DNA Methylation of Specific CpG Sites in the Promoter Region Regulates the Transcription of the Mouse Oxytocin Receptor

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Abstract

Oxytocin is a peptide hormone, well known for its role in labor and suckling, and most recently for its involvement in mammalian social behavior. All central and peripheral actions of oxytocin are mediated through the oxytocin receptor, which is the product of a single gene. Transcription of the oxytocin receptor is subject to regulation by gonadal steroid hormones, and is profoundly elevated in the uterus and mammary glands during parturition. DNA methylation is a major epigenetic mechanism that regulates gene transcription, and has been linked to reduced expression of the oxytocin receptor in individuals with autism. Here, we hypothesized that transcription of the mouse oxytocin receptor is regulated by DNA methylation of specific sites in its promoter, in a tissue-specific manner. Hypothalamus-derived GT1-7, and mammary-derived 4T1 murine cell lines displayed negative correlations between oxytocin receptor transcription and methylation of the gene promoter, and demethylation caused a significant enhancement of oxytocin receptor transcription in 4T1 cells. Using a reporter gene assay, we showed that methylation of specific sites in the gene promoter, including an estrogen response element, significantly inhibits transcription. Furthermore, methylation of the oxytocin receptor promoter was found to be differentially correlated with oxytocin receptor expression in mammary glands and the uterus of virgin and post-partum mice, suggesting that it plays a distinct role in oxytocin receptor transcription among tissues and under different physiological conditions. Together, these results support the hypothesis that the expression of the mouse oxytocin receptor gene is epigenetically regulated by DNA methylation of its promoter.


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These authors contributed equally to this work.

Introduction

Oxytocin (OT) is a nonapeptide, mostly known for its role in enhancing contractions of the uterus during labor and mediating milk release from mammary glands during suckling [1]. Recently, OT activity in the brain was shown to play an important role in mammalian social behavior [2]. OT is produced almost solely in the hypothalamic supraoptic (SON) and paraventricular (PVN) nuclei, and is released either to the periphery via the pituitary gland, or within the brain via multiple pathways [3]. All central and peripheral actions of OT are mediated through one oxytocin receptor (OXTR), which is the product of a single gene [4,5,6]. Activation of the OXTR promotes coupling to two alternative G proteins, Gq and Gi/o both of which lead to activation of proteins in the MAP kinase signaling pathway such as ERK 1/2 and p38 [7,8,9].

Transcription of the rodent Oxt was studied in brain and peripheral tissues where it was found to be partly regulated by estrogens [10,11,12,13,14]. For example, in parallel to the estrogen levels, Otxr peaks in both the uterus and mammary glands before and during labor. However, post partum its levels drop significantly in the uterus, but remain high in the mammary glands [15,16]. Furthermore, rats that underwent gonadectomy, showed a positive response to external estrogen in the uterus [17], but not in the mammary gland [18], suggesting distinct regulatory mechanisms in these two tissues. The mechanism by which estrogen regulates Otxr transcription is elusive, partly because the Otxr promoter of several mammalian species, including that of humans, lacks a palindromic estrogen response element (ERE) [1,4,5,6,19]. Importantly, while Otxr expression can be upregulated by estrogen in tissues, multiple attempts to obtain a similar effect in cultured cells were unsuccessful [12].

DNA methylation is a major epigenetic mechanism that regulates gene transcription [20,21,22]. It involves direct chemical modification of a cytosine, immediately followed by a guanine (CpG). These CpG dinucleotides are highly underrepresented in the genome, and often occur in small clusters known as CpG islands [23]. Hypermethylation of CpG sites in the vicinity of
genes is often associated with suppression of transcription [20,21]. In the present study, we hypothesized that transcription of the mouse Oxtr is regulated by DNA methylation of specific sites in its promoter, in a tissue-specific manner.

Materials and Methods

Ethics statement

All experimental protocols were approved by the Animal Care and Use Committee of the University of Haifa.

DNA constructs

To construct the Unmodified vector, the CMV promoter of the pEYFP-N1 vector (Clontech Laboratories, Mountain View, CA) was replaced by the Oxtr minimal promoter [24], amplified (see Table 1 for primers) from C57BL/6 mouse genomic DNA, using Asel/BamH I digestion. CpG sites 1 and 7 were mutated (C to A) by a PCR-based method [25] to produce the Mut 1 and Mut 7 vectors, respectively. The Del construct was created by PCR amplification of the circular Unmodified vector excluding the 400 bp amplicon region. In this reaction we used primers designed to contain a non-complementary 5’ sequence consisting of an EcoRI site (Table 1). PCR amplifications were performed using the KAPA HiFi™ DNA Polymerase (Kapa Biosystems, Woburn, MA) as follows: 95°C for 2 min, 17 cycles of 98°C for 30 s, 60°C for 30 s, 72°C for 40 s and 72°C for 5 min. PCR products were then treated with Dpn I to eliminate the template DNA and with EcoRI to produce sticky ends. The digested PCR products were then ligated and transformed into DH5α E.coli.

In vitro methylation

20 µg of the examined vectors were methylated in vitro in 200 µl reaction mixture, containing 40 U of CpG methyltransferase (M. SsoI) and 160 mM 5-adenosyl methionine (SAM; New England Biolabs, Ipswich, MA) at 37°C for 3 h, with subsequent inactivation of enzyme at 65°C for 20 min. Due to its low stability at 37°C, SAM was added again to the reaction mixture after 1.5 h. Mock-methylation reactions were performed in the absence of M. SsoI. Following the enzymatic reaction, the different vectors were phenol/chloroform extracted. The efficiency of methylation for each of the constructs was confirmed by the methylation-sensitive restriction enzyme FauI (New England Biolabs, Ipswich, MA) that has one recognition site in the analyzed region and no sites in the sequence of the pEYFP-N1 vector.

Cell cultures and treatment

4T1 mouse mammary carcinoma and GTI-7 immortalized hypophyseal cell lines were kindly provided by Dr. Alon Chen (Weizmann Institute, Israel) and grown in RPMI-1640 and Dulbecco’s modified essential medium (DMEM) media, respectively, both supplemented with 10% fetal calf serum and penicillin/streptomycin. Cells were maintained at 37°C humidified incubator with 5% CO2. Demethylation experiments were performed in 6-well dishes at a density of 4 × 105 cells per well. Cells were incubated with 5-Aza-2’-deoxycytidine (5-AzaC, Sigma, St. Louis, MO) at the indicated concentrations for two days, during which medium was replaced daily. ERK activation was measured in GTI-7 cells following overnight starvation, and subsequent stimulation with 1 µM OT for 10 min. Samples were then harvested in RIPA/SDS buffer (50 mM Tris pH 8, 150 mM NaCl, 5 mM EDTA, 1% w/v NP-40, 0.5% w/v deoxycholic acid, 0.1% w/v SDS, 10 mM NaF, 0.1 mM PMFS and Complete Protease Inhibitor cocktail tablets (Roche, Basel, Switzerland). Protein concentrations were determined with theBradford Assay (Bio-Rad Laboratories, Hercules, CA) and equal amounts of total protein were separated by SDS-PAGE, transferred onto nitrocellulose membranes. Phosphorylation of ERK1/2 was detected by protein immunoblotting using a 1:1000 dilution of rabbit polyclonal phospho-specific ERK antibody, followed by probing for mouse monoclonal total ERK antibody (Santa Cruz Biotechnologies, Santa Cruz, CA). Proteins were visualized by a Chemiluminescence Detection Kit for HRP (EZ ECL, Biological Industries, Beit Haemek, Israel) and quantified using a CCD camera and Quantity One software (XRS, Bio Rad).

Transfection and reporter gene expression

4 × 105 GTI-7 cells were seeded in 6-well plates at 80–90% confluence and allowed to grow overnight. Transfection of the different constructs was carried out using the PolyJet™ transfection reagent (SignaGen Laboratories, Rochville, MD), according to the manufacturer’s instructions. The FCW_tdTomato vector, which expresses the tdTomato red fluorescence protein under the CMV promoter, was co-transfected with the EYFP vectors to control for transfection efficiency and was used to average the levels of EYFP fluorescence over all tdTomato-expressing cells.

Table 1. PCR primer sets.

<table>
<thead>
<tr>
<th>Primers for amplification of:</th>
<th>Sequence</th>
<th>TA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unmodified vector</td>
<td>F: 5’-GGCCATTTAATTTAAACCTTATTGTACAGTGG-3’</td>
<td>59°C, 30 s</td>
</tr>
<tr>
<td>Mut 1 vector</td>
<td>F: 5’-GCCCCCTTGGTGCTGCTATGTTGAACCTAGCAGAAG-3’</td>
<td>60°C, 30 s</td>
</tr>
<tr>
<td>Mut 7 vector</td>
<td>F: 5’-GGACCTGGGGGGTTGTTGGGACGCTGAG-3’</td>
<td>60°C, 30 s</td>
</tr>
<tr>
<td>Del vector</td>
<td>F: 5’-GGAAATCTCTCTCTGTTGTTG-3’</td>
<td>60°C, 30 s</td>
</tr>
<tr>
<td>Oxtr miRNA</td>
<td>F: 5’-GGCGGTAGAGGATGAGG-3’</td>
<td>60°C, 30 s</td>
</tr>
<tr>
<td>Hprt1 miRNA</td>
<td>F: 5’-GGACCTGGGGGGTTGTTGGGACGCTGAG-3’</td>
<td>60°C, 30 s</td>
</tr>
<tr>
<td>Bisulfite treated Oxtr First PCR round</td>
<td>5’-TTTGTGTTTGGTTTTTGTTTGAAGGAGTAAG-3’</td>
<td>54°C, 30 s</td>
</tr>
<tr>
<td>Bisulfite treated Oxtr Second PCR round (Nested PCR)</td>
<td>5’-TTGGAAGTAAGCTCTCTACAACTGATT-3’</td>
<td>54°C, 30 s</td>
</tr>
</tbody>
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TA: Temperatures and time applied for annealing.

doi:10.1371/journal.pone.0056869.t001
DNA Methylation Regulates OXTR Transcription

Animals and tissue processing

Female C57BL/6 mice, 12–16 weeks of age, weighing 20–25 g, were used in all experiments. Animals were housed under diurnal lighting conditions and allowed food and tap water ad libitum. Animals were sacrificed following light anesthesia (Isoflurane, Abbott Laboratories, Abbott Park, IL) by cervical dislocation, and tissues were harvested and immediately frozen in liquid nitrogen and then stored at −80℃ pending analysis.

RNA isolation and qPCR

Total RNA was isolated using the TRI Reagent® kit (Sigma, St. Louis, MO) according to manufacturer’s protocol. 1 μg and 400 ng of total RNA from cell lines and mouse tissues, respectively, underwent reverse transcription to cDNA using the High Capacity RNA to cDNA kit (Applied Biosystems, Foster City, CA). Transcription of the Oxtr was assessed by Real-Time PCR using primers described in Table 1, carried out in triplicates using a 7500 Real-time PCR system (Applied Biosystems) using fluorescent SYBR Green technology. The hypoxanthine-guanine phosphoribosyltransferase (Hprt) gene was used as endogenous control. cDNA equivalent to 15 ng of total RNA was added to a 20 μl total reaction mixture with sequence specific primers at a final concentration of 250 nM.

DNA extraction, bisulfite treatment and sequencing

DNA extraction was carried out using the DNeasy Blood & Tissue Kit (Qiagen, Valencia, CA), according to the manufacturer’s instructions. 200–400 ng of DNA was subjected to a sodium bisulfite treatment using the EpiTect Bisulfite Kit (Qiagen). The Oxtr promoter amplicon was amplified from the bisulfite modified DNA by two rounds of PCR amplification using a hemi-nested primer approach (see Table 1 for primers). In the first round, 3 μl of the modified DNA were amplified in a total volume of 20 μl. Each bisulfite treated sample was amplified in triplicates, which were then pooled together, and 5 μl were subjected to a second round of PCR in a 50 μl total reaction volume. The pooled PCR products of each sample were separated on a 1.5% agarose gel, extracted from the gel with the Illustra GFX™ PCR DNA and Gel Band Purification Kit (GE Healthcare Life Sciences, Piscataway, NJ) and ligated into the pGEM-T-easy vector (Promega, Madison, WI). 2 μl ligation product of each sample were then transformed into DH5α E.coli. At least 20 independent recombinant clones per sample were sequenced at by the NINDS DNA Sequencing Facility (Bethesda, MD) and the methylation data was compiled using the BISMA software (http://biochem.jacobs-university.de/BDPC/BISMA/). The Lower threshold conversion rate was set to 90%, while all the other analysis parameters were set to the BISMA default values. CpG island location was determined using Methyl Primer Express software (Applied Biosystems).

Results

Negative correlation between methylation of the Oxtr promoter and its expression

The mouse Oxtr gene is comprised of four exons and three introns [24]. A CpG island extends from the promoter region (~689) into the coding sequence on the third exon (~2315) (Fig. 1A). The minimal promoter of the gene contains several half-palindromic estrogen receptor elements (half EREs), a palindromic ERE and two SP1 sites (Fig. 1B). We focused our analysis of DNA methylation on a ~400 bp region within the promoter (herein termed amplicon), which contains seven CpG sites. The first CpG site (~932) is located within a half-ERE binding sequence, and the last one, (~735) resides within one of the SP1 binding sequences (Fig. 1C). Both of these transcription factors have been implicated in Oxtr transcription regulation [26]. Thus, DNA methylation in these sites may regulate Oxtr transcription by modulating the binding of the relevant transcription factors to the gene promoter.

To test whether Oxtr transcription is associated with DNA methylation of its promoter, we first used quantitative real-time PCR (qPCR) to compare Oxtr mRNA levels in different mouse-derived cell lines. As shown in Fig. 2A, the GNRH-releasing immortalized GT1-7 neurons express high levels of Oxtr mRNA, ~600-fold higher than the mammary gland-derived 4T1 carcinoma cells. We then used the bisulfite sequencing method to analyze the methylation level of the Oxtr promoter in both cell lines. As depicted in Fig. 2B, the levels of DNA methylation in all seven CpG sites were about 10 fold higher in 4T1 cells compared to GT1-7 cells. The strong negative correlation between the methylation level of the Oxtr promoter and mRNA abundance is consistent with a role of DNA methylation in the transcription regulation of the Oxtr gene. In order to verify that the high Oxtr mRNA levels in GT1-7 cells result in a functional Oxtr protein, we stimulated these cells with OT and measured changes in ERK 1/2 phosphorylation. As shown in Fig. 2C, 10 minutes of OT stimulation caused a marked increase in phosphorylated ERK. Furthermore, exposure of the cells to OT caused a specific elevation in Oxtr mRNA levels (Fig. 2D). Together these results link Oxtr promoter methylation to gene transcription and protein expression.

Demethylation enhances Oxtr mRNA expression in 4T1 cells

To further establish a connection between methylation of the Oxtr promoter and the mRNA levels of Oxtr, we treated both cell lines with the demethylating agent 5-Aza-2’-deoxycytidine (5-AzaC) [27]. GT1-7 cells, which display very low methylation and very high Oxtr expression to begin with, responded to 5-AzaC treatment with a mild reduction in Oxtr mRNA levels (Fig. 3A). The reason for this slight decrease is unclear and may be caused by a general shift in the balance of gene expression in the cells. Conversely, treatment of 4T1 cells with 5-AzaC caused a dose-dependent increase in Oxtr mRNA levels. A maximal ~30 fold increase in mRNA levels was obtained at a concentration of 2 μM 5-AzaC, and a further increase in dose was ineffective (Fig. 3B). The increase in Oxtr mRNA measured in 4T1 cells was accompanied by a significant reduction in the methylation of the Oxtr promoter (Fig. 3C). These results strongly support a relationship between the level of Oxtr promoter methylation and the transcription of the gene.
**In vitro** methylation of the Oxtr promoter reduces reporter gene expression

5-AzaC treatment can affect Oxtr expression either directly through modifying DNA methylation of the gene promoter, or indirectly by modulating other mechanisms of transcriptional regulation. In order to discriminate between these possibilities, we designed a reporter gene assay, where the Oxtr minimal promoter, and the relative location of the PCR amplicon between positions −956 and −541. C) The sequence of the PCR amplicon, including the positions of the seven CpG sites. The ERE harboring CpG site 1 and the SP1 binding sequence harboring CpG site 7 are underlined. doi:10.1371/journal.pone.0056869.g001

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well as the deletion of the whole amplicon (Fig. 4C). When the mean fluorescence values of the treated and untreated versions of each construct were compared, only the Unmodified and Mut 1 constructs displayed significant differences (Fig 4D). Importantly, the mutation at site 7, which is located within the SP1 binding sequence, was as effective as the deletion of the whole amplicon in abolishing the effect of methylation, suggesting that it is the most effective site of methylation in this region. Together these results indicate a direct effect of methylation of the Oxtr promoter on its transcription-inducing activity in cell lines.

Methylation of the Oxtr promoter is modified under different physiological conditions in vivo

After confirming the effect of DNA methylation on the transcription of the Oxtr gene in vitro, we used an in vivo model where Oxtr expression was previously shown to be regulated by hormonal modulation [12,17,18]. First, we compared the methylation of the Oxtr promoter in the uterus and mammary glands of virgin mice. As depicted in Fig. 5A, no significant differences were observed between the two tissues. Interestingly, in both cases we found significantly less methylation at CpG site 2, a phenomenon that differs profoundly from the pattern observed in cell lines (Fig. 3D). Next, we monitored the methylation and transcription of the Oxtr 1 h and 24 h after parturition (early and late post-partum, respectively). As shown in Fig. 5B, Oxtr mRNA levels in the uterus increased by about 10-fold in early post-partum compared to virgin mice and returned to baseline in late post-partum. On the other hand, the Oxtr mRNA levels in the mammary glands increased to the same extent as the uterus in early post-partum but remained high 24 hours after parturition. Analysis of the Oxtr promoter methylation in early post-partum mice revealed opposite changes from virgin mice in both tissues, i.e., methylation is...
increased in the mammary glands and decreased in the uterus (Fig. 5C). Accordingly, in early post-partum mice there is a significant difference in the pattern of Oxtr promoter methylation between the mammary glands and the uterus (Fig. 5D).

The differences between the methylation patterns of virgin and early post-partum mice appear to be CpG-site specific and localized to CpG sites 3–7. Further analysis of the mean methylation averaged over CpG sites 3–7 in the uterus showed a mirror image of the expression levels: mean methylation was reduced during early post-partum and returned to baseline at late post-partum (Fig. 5E). An opposite relationship was observed in the mammary glands, where methylation was elevated both in early and late post-partum mice (Fig. 5E). Finally, examination of the relationship between Oxtr mRNA and mean Oxtr promoter methylation at CpG sites 3–7 across all animals revealed a negative correlation in the uterus, and a positive one in the mammary glands (Fig. 5F). Together these data show that methylation of the Oxtr gene promoter is modified along with its expression levels in a tissue-specific manner in vivo under different physiological conditions.

**Discussion**

In the present study, we explored for the first time the pattern of methylation in the promoter of the murine Oxtr gene and its association with the gene expression in vitro and in vivo. We used two mouse-derived cells lines, the GnRH-releasing GT1-7 immortalized neurons, and the mammary gland-derived 4T1 carcinoma cells to show that DNA methylation has an inhibitory effect on Oxtr transcription. We also revealed distinct modes of correlation between changes in Oxtr promoter methylation and the gene transcription that take place in the mammary glands and uterus around the time of labor. A suppressive effect of DNA methylation on Oxtr expression was found in several studies, which focused on different regions of the human gene [28,29,30]. Mizumoto et al. showed that a regulatory element within the third intron of the gene is significantly more methylated in OXTR-expressing compared to non-expressing tissues [30]. Kusui et al. used human hepatoblastoma cells to show that deletion of a ~400 bp segment within the CpG island extending from the gene promoter, reduces the suppressive effect of methylation by 62% [29]. This result suggests that the remaining effect (38%) may be attributed to the promoter region itself, which is in accordance with our findings. Hypermethylation of the same segment of the human OXTR correlates with low expression levels of the gene in postmortem brain tissues of individuals with autism [28]. Our results differ from these studies by two main aspects: First, we used a mouse model, which is much more accessible for further molecular characterization and manipulation, and second, we analyzed a region of the Oxtr promoter enriched with potential methylated transcription factor binding sites. Assuming that multiple regions of the gene are important for the in vivo effect on Oxtr expression, it is possible that their methylation level is determined in concert by a single regulatory process, which is common to different species and tissues. Alternatively, DNA methylation may act in distinct gene regions in a species- and/or tissue-specific manner. This possibility is supported by our findings.
of opposite modulation of Oxtr promoter methylation between the uterus and mammary glands.

One of the intriguing findings of our study is that CpG sites 1 and 7 are crucial for the overall effect of Oxtr promoter methylation on gene expression (Fig. 4). A mutation in CpG site 7, which is located within a putative SP1 binding sequence, was the most significant one, as it was sufficient to abolish the effect of Oxtr promoter methylation on transcription, similar to deletion of the whole amplicon region. This result raises the possibility that methylation of CpG 7 possibly interferes with the binding of SP1, thus affecting the recruitment of SP1-associated regulatory proteins to the promoter of the Oxtr gene [31]. A similar phenomenon was observed in other studies, where methylation of SP1 binding sites was shown to attenuate its binding to the promoter regions of several genes such as the human extracellular superoxide dismutase [32], the human \( \tau \) receptor adrenergic receptor [33], and the mouse Abcc6 [34] genes, and to impair their cellular or tissue-specific expression.

In addition to CpG site 7, methylation of CpG site 1, located within a half ERE, was also instrumental for the effect of methylation, suggesting a possible interaction between CpG sites 1 and 7. A similar interaction between half ERE and SP1 sites was previously suggested as a mechanism for estrogen–mediated transcription regulation of several dozens of genes including the progesterone receptor, heat-shock protein 27, cathepsin D, uteroglobin and retinoic acid receptor [35,36]. While these genes are all regulated by estrogen, none of them contain palindromic EREs in their promoter regions of several genes such as the human extracellular superoxide dismutase [32], the human \( \tau \) receptor adrenergic receptor [33], and the mouse Abcc6 [34] genes, and to impair their cellular or tissue-specific expression.

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In addition to CpG site 7, methylation of CpG site 1, located within a half ERE, was also instrumental for the effect of methylation, suggesting a possible interaction between CpG sites 1 and 7. A similar interaction between half ERE and SP1 sites was previously suggested as a mechanism for estrogen–mediated transcription regulation of several dozens of genes including the progesterone receptor, heat-shock protein 27, cathepsin D, uteroglobin and retinoic acid receptor [35,36]. While these genes are all regulated by estrogen, none of them contain palindromic EREs in their promoter regions of several genes such as the human extracellular superoxide dismutase [32], the human \( \tau \) receptor adrenergic receptor [33], and the mouse Abcc6 [34] genes, and to impair their cellular or tissue-specific expression.
Figure 5. *Oxtr* promoter methylation changes in vivo. A) The methylation pattern of the *Oxtr* promoter shows no significant difference between the mammary glands (black bars, n = 3) and uterus (gray bars, n = 4) of virgin mice. Nevertheless, a significant difference in methylation was found between CpG sites among all animals. B) *Oxtr* mRNA levels in the mammary glands (black bars, n = 4) and uterus (gray bars, n = 5) are significantly elevated 1 h following parturition (early postpartum). Whereas in the uterus mRNA levels dropped to background 24 h later (late postpartum).
postpartum, n = 3), they remain high in the mammary glands (n = 2). The mean difference in the methylation of the seven examined CpG sites, promoters, plotted as a function of the OXTR promoter, is observed between the mammary glands and the uterus. Mammary glands - Pearson correlation, p < 0.05, R² = 0.48.

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References

Author Contributions
Conceived and designed the experiments: SM HH HG LB SW. Performed the experiments: SM HH RS VS. Analyzed the data: SM HH HS LB SW. Contributed reagents/materials/analysis tools: LB HG. Wrote the paper: HH LB SW.

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postpartum, n = 3), they remain high in the mammary glands (n = 2). The mean difference in the methylation of the seven examined CpG sites, promoters, plotted as a function of the OXTR expression in all animals. A statistically significant positive correlation is seen in the mammary glands (black rectangles), compared to a negative correlation in the case of the uterus (gray circles). Statistics: A) Between conditions - repeated two-way ANOVA F(2)=50.6, p < 0.001, * differ from all others p < 0.005. B) Uterus - one-way ANOVA F(3)=10.1, p < 0.001, * differ from virgins, p < 0.005, * differ from virgins p < 0.01. D) Repeated one-way ANOVA F(1)=6.0, p < 0.05. E) Uterus - one-way ANOVA F(2)=50.6, p < 0.001, * differ virgins p < 0.01. Mammary glands - one-way ANOVA F(1)=11.4, p < 0.005, * differ from all others p < 0.01. F) Mammary glands - Pearson correlation, p < 0.01, R² = 0.61. Uterus - Pearson correlation, p < 0.05, R² = 0.48.

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