**). *LGALS3* expression is significantly reduced by PGE_2

(**G**) while acute treatment of hCG does not affect its expression *in vitro* (**H**). The *LGALS3* expression is gradually increased with time and the inhibitory effect of hCG becomes clear *in vitro* (**I**). *In vivo* treatment of hCG significantly reduces *LGALS3* expression (**J**). Asterisks without bars in A and I show significant differences versus EL/ML phases or control on each culture day. The values on graphs represent mean ± SEM. **P*<0.05, ***P*<0.01, ****P*<0.001.

Figure 3. Expression and regulation of a α2,6-sialyltransferase, *ST6GAL1*, in the human CL and cultured human LGCs. Figure 3A is a scheme showing the structure of α2,6-sialylated sugar chain and the binding affinity of each galectin for it. Galectin-1 cannot recognize α2,6-sialylated galactose catalyzed by ST6GAL1, while galectin-3 can bind to it (**A**). The expression of *ST6GAL1* is significantly high in the CL at LL phase compared to EL and ML phases, and marked increase of *ST6GAL1* is found in the CL at menstruation (**B**). In contrast, *HSD3B1* expression is decreased in the CL at LL and menstrual phases (**C**). The *ST6GAL1* mRNA is significantly reduced by luteotrophic PGE_2 *in vitro* (**D**) as well as hCG both *in vitro* (**E**) and *in vivo* (**F**). Withdrawal of hCG increases *ST6GAL1* expression *in vitro* (**G**). Staining of SNA, an α2,6-sialic acids-recognizing plant lectin, and 3β-HSD on serial sections at ML (**H**, **I**) and LL (**J**, **K**) shows conflicting staining pattern on granulosa lutein cells: SNA-positive cells contain minimal 3β-HSD immunoreactivity (*asterisks* in **H**-**K**). Capillary vessels are positive for SNA (*arrows* in **H** and **J**). Asterisks without bars in B, C, and G show significant differences versus EL/ML phases or LGCs on culture day 7. Asn: asparagine, Gal: galactose, GlcNAc: *N*-acetylglucosamine, LacNAc: *N*-acetyllactosamine, Man: mannose, SA: sialic acid. The values on graphs represent mean ± SEM. **P*<0.05, ***P*<0.01, ****P*<0.001.

Figure 4. Differential expression of 3β-HSD, α2,6-sialic acids, and galectins, in the regressing CL at menstrual phase. Observation of four serial sections from the regressing CL

551 at menstrual phase displays three groups of granulosa lutein cells. Intact cells with intense
552 3β-HSD immunoreactivity lack α2,6-sialic acids and galectin-3 but contain galectin-1 (region
553 "1" in **A**-**D**). Degenerating cells lack 3β-HSD but abundantly contain α2,6-sialic acids and
554 galectin-3 with moderate immunoreactivity for galectin-1 (region "2" in **A**-**D**). Pyknotic cells
555 are rich in 3β-HSD and α2,6-sialic acids but lack immunoreactivities for two galectins (region
556 "3" in **A**-**D**). Galectin-1 and galectin-3 are respectively localized in fibroblasts (*arrowheads* in
557 **C**) and macrophages (*arrowheads* in **D**) in this area. Arrows in A-D indicate theca lutein cells.
558 Inserts in A-D show degenerating (*arrowheads*) and pyknotic cells (*arrows*) observed at higher
559 magnification. **E** summarizes the expression of 3β-HSD, α2,6-sialic acids, and galectin in
560 granulosa lutein cells in the regressing CL.
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Table 1. Primers used for qPCR analysis

Genes name	Protein name	Accession no.	Forward	Reverse	Product size (bp)
G6PD	G6PD	NM_000402	CGGAAACGGTCGTACACTTC	CCGACTGATGGAAGGCATC	155
LGALS1	galectin-1	NM_002305	CGAGTGCGAGGCGAGGTG	CGTTGAAGCGAGGGTTGAAGTG	100
LGALS3	galectin-3	NM_002306	GGCCACTGATTGTGCCTTAT	TCTTTCTTCCCTTCCCCAGT	224
ST6GAL1	ST6GAL1	NM_173216	CTGCCCAAGGAGAGCATTAG	TGTTGGAAGTTGGCTGTGG	158
HSD3B1	3β-HSD	NM_000862	CCATGAAGAAGAGCCTCTGG	GTTGTTCAGGGCCTCGTT TA	202

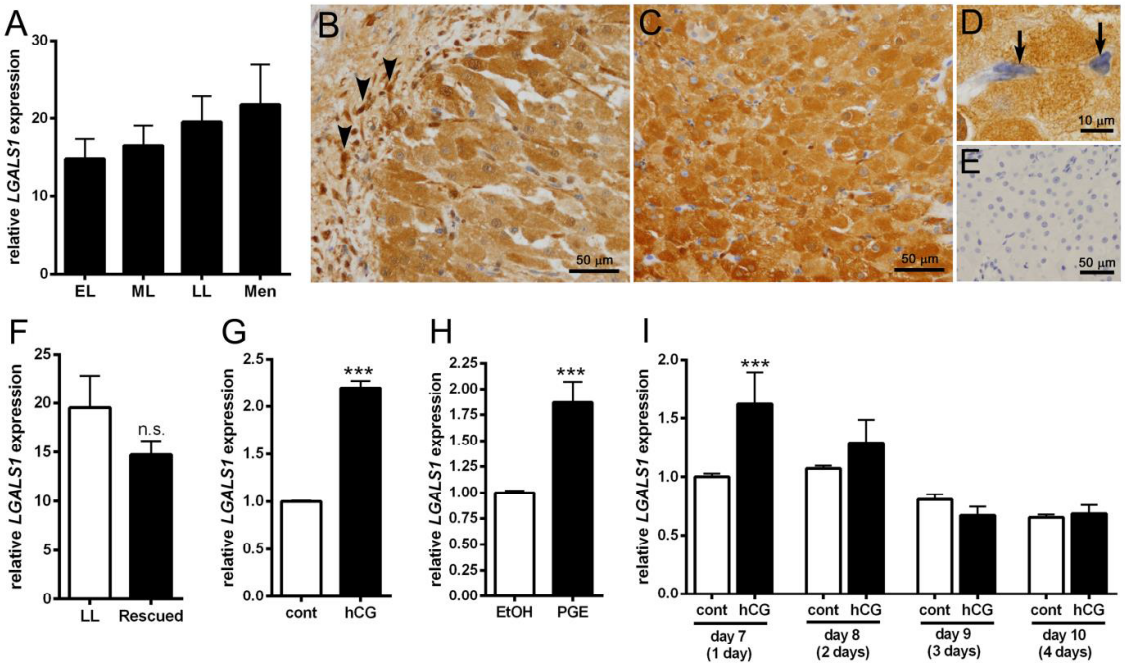


Figure 1 Nio-Kobayashi et al.

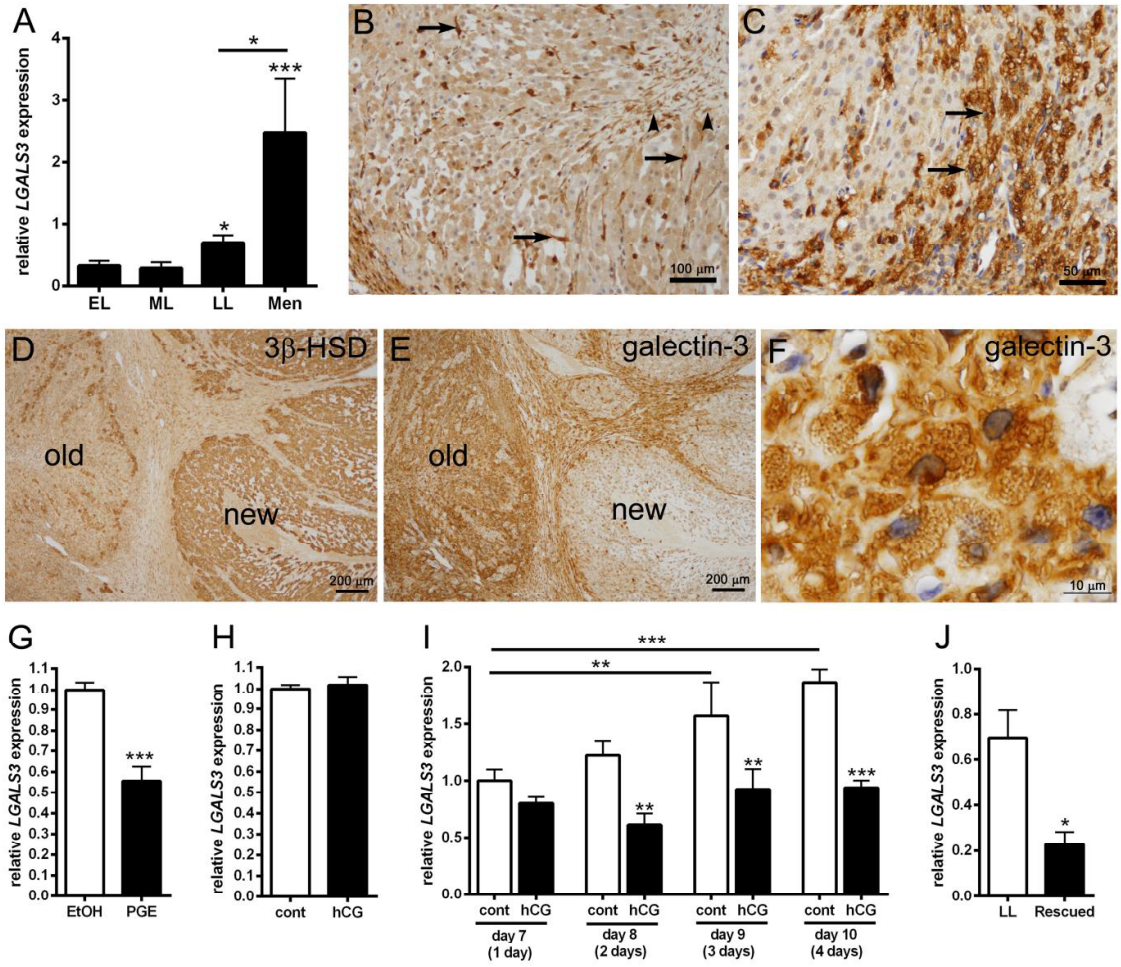


Figure 2 Nio-Kobayashi et al.

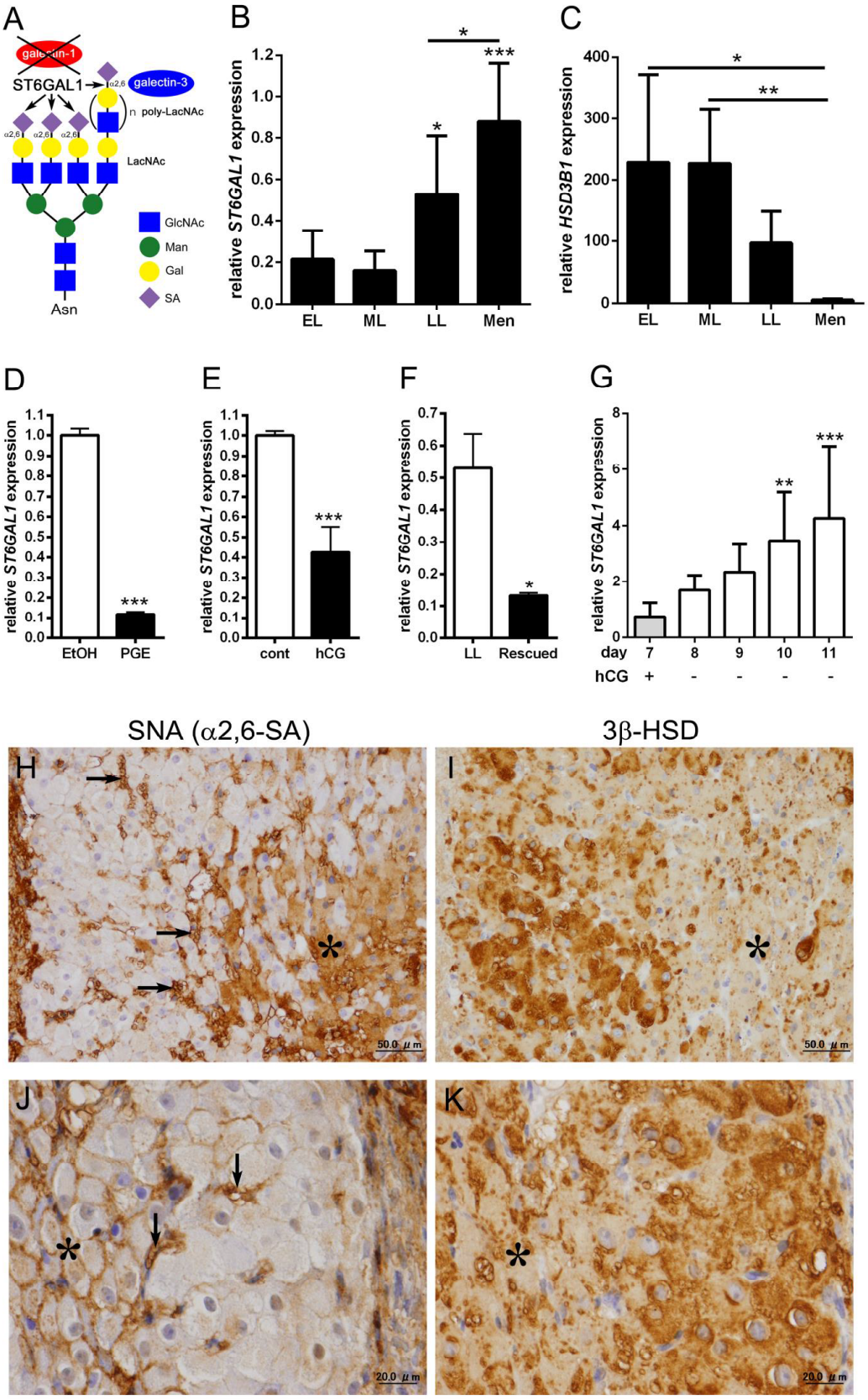
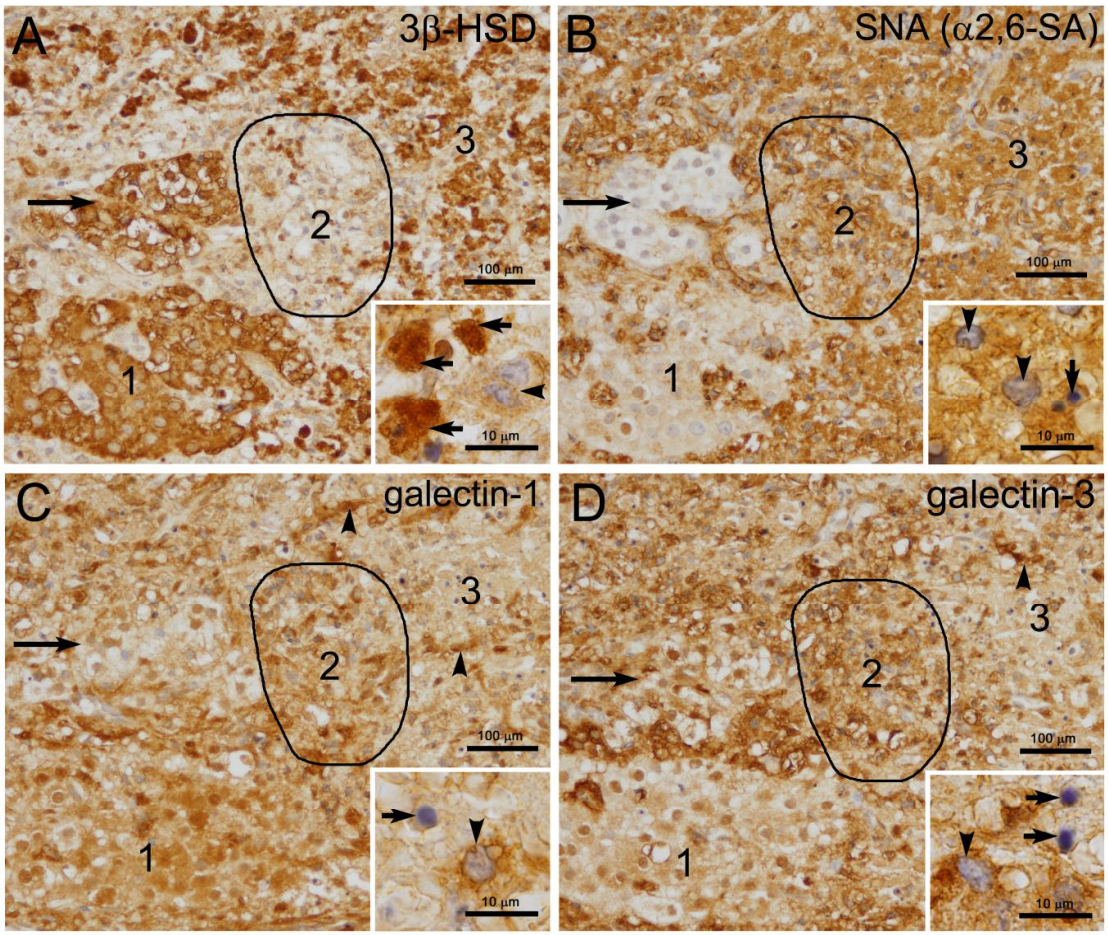


Figure 3 Nio-Kobayashi et al.



E

	Intact	Pyknotic	Degenerating
3β-HSD	+++	+++	–
α2,6-SA	–	+++	+++
galectin-1	++	–	+
galectin-3	–	–	+++

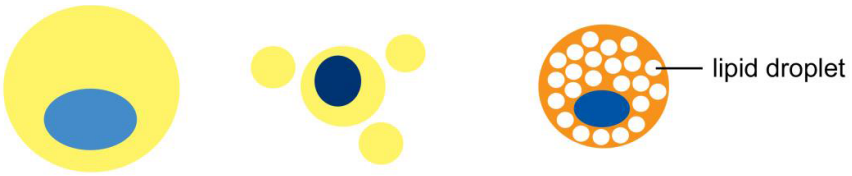


Figure 4 Nio-Kobayashi et al.