

# Evidence for oestrogenic regulation of heat shock protein expression in human endometrium and steroid-responsive cell lines

Pei-Zhong Tang<sup>1</sup>, Michael J Gannon<sup>2</sup>, Alison Andrew<sup>3</sup> and David Miller<sup>3</sup>

*Endocrinology Reproductive Research Branch<sup>1</sup>, Institute of Child Health and Human Development, NIH, Bethesda, MD, USA; Academic Unit of Obstetrics and Gynaecology<sup>2</sup>, Leeds General Infirmary, Leeds UK; Institute of Pathology<sup>3</sup>, Department of Clinical Medicine, University of Leeds, Leeds, UK*

Tang P-Z, Gannon MJ, Andrew A, Miller D. Evidence for oestrogenic regulation of heat shock protein expression in human endometrium and steroid-responsive cell lines. *Eur J Endocrinol* 1995;133:598–605. ISSN 0804-4643

Gene amplification with target-specific primers (reverse-transcription polymerase chain reaction (RT-PCR)) was used to monitor the relative expression of oestrogen and progesterone receptor mRNAs alongside the mRNAs for heat shock proteins HSP 90 $\alpha$ , HSP 90 $\beta$  and HSP 70 $\alpha$  in normal samples of human endometrial tissue over the whole menstrual cycle and in short-term cultures of steroid-responsive (T47-D) and unresponsive (HRT-18) cell lines exposed to oestradiol and progesterone over a 24-h incubation period. In endometrium, oestrogen and progesterone receptors followed the expected patterns of expression at the protein level during the menstrual cycle and also showed a positive correlation of expression with each other throughout ( $r = 0.514$ ). Of the HSPs only HSP 90 $\alpha$  expression correlated positively with oestrogen receptor ( $r = 0.687$ ), while HSP 70 $\alpha$  expression, which peaked in the late secretory stage, displayed a significantly inverse correlation with HSP 90 $\beta$  expression ( $r = -0.526$ ). All  $p$  values  $< 0.05$ . In T47-D cell cultures, oestrogen receptor expression was stimulated transiently by oestradiol ( $10^{-7}$  mol/l) and more persistently by progesterone ( $10^{-7}$  mol/l). Progesterone receptor expression was depressed by progesterone and weakly stimulated by oestradiol. HSP 70 $\alpha$  and HSP 90 $\alpha$  expression were stimulated by oestradiol. Progesterone generally depressed HSP 90 $\alpha$  expression and simultaneous addition of both oestradiol and progesterone to the culture medium was antagonistic to HSP 90 $\alpha$  expression. No clear effect of agonist addition on HSP mRNA expression was apparent in the HRT-18 cultures. A possible mechanism for observed oestrogenic effects on HSP expression is put forward.

*David Miller, Institute of Pathology, Department of Clinical Medicine, Algernon Firth Building, University of Leeds, Leeds, LS2 9JT UK*

Although heat shock proteins (HSP) are ubiquitous components of all living systems (1), their roles are only now becoming clearer. Members of the 70 kD family of proteins, for example, are molecular chaperones that serve to maintain nascently synthesized polypeptides in a stable form prior to their achieving a final conformation (2, 3). It has been suggested that heat shock up-regulates the expression of HSP 70 in response to the cytotoxic potential of thermally denatured polypeptides (4). A chaperoning role for HSP 90 has also been demonstrated (5) and an association with steroid receptors in their inactive 8S form has been inferred by *in vitro* studies in cell-free extracts (6, 7). Indeed, reports have indicated that HSP 70 and HSP 56 are also present in the untransformed steroid receptor (8, 9).

As there is considerable interest in the association of HSPs with steroid receptors, and the possibly related phenomenon of HSP 90 $\alpha$  overexpression in human steroid responsive malignancies (10), it is surprising how little is known about the regulation of HSP expression in steroid responsive tissues under physiological conditions. We therefore monitored the mRNA

expression of HSP 90 $\alpha$ , HSP 90 $\beta$  and HSP 70 and the oestrogen and progesterone receptors in 53 samples of human endometrial tissues, covering the whole menstrual cycle, using a sensitive gene amplification assay capable of reliably revealing relative changes in their expression. Endometrial tissue is particularly well suited to this task because it is both readily available and has been characterized extensively with respect to oestrogen and progesterone receptor expression. For comparison, we turned to an *in vitro* cell model for help in explaining our observations.

## Materials and methods

### Reagents

All reagents were of molecular biological grade unless stated otherwise. For RNA isolation, a guanidinium isothiocyanate/phenol solvent as described previously (11) was used throughout. All salts and deoxynucleotides were purchased from Sigma, UK; *Taq* DNA polymerase was obtained from Northumbria Biochemicals (UK) Ltd;

RNAse-free DNase 1 and Maloney murine leukaemia virus (MMLV) reverse transcriptase were purchased from GIBCO-BRL (UK); RNAse inhibitor was purchased from Promega (UK); and [ $^{35}\text{S}$ ] dATP was purchased from New England Nuclear (UK). A Hybaid<sup>TM</sup> halogen thermal cycler was used for all polymerase chain reaction (PCR) runs. Dimethyl sulphoxide (DMSO) was from Sigma, UK.

#### *Subject groups and corresponding tissue samples*

Ethical approval was obtained from the Leeds East and Leeds West Area Health Authorities for the purpose of this study. All samples were obtained from patients already attending gynaecological clinics at Leeds General Infirmary, St James's University Hospital and the Friarage Hospital, Northallerton. Consent for the use of residual tissue was obtained from women who were under investigation for menorrhagia and who had regular periods. Routine endometrial biopsy was carried out by aspiration with a disposable pipette. Only samples from patients with no recent use of hormonal drugs were processed further. Sample dating within the menstrual cycle was based on two criteria: the onset of the last period and direct histological examination by two experienced pathologists. Samples in which staging could not be matched were discarded, leaving 53 that were divided into eight groups as follows: days 1–5 (menstruation, N=8), henceforth referred to as M(1–5); 6–8 (early proliferative, N=6); 9–11 (mid-proliferative, N=9); 12–14 (late proliferative, N=8); 15–18 (early secretory, N=5); 19–22 (mid-secretory, N=5); 23–26 (late secretory, N=8); 27–M(pre-menstruation, N=4).

#### *Cell culture*

Oestrogen and progesterone receptor-positive T47-D cells derived from a human breast cancer tumour and receptor-negative HRT-18 cells derived from a human rectal carcinoma were purchased from the European Cell Culture Collection at Porton Down, Salisbury (ECACC). HRT-18 cells were initially cultured in RPMI 1640 buffer without phenol red (Gibco), supplemented with 10% fetal calf serum (FCS; Life Technologies Ltd.), L-glutamine, penicillin and streptomycin. For T47-D cells, 0.008% bovine insulin, 1 mmol/l oxaloacetic acid and 0.5 mmol/l sodium pyruvate supplemented the above medium. Cells were plated out in Falcon 175-cm<sup>2</sup> flasks and grown in a humidified CO<sub>2</sub> (5%) incubator at 37°C. After 5 days of growth, cells were transferred into identical media except for the inclusion of 10% charcoal-stripped FCS. After a further 5 days of growth under steroid-free conditions, cells were diluted out to a final density of approximately  $2 \times 10^6$  cells per plate and allowed to attach to the plastic substrate for 12 h prior to the experimental procedures outlined below.

#### *Addition of progesterone and oestradiol*

All reagents were of cell culture standard from Sigma (UK). Oestradiol was initially dissolved in DMSO to 1 mol/l prior to its addition to the culture medium at a final concentration of  $10^{-7}$  mol/l. Progesterone was added directly to a final concentration of  $10^{-7}$  mol/l. Reagent-free control flasks were included for each cell line to derive the basal level of gene expression. Cultures were sampled at 0, 1, 3, 6, 12 and 24 h after the addition of agonists and processed for RNA isolation as follows.

#### *Isolation of RNA*

The method was based on that of Ref. 11 with modifications. Briefly, approximately 50–100 mg of endometrial tissue from individual samples was homogenized in solvent and the precipitated RNA was treated with 4  $\mu\text{g}$  of DNase 1 in 100  $\mu\text{l}$  of TE buffer containing 10 mmol/l MgCl<sub>2</sub>, 1 mmol/l DTT and 40 units of RNase inhibitor for 30 min at 37°C to digest any contaminating DNA. The RNA was reprocessed in solvent and both its purity and quantity were assessed by measuring the optical density at 260 nm. For cell cultures, solvent (1 ml) was added directly to the flasks after the medium had been removed and the cells washed twice with phosphate-buffered saline (PBS). The RNA was isolated as for tissues.

#### *Reverse transcription*

Prior to reverse transcription of RNA, approximately 1  $\mu\text{g}$  of each preparation was tested to ensure that DNA had been removed by PCR with primers flanking a short region on the  $\beta$ -actin gene (12). Reverse transcription was carried out essentially as described previously (13) except that 5  $\mu\text{l}$  of cDNA product per sample was amplified in a duplexed PCR reaction containing 25 pmol of  $\beta$ -actin primers and primer flanking regions on the human gene for HSP 90 $\alpha$  (14), HSP 90 $\beta$  (15), HSP 70a (16), oestrogen receptor (12) or progesterone receptor (17) (see Table 1). For quantification of PCR products, [ $^{35}\text{S}$ ]dATP (0.04 mmol/l; 4  $\mu\text{Ci}$ ) was included in the reaction mix. The PCR conditions included an initial denaturation step at 95°C for 5 min, followed by 36 cycles of 1 min denaturation at 95°C, 1 min of annealing at 57°C and a 1-min extension at 72°C. A final extension at 72°C for 5 min was carried out prior to analysis. A number of reactions were carried out following substitution of  $\beta$ -actin primers with primers specific for an alternative "housekeeping" gene, glyceraldehyde-3-phosphate dehydrogenase (GA3PDH) (18). This enabled us to evaluate the constancy of  $\beta$ -actin expression in endometrial tissues and hence its effectiveness as an internal reference.

#### *Electrophoresis and quantification of products*

Sample aliquots were resolved on 1-mm thick 5%

Table 1. Primer sequences used in the polymerase chain reaction.<sup>a</sup>

Gene	Primer sequences		Product size (bp)
	5'-sense-3'	5'-antisense-3'	
$\beta$ -Actin	ATCATGTTTGAGACCTTCAA	CATCTCTTGCTCGAAGTCCA	318
ER	GGAGACATGAGACTGCCAAC	CCAGCAGCATGTCGAAGATC	438
PR	GCAGATCCACAGGAGTT	TGAGCTCGACACAACCTCC	196
HSP 90 $\alpha$	ACAAGCACATATGGCTGGACAGCA	TTCAGTTACAGCAGCACTGGTATC	342
HSP 90 $\beta$	ACCAGCACCTACGGCTGGACAGCC	GATCTCATCAGGAAGTGCAGCATT	348
HSP 70a	CTAGCCTGAGGACTGGCTGCGACA	GTTCCCTGCTCTGTCTGGCTCGG	205
GA3PDH	GAGTCAACGGATTGTGGTCGT	GGTGCCATGGAATTTGCCAT	156

<sup>a</sup> ER = oestrogen receptor; PR = progesterone receptor; GA3PDH = glyceraldehyde-3-phosphate dehydrogenase.

polyacrylamide gels in 50 mmol/l TRIS and 5 mmol/l EDTA. After 60 min of electrophoresis at 120 V, PCR product bands were visualized by drying and exposing the gels to autoradiographic film for 48 h at room temperature. Corresponding bands on the gel were excised carefully and their radioactivity counted in an LKB liquid scintillation counter. Raw data (cpm/band) were converted into ratios relative to the  $\beta$ -actin (or GA3PDH) internal control.

The main advantage of this relativistic assay over more absolute assays is that it is simple to perform and, provided that products are obtained in the linear range of the PCR, it is unnecessary for the precise quantity of template mRNA/cDNA to be determined beforehand. The efficiency of reverse transcription to cDNA for each mRNA under investigation and hence the overall reproducibility of the method was assessed using data accumulated from frozen-stored samples of normal endometrial tissue processed for duplex RT-PCR at three different times over an 18-month period, in quadruplicate. The high degree of reproducibility in ratios obtained relative to  $\beta$ -actin for each target (< 5% and < 3% deviation for inter and intra-batch mean ratios, respectively) assured us that sample processing was equally efficient and that any subsequent variations were due to actual differences and not to processing artefacts.

### Statistical analysis

Relationships between the expression of all genes under investigation were determined by correlation *t*-test analysis.

### Results

All data refer to cDNA levels for target mRNAs relative to  $\beta$ -actin mRNA, unless stated otherwise.

### Endometrial tissue

Although progesterone receptor (PR) levels were generally lower than oestrogen receptor (ER) the relative expression of these receptors was broadly similar

throughout the cycle, reaching maximal levels during the late proliferative/early secretory stages (Fig. 1A; days 12–14). Expression of HSP 70a rose almost constantly throughout the cycle, reaching a peak in the late secretory stage (Fig. 1B; days 23–26). There was no similarity in the expression of the two HSP 90 genes, with HSP 90 $\beta$  levels being generally much lower than the corresponding values for HSP 90 $\alpha$ . Moreover, while HSP 90 $\alpha$  expression followed a broadly similar pattern to that observed for both ER and PR, plateauing at the late-proliferative/early secretory stage (Fig. 1C; days 12–18), HSP 90 $\beta$  expression fell gradually throughout the cycle (Fig. 1D).

The scatter plots shown in Fig. 2 represent the closest correlations of expression found from the mean data (total *N* = 53) for each time point in the cycle. The best of these was for ER relative to HSP 90 $\alpha$  expression (Fig. 2A; *p* < 0.05, *r* = 0.687), followed by that between ER and PR expression (Fig. 2C; *p* < 0.05, *r* = 0.514). An inverse correlation of expression was found between HSP 70a and HSP 90 $\beta$  expression (Fig. 2D; *p* < 0.05, *r* = 0.526). The weakest correlation was between PR and HSP 90 $\alpha$  expression, as suggested by Fig. 1A, C, although this just failed to reach significance for averaged data (Fig. 2B; *p* > 0.05, *r* = 0.245).

### Cell culture

The outcome of a time course of exposure of T47-D cells and HRT-18 cells to oestradiol ( $10^{-7}$  mol/l) and progesterone ( $10^{-7}$  mol/l) over a 24-h period is shown in Figs. 3 and 4. In contrast with T47-D cells, the HRT-18 cell line does not contain either ER or PR mRNAs, as indicated by the failure to detect the appropriate PCR products from these cells (not shown). Hence, only the effect on ER and PR expression in T47-D cells was measurable (Fig. 3). Oestradiol weakly stimulated both ER and PR expression, with the latter being maximally stimulated 6 h after the addition of agonist and for the duration of the experiment thereafter (Fig. 3). Progesterone had no effect on ER expression (Fig. 3A), while PR expression was depressed overall (Fig. 3B).

The HRT-18 cells were used to "bench-mark" sex

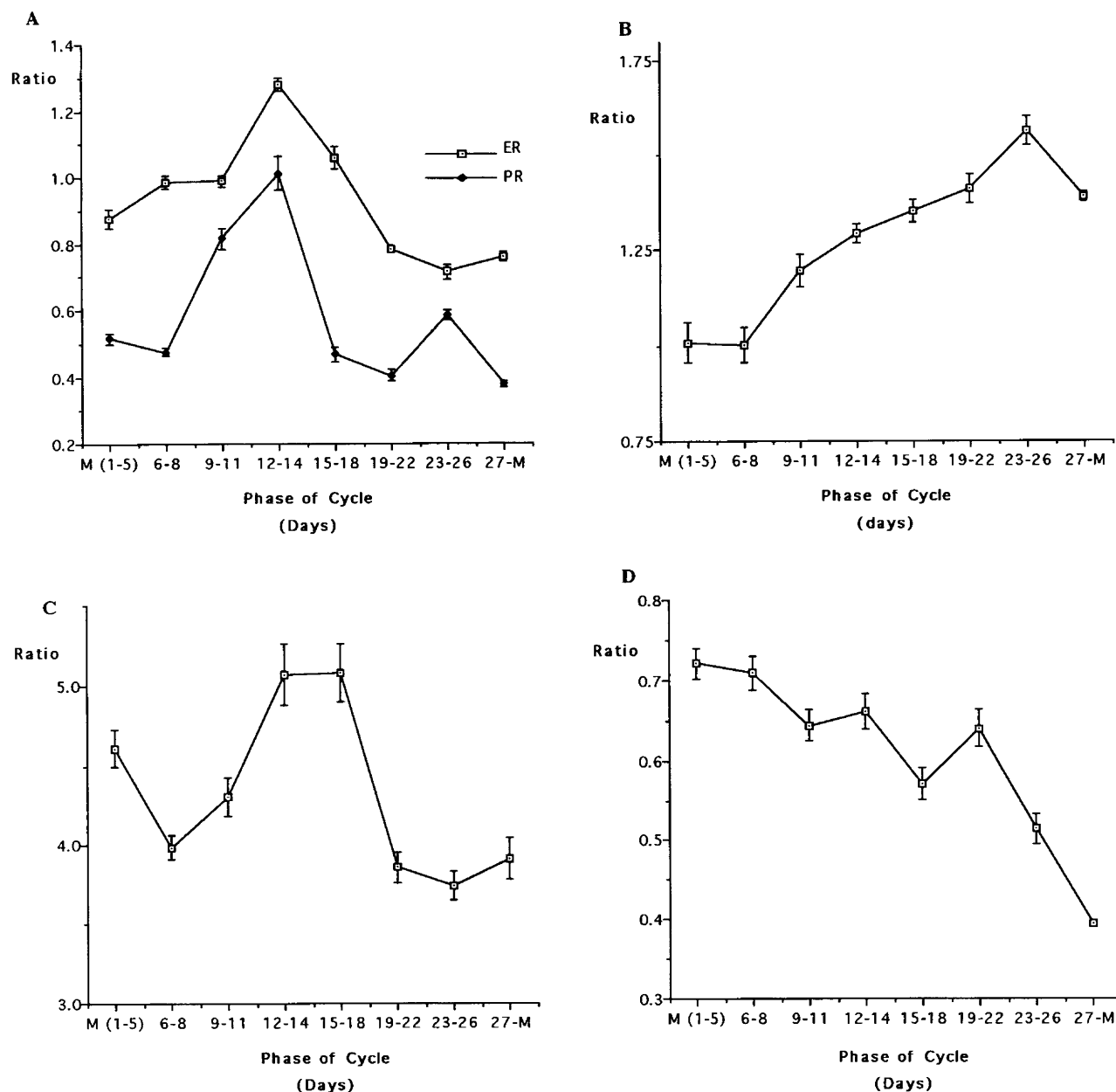


Fig. 1. Mean  $\pm$  SEM ratios for cDNA corresponding to (A) oestrogen receptor (ER) and progesterone receptor (PR), (B) HSP 70a, (C) HSP 90 $\alpha$  and (D) HSP 90 $\beta$  in normal cycling human endometria. All ratios are relative to  $\beta$ -actin.

steroid-mediated changes in HSP expression in a receptor-negative environment. Comparing the two cell lines indicated that HSP 70a expression was stimulated in responses to oestradiol in T47-D cells only, while there was no discernable difference in the response to progesterone in either cell line (Fig. 4A,B). Expression of HSP 90 $\alpha$  was also stimulated by oestradiol in T47-D cells and remained higher over the 24 h incubation period (Fig. 4C). In contrast, stimulation was weak and transient in the HRT-18 cells (Fig. 4D). Progesterone rapidly suppressed T47-D HSP 90 $\alpha$  expression in the first 3 h of incubation, before

rising back to control levels by 6 h (Fig. 4C). When both agonists were added simultaneously to the culture medium, they appeared to act antagonistically. There was no observable change in HSP 90 $\beta$  expression following the addition of either agonist to both cell lines (Fig. 4E,F).

#### *Efficacy of $\beta$ -actin as internal reference in duplexed reactions*

We initially used  $\beta$ -actin as our internal reference, as

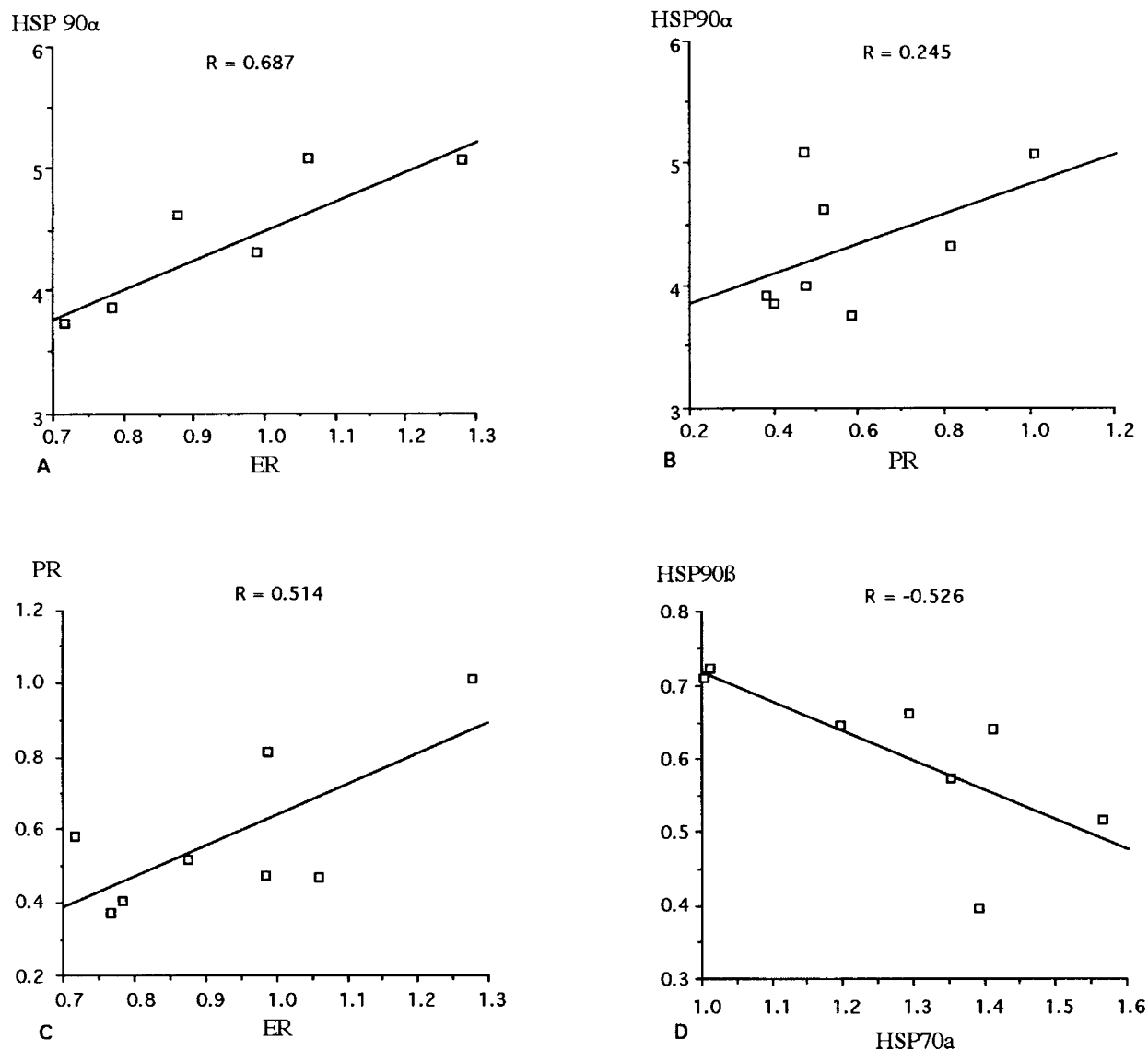


Fig. 2. Scatter plots of mean ratios for cDNA levels and correlation coefficients of: (A) HSP 90 $\alpha$  vs oestrogen receptor (ER) ( $r = 0.687$ ); (B) HSP 90 $\alpha$  vs progesterone receptor (PR) ( $r = 0.245$ ); (C) PR vs ER ( $r = 0.514$ ); (D) HSP 90 $\beta$  vs HSP 70a ( $r = -0.526$ ). All  $p$  values  $< 0.05$  except for (B). Total number of samples (N) = 53. All ratios are relative to  $\beta$ -actin.

has been the common practice in many relativistic studies owing to its fairly constant level of expression in numerous cells and tissues (12, 19, 20). However, because there is some concern about its overall stability, we cross-checked its efficacy by comparison with GA3PDH expression in a selection of endometrial tissues comprising four normal and eight abnormal (carcinoma in situ) samples. These had widely divergent HSP 90 $\alpha$ / $\beta$ -actin ratios, which were plotted against the corresponding HSP 90 $\alpha$ /GA3PDH ratios, and indicated a strongly positive correlation coefficient ( $r = 0.873$ ,  $p < 0.005$ ) (data not shown). This not only demonstrates the suitability of  $\beta$ -actin as a reference in this context, but that levels of HSP 90 $\alpha$  are consistently

higher in abnormal samples of endometrial tissue (to be detailed at a later date).

## Discussion

Ours is the first report to deal with HSP gene expression in normal cycling endometrium under physiological conditions at the mRNA level, although protein expression has been described in human endometrial tissues under heat shock conditions (21). The small samples of tissue that are available following endometrial biopsy generally preclude Northern or Western blot analysis. We therefore resorted to a highly sensitive RT-PCR method for this study, which can reveal relative

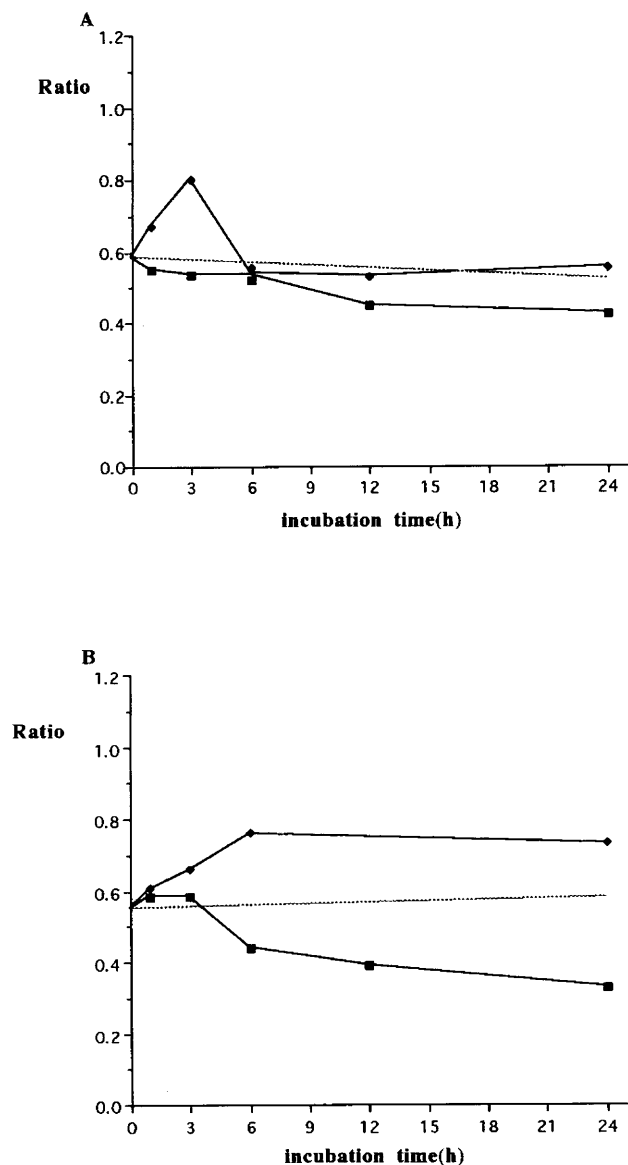


Fig. 3. Effect on (A) oestrogen receptor (ER) and (B) progesterone receptor (PR) expression of  $10^{-7}$  mol/l oestradiol (◆) and  $10^{-7}$  mol/l progesterone (■) in T47-D cells. Baseline controls are indicated by a broken line. All ratios are relative to  $\beta$ -actin.

differences in the expression of mRNAs without actually quantifying them. Analysing samples from 53 women at various stages in the cycle showed varying patterns of mRNA expression for HSP 90 $\alpha$ , HSP 90 $\beta$ , HSP 70A, ER and PR, consistent with stage-related changes in gene expression. We believe that these changes are representative for the following reasons.

Natural variations in the level of ER and PR proteins in the normal cycling endometrium have been documented extensively (22–25) and it is widely recognized that ER and PR levels are higher in proliferative than in secretory stages of the cycle, with the highest levels measured in the late proliferative

stage (22, 23). Our RT-PCR-based data concord reasonably with these reports, indicating its reliability for both ER and PR levels and, by extension, the observed levels of HSPs in these tissues. Exact matches are unlikely to be obtained, however, because maximal mRNA expression may precede that obtained for actual receptor proteins owing to possible delays in translation.

Our observations suggesting an oestrogenic effect on HSP 90 $\alpha$  expression in vivo (endometrial tissues) and in vitro (cell cultures) are supported by several reports dealing with the stimulation of expression of this HSP (and its homologues) in response to exogenous steroids. For example, tissue-specific up-regulation of chick HSP 108 (which is 50% homologous to yeast HSP 90) has been reported in response to oestradiol, progesterone and dexamethasone (26). More recently, of the two recognized genes of murine HSP 90, HSP 86 (corresponding to human HSP 90 $\alpha$ ) has been shown to be much more sensitive to oestrogenic stimulation than HSP 84, which is homologous to human HSP 90 $\beta$  (27). HSP 90 $\alpha$  is overexpressed in human breast and ovarian cancers, giving rise to speculation about its use as a prognostic indicator (10, 20, 28). Northern analysis has revealed that HSP 90 $\alpha$  is up-regulated by oestradiol in MCF-7 and T47-D cell lines but not in ER-negative MDA-MB-231 cells. Finally, a positive correlation of expression between this HSP and ER has been reported in breast cancer (29). These results suggest that only HSP 90 $\alpha$  is sensitive to steroids and that its regulation by oestradiol, for example, is only possible in tissues that harbour their receptors. However, reports of HSP 70 up-regulation in response to steroids are more ambiguous, with some workers reporting no differences (30) and others reporting increases, again in a tissue-specific pattern (31).

The gradual rise in HSP 70a (the inducible isovariant of this family) expression observed during the cycle is likely to be due to the increase in growth and molecular chaperoning of cellular molecules during the endometrial thickening that occurs as the glandular epithelium develops. The inverse correlation of expression with HSP 90 $\beta$  may be significant in this respect, but we are presently unable to confirm this. The seemingly wide divergence in both HSP 70a and HSP 90 $\beta$  expression during menstruation compared with the late proliferative stages is possibly a result of the physico-biochemical effects of menstruation and not to any hormonal changes relevant to the cycle per se.

Although the weak responses to agonists in HRT-18 cells are probably a result of non-steroidal effects of the agonists themselves, it could be argued that in the context of this study the HRT-18 cells are inappropriate for comparing steroidogenic responses in receptor-negative cells because equivalent breast-derived cell lines are available. There were indications, however, that breast-derived substitutes harbour PR (if not ER) mRNA while the rectal cells contain neither and,

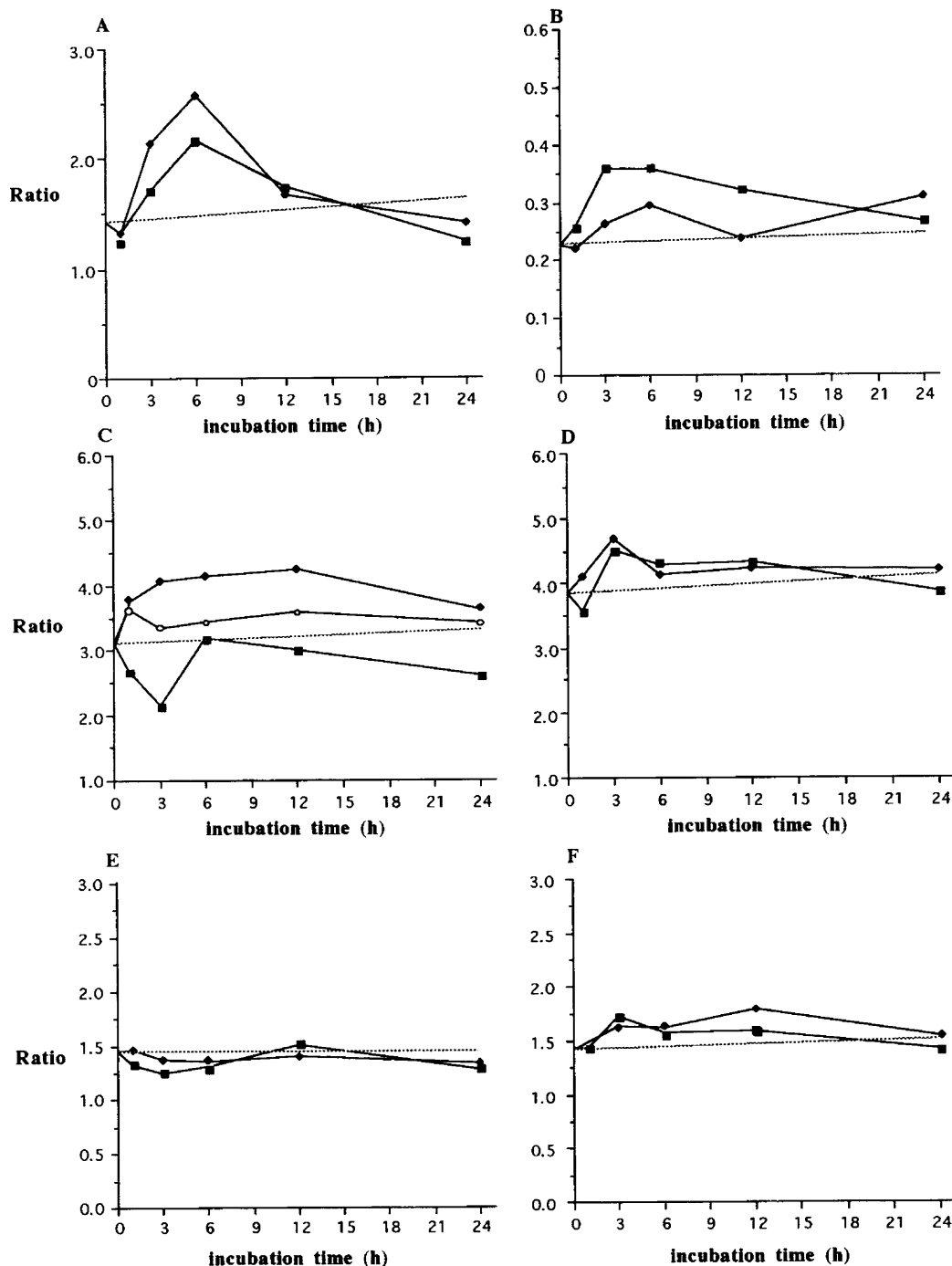


Fig. 4. Effect on HSP 70a (A, B), HSP 90α (C, D) and HSP 90β (E, F) expression of  $10^{-7}$  mol/l oestradiol (◆) and  $10^{-7}$  mol/l progesterone (■) in T47-D cells (A, C, E) and HRT-18 cells (B, D, F). Concurrent addition of both oestradiol and progesterone is indicated by open circles in (C) only. Baseline controls are indicated by a broken line. All ratios are relative to β-actin.

because it was considered important to use a system that has no receptors at the mRNA level, it can equally be argued that most cells or tissues fulfilling these criteria, including HRT-18 cells, could have been used.

For an explanation of the selective oestrogenic effects on HSP expression it was reasonable to look for

oestrogen response element (ERE)-like sequences in the genes themselves. Based on the available sequence data (14, 16) an imperfect ERE-like sequence in the 5' untranslated regions of the HSP 70a gene ( $5'$ -GGTCTnnnTGACG- $3'$ ) and, more convincingly, in the HSP 90α gene ( $5'$ -GGTCAnnnTAGCC- $3'$ ) is available. If

these ERE-like sequences can be shown to bind ER, then this may explain why specific HSP genes are up-regulated by oestradiol in an ER-dependent tissue/cell-specific pattern. Because HSP 90 $\beta$  has no ERE-like sequences, it would not be expected to respond to the presence of oestradiol.

We believe that the oestrogenic effects on HSP 90 $\alpha$  expression observed in this study are related to the putative functional association between this protein and steroid receptors in that the dynamic equilibrium of that association (in oestrogen-responsive tissues) may be under the control of ER. Indeed, the unusually high levels of HSP 90 $\alpha$  reported in endometrial and breast carcinomas may serve to limit ER-mediated cellular proliferation by binding to excess ER and work is currently in progress aimed at testing this hypothesis.

## References

1. Nover L, Scharf K-D. Heat shock proteins. In: Nover L, editor. Heat shock response. New York: CRC Press, 1991:373–408
2. Craig EA, Weissman JS, Horwich AL. Heat-shock proteins and molecular chaperones—mediators of protein conformation and turnover in the cell. *Cell* 1994;78:365–72
3. Gatenby AA, Viitanen PV. Structural and functional aspects of chaperonin-mediated protein folding. *Ann Rev Plant Physiol Plant Mol Biol* 1994;45:469–91
4. Parsell DA, Lindquist S. The function of heat-shock proteins in stress tolerance-degradation and reactivation of damaged proteins. *Ann Rev Genet* 1993;27:437–96
5. Parsell DA, Kowal AS, Singer MA, Lindquist S. Protein disaggregation mediated by heat-shock protein HSP 104. *Nature* 1994, 372:475–8
6. Pratt WB. Control of steroid-receptor function and cytoplasmic-nuclear transport by heat-shock proteins. *Bioassays* 1992;14: 841–8
7. Bagchi MK, Tsai M-J, O'Malley BW, Tsai SY. Analysis of the mechanism of steroid hormone receptor-dependent gene activation in cell-free systems. *Endocr Rev* 13:525–535.
8. Catelli MG, Binart N, Jung-Testas I, Renoir IM, Baulieu EE, Feramisco JR, et al. The common 90kDa protein of non-transformed steroid receptors is a heat shock protein. *EMBO J* 1985;4:3131–40
9. Smith DF, Toft DO. Minireview: steroid receptors and their associated proteins. *Mol Endocrinol* 1993;7:4–11
10. Jameel A, Law M, Coombers RC, Luqmani YA. Significance of heat shock protein 90 as a prognostic indicator in breast cancer. *Int J Oncol* 1993;2:1075–80
11. Siebert P, Chenchik A. Modified acid guanidinium thiocyanate–phenol–chloroform RNA extraction method which greatly reduces DNA contamination. *Nucl Acids Res* 1993;21:2019–20
12. Fuqua SAW, Falette NF, McGuiire WL. Sensitive detection of estrogen receptor RNA by polymerase chain reaction assay. *J Natl Cancer Inst* 1990;82:858–61
13. Miller D, Tang P, Skinner C, Lilford R. Differential RNA fingerprinting as a tool in the analysis of spermatozoal gene-expression. *Hum Reprod* 1994;9:864–9
14. Hickey E, Brandon SE, Smale G, Lloyd D, Webber LA. Sequence and regulation of a gene encoding a human 89-kilodalton heat shock protein. *Mol Cell Biol* 1989;9:2615–26
15. Rebbe NF, Hickman WS, Ley TJ, Stafford DW, Hickman S. Nucleotide sequence and regulation of human 90-kDa heat shock protein gene. *J Biol Chem* 1989;264:15006–11
16. Hunt C, Morimoto RI. Conserved features of eukaryotic hsp-70 genes revealed by comparison with the nucleotide sequence of human hsp-70. *Proc Natl Acad Sci USA* 1985;82:6455–9
17. Misrahi M, Atger M, D'Auriol L, Loozeelt H, Meriel C, Fridlansky F, et al. Complete amino acid sequence of the human progesterone receptor deduced from cloned cDNA. *Biochem Biophys Res Commun* 1987;143:740–8
18. Paludan K, Thestrup-Pedersen K. Use of the polymerase chain reaction in quantification of interleukin-8 mRNA in minute epidermal samples. *J Invest Dermatol* 1992;99:830–5
19. Dallman MJ, Porer ACG. Semi-quantitative PCR for the analysis of gene expression. In: McPherson MJ, Quirke P, Taylor GR, editors. PCR: a practical approach. Oxford: IRL Press, 1992:215–24
20. Mileo AM, Fanuele M, Battaglia F, Scambia G, Benedetti-Panici P. Selective over-expression of mRNA coding for 90kDa stress-protein in human ovarian cancer. *Anticancer Res* 1990;10:903–6
21. Ron A, Birkenfeld A. Stress proteins in the human endometrium and decidua. *Hum Reprod* 1987;2:277–80
22. Bayard F, Dalmilano S, Robel P, Baulieu EE. Cytoplasmic and nuclear oestradiol and progesterone receptors in human endometrium. *J Clin Endocrinol Metab* 1978;46:635–48
23. Bergqvist A, Ekman R, Ljungberg O. Binding of estrogen and progesterone to human endometrium in the different stages of the menstrual cycle. *Am J Clin Pathol* 1985;83:444–9
24. Garcia E, Bouchard P, Bruix DE, Berdah J, Frydman R, Schaison G, et al. Use of immunocytochemistry of progesterone and estrogen receptors for endometrial dating. *J Clin Endocrinol Metab* 1988;67:80–7
25. Lessey BA, Killan AP, Metzger DA, Haney AF, Greene GL, McCarty KS. Immunohistochemical analysis of estrogen and progesterone receptors throughout the menstrual cycle. *J Clin Endocrinol Metab* 1988;67:334–40
26. Baez M, Sargan DR, Elbrech A, Kulomma MS, Zarucki-Schultz T, Tsai M-J, et al. Steroid hormone regulation of the gene encoding the chicken heat shock protein HSP 108. *J Biol* 1987;262:6582–8
27. Shyamala G, Gauthier Y, Moore SK, Catelli MC, Ullrich SJ. Oestrogenic regulation of murine uterine 90-kilodalton heat shock protein gene expression. *Mol Cell Biol* 1989;9:3567–70
28. Jameel A, Skilton RA, Campbell TA, Chamder SK, Coombers RC, Luqmani YA. Clinical and biological significance of HSP89 alpha in human breast cancer. *Int J Cancer* 1992;50:409–15
29. Shyamala G, Schweitzer M, Ulrich SJ. Relationship between 90kDa heat shock protein, estrogen receptor and progesterone receptor in human mammary tumours. *Breast Cancer Res Treat* 1993;26:95–100
30. Olazabal UE, Pfaff DW, Mobbs CV. Sex differences in the regulation of heat shock protein 70kDa and 90kDa in the rat ventromedial hypothalamus by estrogen. *Brain Res* 1992;596: 311–4
31. Krawczyk Z, Schmid W, Harkonen P, Wolnirzek P. The ERE-like sequence from the promoter region of the testis specific hsp-70 related gene is not estrogen responsive. *Cell Biol Int Rep* 1992;16:937–48

Received January 30th, 1995

Accepted June 29th, 1995