Oregon Health & Science University
School of Medicine

Scholarly Projects Final Report

Title

Stromal Cell Influence on Estradiol-Responsive Gene Expression in the Endocervical Epithelium of Rhesus Macaques

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Project Course

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Scholarly Project Final Report

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Project/Research Question

Does the presence of endocervical stromal cells alter hormone-responsive gene expression in Rhesus macaque endocervical epithelial cells?

Sub questions:

1. Does co-culture with stromal cells change epithelial gene expression? (−stromal −E2 vs +stromal −E2)
2. Does treatment with estradiol change epithelial gene expression in monoculture? (−stromal −E2 vs −stromal +E2)
3. Does treatment with estradiol change epithelial gene expression in co-culture? (+stromal −E2 vs +stromal +E2)
4. Is epithelial gene expression different in E2-treated monoculture and E2 treated co-culture? (−stromal +E2 vs +stromal +E2)

Type of Project

Research Study

Key words

Endocervical epithelium
Stromal cell
Estradiol signaling
Cervical mucus regulation
CFTR
PGR
ESR2
qPCR gene expression

Next Steps

Several avenues of future research could further clarify the role of stromal-epithelial interactions in modifying cervical epithelial gene expression.

First, expanding the number of biological replicates would improve statistical power and help confirm the transcriptional patterns observed in this study. Additional experiments examining a broader panel of genes involved in mucus production, ion transport, and hormone signaling could also provide a more comprehensive view of how stromal cells influence epithelial physiology.

Second, evaluating gene expression at the protein level would help determine whether the transcriptional changes observed translate into functional differences in epithelial signaling pathways and mucus regulation. Functional assays examining mucus hydration, ion transport activity, or epithelial barrier properties may provide additional insight into how stromal signaling influences cervical mucus physiology.

Finally, future work could investigate the molecular mechanisms of stromal-epithelial signaling, including the role of stromal-derived growth factors, extracellular matrix signaling, and steroid receptor crosstalk. Identifying these signaling pathways may help clarify how stromal cells regulate epithelial responses to hormonal cues within the cervical microenvironment.

Together, these future studies may help refine in vitro models of cervical epithelial biology and improve understanding of hormone-regulated processes that influence fertility and reproductive tract barrier function.

Introduction

Despite widespread use of hormonal contraception, patient concerns regarding safety and side effects contribute to ongoing demand for effective non-hormonal alternatives[1]. For many patients, dissatisfaction with hormonal methods leads to non-use despite the desire to avoid pregnancy[2]. With unplanned pregnancies constituting nearly half of all pregnancies in the United States[3], the development of additional non-hormonal contraceptive strategies remains an important clinical need.

Methods of predicting fertility during the menstrual cycle constitute most current non-hormonal contraceptive methods outside of sterilization and barrier use[1]. One such predictive method is the tracking of changes in the viscosity of cervical mucus[4] as cervical mucus plays a central role in reproductive physiology by regulating the passage of sperm and microorganisms through the cervical canal. To achieve this regulation, the physical properties of cervical mucus change across the menstrual cycle in response to fluctuating steroid levels, particularly estradiol and progesterone. During the periovulatory phase, estradiol promotes a hydrated and penetrable mucus environment that facilitates sperm transport, and during the luteal phase, progesterone promotes a denser mucus structure that limits sperm transport[5].

These hormone-dependent changes are mediated through transcriptional regulation of epithelial genes involved in mucus production, ion transport, and steroid hormone signaling, and a number of epithelial genes that have been implicated in regulating cervical mucus properties may be considered for druggable targets for non-hormonal contraception[4]. For example, the cystic fibrosis transmembrane conductance regulator (CFTR) plays a role in epithelial ion transport and fluid secretion, processes that contribute to mucus hydration[6]. Dysfunction of CFTR is associated with increased cervical mucus viscosity and lack of cyclical changes, contributing to subfertility[7]. Additionally, steroid hormone receptors, including the progesterone receptor (PGR) and estrogen receptors (ESR1, ESR2), are key mediators of hormone-responsive epithelial gene expression and provide a framework for evaluating transcriptional responses in this system[8].

To examine hormone-responsive expression of such genes, many *in vitro* studies of cervical epithelial biology rely on endocervical epithelial monoculture systems. However, epithelial cells *in vivo* exist within a complex tissue environment that includes stromal cells, extracellular matrix components, and paracrine signaling networks. It has been demonstrated in several reproductive tissues, including vaginal and uterine epithelium, that stromal cells play an important role in mediating epithelial responses to steroid hormones through stromal-epithelial signaling interactions[9-12]. Despite the recognized importance of stromal-epithelial interactions in reproductive biology, relatively few studies have examined how stromal cells influence hormone-responsive gene expression in the endocervical epithelium. Given that most *in vitro* cervical epithelial models lack stromal components, such models may not fully replicate physiologic regulation of epithelial transcription.

The objective of this study was to evaluate whether the inclusion of endocervical stromal cells alters epithelial estradiol-responsive gene expression in endocervical epithelial cultures. Using primary rhesus macaque endocervical epithelial cells cultured either alone or in co-culture with primary endocervical stromal cells, we examined the expression of genes involved in mucus physiology and steroid hormone signaling, including CFTR, PGR, and ESR2, under conditions with and without estradiol treatment.

Methods

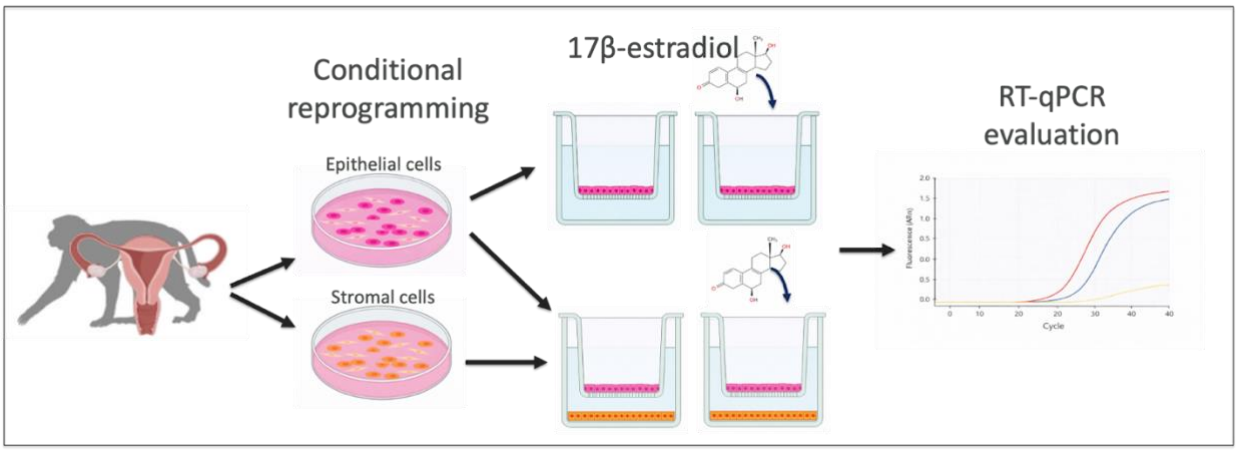

Figure 1. Experimental methods

Primary Cell Isolation, Culture and Hormone Treatment

Endocervical tissues from reproductive age Rhesus macaques were obtained from a tissue disbursement program at the Oregon National Primate Research Center administered by the Division of Comparative Medicine (DCM). The cervix was isolated and digested; epithelial and stromal tissue were separated and expanded separately. Endocervical epithelial cells were co-cultured and maintained using a conditionally reprogrammed culture system as previously described[13]. Stromal cells were cultured and maintained under standard conditions in complete growth media (Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum, penicillin–streptomycin (1%), gentamicin (10 µg/mL), and amphotericin B (0.25 µg/mL). To establish monoculture, epithelial cells were then seeded at $4x10^5$ cells on permeable supports (12 mm diameter, 0.4 µM pore size, polyester Costar Transwell; Corning, Action, MA, USA) in serum-free media (Reprolife CX; Lifeline Cell Technology, Federick, MD, USA) supplemented with calcium (Ca+2) (total concentration = 0.4 mM; Sigma Aldrich, St. Louis, MO, USA) and differentiated at an air-liquid interface (ALI).

To establish co-culture conditions stromal cells were plated at $5x10^5$ cells/well along the bottom wells of the transwell plate. To establish hormone conditions epithelial monocultures and stromal-epithelial co-cultures were then treated with 10^{-8} M 17β-estradiol (E2; Sigma Aldrich) or vehicle control for seven days.

RNA Isolation and Quantitative PCR

Following hormone treatment, total RNA was extracted from epithelial cells with TRIzol (TRIzol RNA+ Mini-Kit, Invitrogen, Gaithersburg, MD, USA). cDNA was then generated from 1µg of RNA using the Superscript III First Strand cDNA Synthesis kit (Invitrogen, Waltham, MA, USA). Quantitative PCR was performed using Power SYBR Green PCR Master Mix (Invitrogen) according to the manufacturer's instructions. Reactions were performed in technical triplicate, and amplification was detected using SYBR Green fluorescence. Gene expression was evaluated for CFTR, PGR, and ESR2 and expression levels were normalized to the reference gene GAPDH.

Statistical Analysis

All statistical analyses were performed using GraphPad Prism version 11.0.0 (GraphPad Software, San Diego, CA).

Each experimental condition included three independent biological replicates, with three technical replicates. Prior to statistical analysis, technical replicates were averaged to generate a single value for each biological replicate.

For reactions in which no amplification was detected, the Ct value of the technical replicate was assigned a value of 40 for downstream analysis. A biological replicate was assigned Ct = 40 only when amplification was undetectable in all three technical replicates.

Relative gene expression was calculated using the comparative ΔΔCt method, and statistical testing was performed on ΔCt values, as these more closely approximate normal distributions compared with fold-change values. Pairwise comparisons between experimental conditions were conducted using two-sided Welch t tests, so as not to assume equal variances between groups. Statistical significance was defined as $p<0.05$. Analyses were exploratory, and no adjustment was made for multiple comparisons because the genes analyzed (CFTR, PGR, and ESR2) were selected a priori based on their established roles in cervical mucus physiology and steroid hormone signaling.

Results

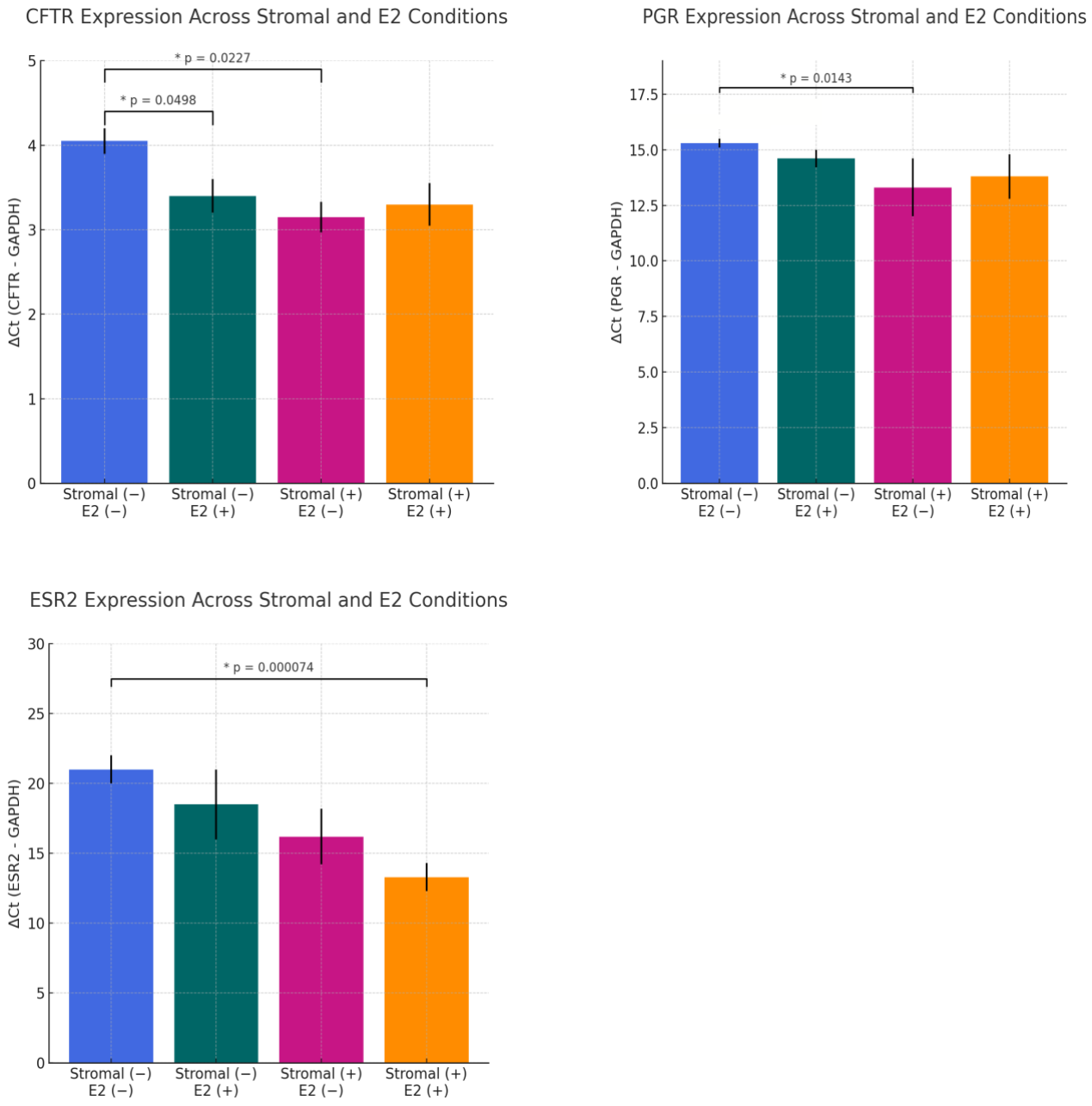

Figure 2. Effect of Stromal Cells and Estradiol on CFTR, PGR, and ESR2 Gene Expression in Primary Rhesus Macaque Endocervical Epithelial Cells. Pairwise comparisons between all stromal (±) and estradiol (±) conditions were performed using Welch's t-tests. Only statistically significant p-values are shown.

Gene	Experimental Condition		Mean ΔCt ± SD	ΔΔCt [vs (−)Stromal, (−)E2]	Fold change ($2^{-\Delta\Delta Ct}$)	p-value
	Stromal	E2				
CFTR	-	-	4.05 ± 0.30	0	1	
	-	+	3.40 ± 0.30	−0.68	1.6	**0.049**
	+	-	3.12 ± 0.10	−0.93	1.9	**0.023**
	+	+	3.31 ± 0.52	−0.74	1.7	0.115
PGR	-	-	15.3 ± 0.24	0	1	
	-	+	14.6 ± 0.36	−0.65	1.6	0.068
	+	-	13.3 ± 0.55	−1.9	3.8	**0.014**
	+	+	13.8 ± 2.10	−1.5	2.7	0.352
ESR2	-	-	20.9 ± 0.54	0	1	
	-	+	18.6 ± 3.52	−2.3	4.9	0.377
	+	-	16.0 ± 3.06	−4.8	29.8	0.105
	+	+	13.5 ± 0.55	−7.4	174.5	**<0.0001**

Table 1. Relative Gene Expression of CFTR, PGR and ESR2 in Endocervical Epithelial Cells Across Stromal and Estradiol Conditions. Gene expression was normalized to GAPDH. ΔΔCt and fold change were calculated relative to epithelial monoculture without estradiol treatment. Lower ΔCt values correspond to higher gene expression. Samples without detectable amplification were assigned Ct = 40. Statistical comparisons were performed using Welch's t test.

Gene	Experimental Condition		Mean ΔCt ± SD	ΔΔCt [vs (+)Stromal (−)E2]	Fold change ($2^{-\Delta\Delta Ct}$)	p-value
	Stromal	E2				
CFTR	+	-	3.12 ± 0.10	0	1	
	+	+	3.31 ± 0.52	0.19	0.88	0.595
PGR	+	-	13.3 ± 0.55	0	1	
	+	+	13.8 ± 2.10	0.47	0.72	0.739
ESR2	+	-	16.0 ± 3.06	0	1	
	+	+	13.5 ± 0.55	−2.5	5.85	0.284

Table 2. Effect of Estradiol Treatment on Endocervical Epithelial Gene Expression of CFTR, PGR and ESR2 in Stromal-Epithelial Co-culture. Gene expression was normalized to GAPDH. ΔΔCt and fold change were calculated relative to stromal-epithelial co-culture without estradiol treatment. Lower ΔCt values correspond to higher gene expression. Samples without detectable amplification were assigned Ct = 40. Statistical comparisons were performed using Welch's t test.

CFTR

Expression of the ion channel gene CFTR differed across stromal and estradiol treatment conditions. In the absence of estradiol, epithelial cells cultured with stromal cells demonstrated higher CFTR expression compared with epithelial monoculture. Mean CFTR ΔCt values were 4.05 ± 0.30 in epithelial monoculture and 3.12 ± 0.10 in epithelial cells cultured with stromal cells (n = 3 per condition), corresponding to an approximately 1.9-fold increase in CFTR expression (p=0.0227) (Table 1, Figure 2).

Estradiol treatment increased CFTR expression in epithelial monoculture, but not in stromal-epithelial co-culture. Epithelial cells cultured alone demonstrated a 1.6-fold increase in CFTR expression following E2 exposure compared with untreated monoculture (p=0.0498) (Table 1, Figure 2). The magnitude of CFTR upregulation observed in epithelial monoculture following estradiol treatment was similar to the increase observed in stromal co-culture under hormone free conditions.

In contrast, estradiol treatment did not significantly alter CFTR expression in epithelial cells co-cultured with stromal cells (Fold change=0.877, p=0.5951) (Table 2). Under estradiol-treated conditions, CFTR expression levels were similar between epithelial monoculture and stromal co-culture conditions (Fold change=1.044, p=0.8659) (Table 1). Notably, the combined stromal + E2 condition did not produce a greater increase in CFTR expression than either stromal co-culture or estradiol treatment alone.

PGR

Expression of PGR, which encodes the progesterone receptor, also differed between monoculture and co-culture conditions. In the absence of estradiol, epithelial cells cultured with stromal cells exhibited higher PGR expression when compared with epithelial monoculture, corresponding to an approximately 3.8-fold increase in PGR expression in the stromal co-culture condition (p= 0.0143) (Table 1, Figure 2.

Estradiol treatment did not significantly alter epithelial PGR expression within either monoculture or stromal co-culture conditions. In epithelial monoculture, estradiol treatment was associated with a modest increase in PGR expression (fold change=1.6), although this difference did not reach statistical significance (p=0.068) (Table 1). In addition, when comparing presence versus absence of stromal cells, PGR expression levels did not significantly differ between monoculture and co-culture when estradiol was present (fold change=1.67, p=0.573) (Table 1).

ESR2

Expression of ESR2, which encodes estrogen receptor beta, demonstrated a distinct pattern across experimental conditions wherein ESR2 transcript detectability increased as stromal cells and estradiol were introduced into the culture system, with the highest expression observed when both stromal cells and estradiol were present (Table 1, Figure 2).

In epithelial monoculture without estradiol treatment, amplification of ESR2 transcripts was not detected in any biological replicate. ESR2 transcripts became detectable in 2 of 3 biological replicates when estradiol was added in the absence of stromal cells. Detection of ESR2 transcripts also increased when epithelial cells were cultured with stromal cells in the absence of estradiol, with detectable amplification observed in 1 of 3 biological replicates. In contrast, ESR2 transcripts were detectable in 3 of 3 biological replicates only when stromal cells and estradiol were both present.

To allow quantitative comparison across conditions, samples in which amplification was not detected after 40 PCR cycles were assigned a Ct value of 40 prior to calculation of relative expression. Under these conditions, epithelial ESR2 expression was markedly increased in the stromal + E2 condition, corresponding to an approximately 174-fold increase in expression, relative to epithelial monoculture without estradiol (p=0.000074) (Table 1, Figure 2).

Discussion

This study examined whether the presence of endocervical stromal cells alters hormone-responsive epithelial gene expression in endocervical epithelial cultures. Using a co-culture model of primary rhesus macaque endocervical epithelial and stromal cells, we found that the presence of stromal cells was associated with differences in gene expression in a gene-specific manner. Three principal observations emerged from this analysis.

First**,** stromal cell presence was associated with increased epithelial expression of genes involved in cervical mucus physiology and hormone signaling. In the absence of estradiol, both CFTR and PGR expression were higher in epithelial cells cultured with stromal cells than those cultured alone. The increase in CFTR expression observed with stromal co-culture parallels estrogen-associated epithelial regulation despite the absence of exogenous hormone, while the corresponding increase in PGR expression suggests that stromal cell presence may increase epithelial capacity for hormone receptor-mediated signaling.

Second, stromal cell presence was associated with differences in epithelial CFTR expression across estradiol treatment conditions. Although both estradiol exposure and stromal co-culture were independently associated with increased CFTR expression, no additional increase was observed when these conditions were combined. This pattern suggests that stromal and estradiol effects on CFTR expression are not additive under the conditions tested, but does not distinguish between whether estradiol fails to further increase stromal-associated expression or whether stromal-associated effects are diminished in the presence of estradiol.

Third, stromal cell presence was associated with marked differences in epithelial ESR2 expression across experimental conditions. Epithelial ESR2 expression was absent in monoculture, partially detected in association with either stromal co-culture or estradiol exposure, and fully detected across all replicates when both conditions were present. Consistent with this pattern, ESR2 expression was significantly increased only in the stromal co-culture with estradiol condition compared with epithelial monoculture without estradiol. These findings suggest that stromal-epithelial interactions may be associated with regulation of epithelial estrogen receptor expression in a hormone-dependent context. Future studies examining ESR1 expression may help determine whether this pattern is conserved across estrogen receptors or specific to ESR2.

This study has several limitations. The sample size for each condition was limited to three biological replicates, which may reduce statistical power and increase sensitivity to biological variability. In addition, ESR2 transcript abundance was low in several conditions, and undetectable amplification reactions were conservatively assigned Ct = 40 for analysis. While this approach allows quantitative comparison across conditions, it may contribute to large fold-change estimates when baseline expression is near the detection threshold. Furthermore, because relative expression was calculated using the ΔΔCt method, differences in baseline gene expression may influence the magnitude of observed fold changes. Elevated baseline expression in stromal co-culture conditions may reduce the apparent fold-change response to estradiol even when absolute transcript levels differ. This limitation may be addressed in future RT-qPCR experiments by incorporating standard curve–based quantification of transcript abundance.

Despite these limitations, differential expression noted in co-culture suggests that further studies to determine the methodological benefit of co-culture are warranted, as well as mechanistic studies to further understand stromal-epithelial cell interactions in the cervix.

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Conclusions

The presence of stromal cells modified epithelial hormone-responsive gene expression in endocervical epithelial cultures. These findings suggest that incorporating stromal components into epithelial culture models may improve the physiologic relevance of in vitro models of cervical epithelial biology.

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