Use of real-time quantitative RT-PCR (qRT-PCR) for assessment of chemical effects on receptivity of human endometrium for embryo implantation

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Introduction

The identification of reproductive toxicants and their mechanisms of action is a major scientific challenge during safety assessment of chemicals. The aim of the *ReProTect*-project is the development of new *in vitro* tests required under the new European chemicals legislation (REACH). We investigate effects of chemicals interfering with the receptivity of human endometrium for embryo implantation [1]. qRT-PCR is applied as highly sensitive method to detect changes in gene expression of numerous pre-selected target genes (s. Table) modulated by drugs and xenobiotics. The most pivotal target is the endometrial progesterone receptor (PR), since embryo implantation is orchestrated by the ovarian steroid progesterone. As models the human endometrial Ishikawa cell line [2] and human endometrial explants cultures [3] are investigated. A similar approach was described to detect modification of mRNA expression after anabolic treatment of farm animals [4].

Gene		Amplicon (bp)	UPL probe	Sequence ID
Target genes				
Leukemia inhibitory factor	LIF	64	26	NM_002309
Cyclooxygenase-2	COX2	70	2	NM_000963
Estrogen receptor alpha	ERa	90	24	NM_000125
Progesterone receptor	PR	74	11	NM_000926
VEGF receptor 2	KDR	66	18	NM_002253
Calcitonin	CALCA	71	15	M26095
CRH receptor 1	CRHR1	78	13	NM_004382
CRH receptor 2	CRHR2	70	9	NM_001883
IGF binding protein	IGFBP1	73	58	NM_000596
Annexin 1	ANXA1	66	80	NM_000700
Glucocorticoid receptor	GR	93	40	NM_000176
Interleukin-15	IL-15	70	78	NM_000576
Prolactin	PRL	60	18	NM_000948
Reference genes				
Glucose-6-phosphate dehydrogenase	G6PDH	107	5	NM_000402
Delta (5)-aminolevulinate-synthase	ALAS1	69	36	NM_199166
Porphobilinogen deaminase	PBGD	92	18	X04808
Hypoxanthine phosphoribosyltransferase	HPRT	102	73	L29382
Phosphoglycerate kinase 1 (PGK1)	PGK1	75	72	NM_000291
RNA polymerase II	RPII	73	1	NM_000937
ß2-microglobulin	ß2M	86	42	AB021288
ß-Actin	ACTb	97	64	NM_001101

Methods

Ishikawa endometrial adenocarcinoma cells (ECACC) were grown for 3 days to subconfluency and subsequently co-incubated for 0.5 -24 hrs with test substances. Endometrial tissues were obtained by aspiration curettage from premenopausal women, chopped into pieces of 1-2 mm/side and co-incubated with test chemicals for 0.5 -6 hrs. qPCR assays from the Universal Probe Library (Roche) were used on a LightCycler 480 instrument in order to being flexible in covering various potential toxicological endpoints. Data analysis was performed by calibrator-normalized relative quantification with efficiency correction. Reference genes were selected by NormFinder and geNORM. Standard curves were established by addition of standard DNA generated by conventional PCR to the sample matrix.

Results of qPCR analysis

For relative quantification high quality standard curves were established by addition of appropriate DNA standards to pooled patient samples. High scattering of replicates was obtained when diluted patient samples were used for standard curve generation (Fig. 1). In endometrial explants mRNA levels of PR and ERα were reduced and those of LIF and COX-2 increased after 6 hrs of culture in the absence of test compounds (Fig. 2). Preliminary data indicate that PR and ERα downreglation is antagonised by estrogenic compounds in proliferative endometrium (not shown). In Ishikawa cells PR mRNA was significantly upregulated by 10^{-6} M diethylstilbestrol (DES; Fig. 3) and a quantitative dose-response-relationship was established for this effect (Fig. 4). Non-estrogenic test compounds did not alter PR mRNA levels. Different time courses were found for the expression of selected target genes in Ishikawa cells. Whereas PR and ERα mRNA levels increased continuously over 24 hrs, LIF mRNA expression peaked after 1-2 hrs after addition of progestagenic test compounds (Fig. 5).

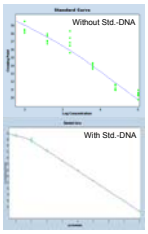

Fig. 1: LIF standard curves ± DNA standard

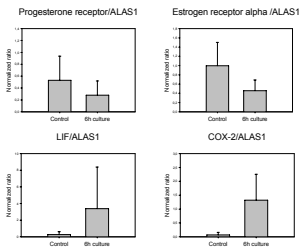

Fig. 2: mRNA levels of various target genes before and after 6 hrs of tissue culture

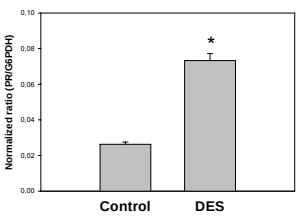

Fig. 3: Upregulation of PR mRNA by 10^{-6} M DES in Ishikawa cells (4 hrs)

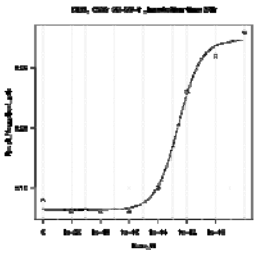

Fig. 4: Dose-dependent up-regulation of PR mRNA by DES in Ishikawa cells (24 hrs)

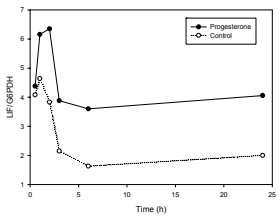

Fig. 5: Time course of LIF mRNA in Ishikawa cells over 24 h culture

Summary

(1) The Universal Probe Library is an useful and cost-efficient tool to make a broad spectrum of qPCR assays rapidly available.
(2) A critical aspect is the generation of standard curves for low and medium expressed genes (e.g. LIF).
(3) qPCR analysis with calibrator normalised relative quantification is a sensitive and rapid method for detecting chemical effects on mRNA expression of pre-selected target genes.
(4) Dose-response-relationships and time courses of substance effects can be evaluated by the described method.

References

[1] Strowitzki et al. (2006) Hum Reprod Update 12:617; [2] Overk (2005) J Agr Food Chem 53:6246; [3] Stavreus-Evers (2003) Reprod Biomed Online 7:243; [4] Reiter (2007) Anal Chim Acta 586:73

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