ESTROGENIC REGULATION OF CLUSTERIN mRNA IN NORMAL AND MALIGNANT ENDOMETRIAL TISSUE

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Clusterin is a heterodimeric, 80kDa, glycoprotein that is synthesized in a wide variety of tissues in response to a number of diverse stimuli, including hormone ablation. We have investigated the regulation of clusterin expression by estradiol and anti-estrogens in RUCA-I rat endometrial adenocarcinoma cells in vitro and in vivo. We have also compared clusterin expression in endometrial tumors and in normal uterine tissue. Estradiol treatment significantly increases the steady state mRNA levels of clusterin in RUCA-I cells cultured on a reconstituted basement membrane, with a maximal induction 24 hr after estradiol treatment. The inductive effects of estrogen on clusterin mRNA steady state levels in vitro are significantly more pronounced than the effects on fibronectin mRNA levels, an estrogen-repressed gene in RUCA-I. In vivo, induction of clusterin expression in primary and metastatic endometrial adenocarcinoma is also dependent on the presence of estradiol, in marked contrast to expression of clusterin in the normal endometrium of the same animals. These data suggest that clusterin mRNA expression in rat endometrial adenocarcinoma cells is tightly regulated by estrogens and anti-estrogens in vitro and in vivo, and that there is a complex mechanism of regulation of clusterin expression in the normal and cancerous endometrium. Int. J Cancer 76:684–688, 1998.
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Clusterin, also known as testosterone-repressed message-2 (TRPM-2), sulphated glycoprotein-2 (SGP-2) and apolipoprotein J (ApoJ), was first isolated from the ram rete testis fluid (Blaschuk et al., 1983) The 80 kDa, heterodimeric glycoprotein and its mRNA have been identified in many tissues and has been identified in a variety of species (Jenne and Tschopp, 1992). In many hormone-dependent tissues, such as prostate and mammary gland, clusterin expression is induced after hormone ablation and appears to be involved in the apoptotic processes associated with tissue remodelling (Tenniswood et al., 1992). For example, clusterin expression is induced following hormone ablation in regressing, androgen-responsive, Shionogi mouse mammary tumors (Akakura et al., 1996). In this model system, the expression of clusterin remains elevated when the tumors become hormone-independent and fail to initiate apoptosis after hormone deprivation (Akakura et al., 1996). In MCF-7 breast cancer cells it has been shown that estrogen withdrawal in vivo (Kyprianou et al., 1991 and treatment with vitamin D3 analogs (Simboli-Campbell et al., 1996) induce apoptosis and increase clusterin expression. In the uterus, hormone ablation induces apoptosis of the epithelial tissue compartment and the expression of clusterin in the luminal and glandular epithelial cells (Brown et al., 1995). The expression of clusterin in the mouse uterus, while it requires estrogens, appears to be induced primarily in response to the fall in progesterone levels, which is usually associated with the tissue remodeling that occurs after regression (Brown et al., 1995).

We have established an estrogen receptor positive, rat endometrial adenocarcinoma cell line, RUCA-I (Schütze et al., 1992). In vitro, despite relatively high levels of estrogen receptor, this cell line only responds to treatment with estradiol if cultured on a reconstituted basement membrane (Vollmer et al., 1995a). When grown on the appropriate substratum these cells exhibit estrogenic inducibility of proliferation and complement C3 expression and repression of fibronectin expression by estrogens (Vollmer et al., 1995b). When injected into the flank of syngeneic animals, the

RUCA-1 cell line gives rise to estrogen sensitive, metastasizing endometrial adenocarcinoma, which responds to pure antiestrogens, such as ZK 119,010, ICI 164,384, and ICI 182780, and to antiestrogens exhibiting partially agonistic activities, such as tamoxifen (Vollmer and Schneider, 1997). RUCA-I cells, cultured on a reconstituted basement membrane or grown in vivo, therefore represent an excellent endometrial derived cell culture model to study estrogenic regulation of gene expression in vivo and in vitro.

Since the clusterin promoter contains a complex array of potentially important hormone response elements (Wong et al., 1993), and since increased clusterin expression has been found in various normal and malignant processes, we have investigated the estrogen sensitivity of clusterin expression in the RUCA-1 rat endometrial tumor model. We have demonstrated that clusterin expression in this model system is estrogen-sensitive, both in vitro and in vivo, and we provide evidence that regulation of clusterin expression in the tumors in vivo is diametrically reversed when compared to the expression in the normal uterus of the host animal.

MATERIAL AND METHODS

Hormones

The 17β-estradiol and tamoxifen were obtained from Sigma (Deissenhofen, Germany), ICI 164,384 was provided by Dr. A.E. Wakeling (Zeneca, Macclesfield, UK), ICI 182,780 was synthesized at Schering (Berlin, Germany).

Cell culture

Prior to experimental use, RUCA-I cells (from frozen stocks of passage 30) were cultured for one passage in DMEM/F12 medium without phenol red containing 10% fetal calf serum, and for 2 passages in the above medium containing 5% dextran-coated charcoal treated FCS. For routine experiments 2.5—3.0 × 10^5 cells were seeded on 300 μl of ECM-substrate (Harbour matrix; Cell Systems, Remagen, Germany) per well of a 24-well plate in the presence of 2 ml serum-free defined medium (DMEM/F12 supplemented with 2 μg/ml insulin, 4 mM glutamine, 40 μg/ml transferrin, 10^{-8} M hydrocortisone, 2 × 10^{-8} M sodium selenite, and 1 μg/ml putrescine). The cells were maintained in a humidified 5% CO_2 atmosphere at 37°C and medium was changed daily.

Hormonal treatment of RUCA-I cells in vitro

Prior to hormonal treatment, 2.5—3.0 × 10^5 RUCA-1 cells were seeded on ECM-substrate and cultured in serum free defined medium for 24—48 hr. The cells were then incubated for 2—48 hr with 10^{-7} M estradiol in the absence or presence of 5 × 10^{-7}M ICI 164,384; 5 × 10^{-7}M ICI 182,780; or 10^{-6} M tamoxifen. These

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concentrations were chosen to ensure that the estrogen and anti-estrogens were present in approximately equipotent concentrations based on their relative binding affinities to the estrogen receptor (Wakeling et al., 1991). Control cultures received ethanol, which was used as vehicle. Medium was changed daily and hormonal treatment was repeated.

In vivo growth of RUCA-1 cells

RUCA-I cells (from frozen stocks of passage 30) were cultured for one passage on plastic in DMEM/F12 medium without phenol red containing 10% charcoal stripped FCS, and harvested by trypsinization. Seven to eight week-old female DA/Han rats were ovariectomized under ether anesthesia 48 hr prior to the s.c. injection of 10^6 RUCA-I cells into the right flank. The animals were then randomized into 5 groups of 4 animals: untreated control females; ovariectomized females; ovariectomized females receiving a single depot injection of estradiol undecylate, 50 μg/rat in 100 μl (Progynon Depot 100, Schering), a dose known to be sufficient to stimulate EnDA-tumor growth in ovariectomized DA/Han rats for at least 6 weeks (Horn et al., 1994); intact females receiving 5 mg/kg/day ICI 182,780 dissolved in 20% benzyl benzoate in castor oil; and intact females receiving 20 mg/kg/day tamoxifen dissolved in 20% benzyl benzoate in castor oil. The anti-estrogens were administered 6 days a week for 5 weeks starting one day after tumor cell injection. Tumor growth was monitored weekly by caliper measurement. After 5 weeks the animals were killed, the tumors, ipsilateral lymph nodes and uteri were excised and the wet weights were determined. In the control group, animals were selected at random and not at specific times in the estrus cycle. The statistical analysis of tumor and tissue wet weights was performed using Student's t-test.

RNA preparation and Northern blot hybridization

RNA was extracted from tissues (200—500 mg), after pulverization in liquid nitrogen or from cell cultures (pools of cells from 6 wells) using guanidinium isothiocyanate and centrifugation through cesium chloride (Chirgwin et al., 1979.). After determining the RNA concentration by UV absorption at 280 nm, total RNA (10—20 μg) were electrophoresed on 2.2 M formaldehyde gels, and transferred to nylon membranes by vacuum blotting (Pharmacia, Freiburg, Germany).

After prehybridization the membrane was first hybridized at 42°C overnight with the ^{32}P-labeled clusterin probe (4×10^8—12×10^8 cpm/μg plasmid, 12×10^6 cpm total). The membranes were washed once at room temperature for 5 min, once at 42°C for 5 min and 3 times at 50°C for 15 min. The membranes were then exposed for 24—48 hr to Kodak XOmat film using intensifying screens. After stripping with $0.1 \times$ SSC for 15 min at 70°C, the membranes were was rehybridized with a ^{32}P-labeled control probe recognizing the 18S ribosomal RNA (4×10^8—1.2×10^8 cpm/μg plasmid, 3.2×10^5 cpm total).

The 350 bp Sst I/Sac I fragment (representing positions 157—506 of the rat clusterin cDNA) or the 316 bp Xba fragment (representing positions 1—316 of the rat clusterin cDNA) or the entire rat clusterin cDNA were used as hybridization probes.

Complement C3 and fibronectin cDNA probes were also radiolabeled as described previously. In all cases agarose gel purified restriction cDNA fragments were radiolabeled with ^{32}P-dCTP using a multi-prime labeling kit (GIBCO/BRL, Eggenstein, Germany). Fibronectin was used as a comparative probe for in vitro studies, complement C3 was used for the in vivo studies.

Estrogenic regulation of clusterin expression in vitro

To investigate the potential estrogenic regulation of clusterin, RUCA-I cells were cultured on a reconstituted basement membrane and treated with estradiol or the pure anti-estrogen ICI 164,384 for 2—48 hr (Fig. 1). The steady state level of clusterin mRNA does not alter significantly over the 48 hr time course when incubated in the presence of ethanol alone. Estradiol treatment of RUCA-I cells induces a time-dependent increase of steady state level of clusterin mRNA, reaching a maximum at around 24 hr. Treatment of cells with the pure anti-estrogen ICI 164,384 substantially reduces the steady state level of clusterin mRNA to those seen in the untreated control cultures. The effects of estradiol and the antiestrogen, ICI 164,384, on clusterin mRNA expression were more pronounced than the effects on the expression of fibronectin mRNA, an estradiol repressed protein in RUCA-I cells (Vollmer et al., 1995b) (Fig. 1). The expression of fibronectin mRNA is transiently repressed by estradiol, reaching a nadir at approximately 20 hr (Fig. 1). ICI 164,384 does not appear to significantly affect the steady state fibronectin mRNA, although there is a slight increase 48 hr following treatment (Fig. 1). In summary, at these doses of estradiol (10^{-7}M) and ICI 164,384 (5×10^{-7}M) clusterin gene expression appears to be significantly more responsive to these hormones than fibronectin in RUCA-I cells.

Hormonal regulation of clusterin mRNA expression in vivo

To determine whether the steady state level of clusterin mRNA is regulated by estrogens in vivo, syngeneic DA/Han rats were injected s.c. with 10^6 RUCA-I cells and randomized in 5 experimental treatment groups. Primary tumors developed within 30 days in all groups at the site of injection. These tumors subsequently metastasized to the axillary lymph nodes and lung. Tumors grown in ovariectomized animals or in intact animals treated with ICI 182,780 grew significantly more slowly than untreated controls demonstrating significantly reduced tumor weight ($p < 0.01$) (Fig. 2a) when compared to untreated controls. In these groups, the uterine weight of the host animals (Fig. 2b) and the weight of the ipsilateral axillary lymph nodes (Fig. 2c) were also reduced when compared to untreated controls ($p < 0.01$). Administration of estradiol to these animals restores the growth parameters such that they are not significantly different from those seen in untreated controls (Fig 2a, b, c), demonstrating the importance of estradiol for the growth of RUCA-I cells in vivo. Treatment with tamoxifen had no significant effect on the weight of the RUCA-I primary tumors (Fig. 2a) compared to controls, and while tamoxifen also clearly reduced uterine weight ($p < 0.01$) (Fig. 2b), it demonstrated a distinctly agonistic effect on the weight of the axillary lymph
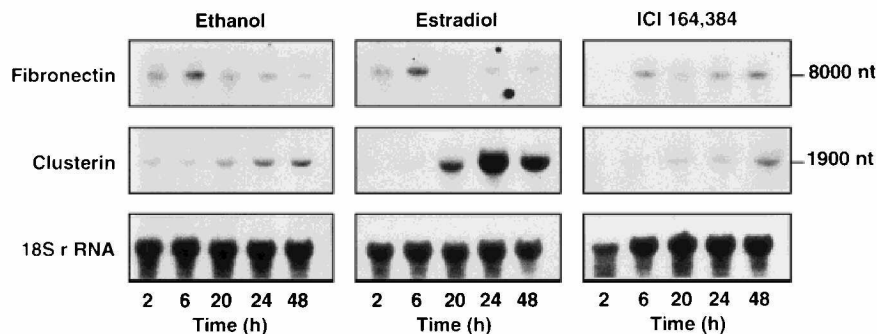

FIGURE 1 – Steady state levels of clusterin and fibronectin mRNAs in RUCA-I cells. RUCA-I cells were cultured on a reconstituted basement membrane and treated with estradiol (10^{-7}M), ICI 164,384 (5×10^{-7}M) or ethanol (control) for 2—48 hr. RNA was extracted, electrophoresed on formaldehyde gels, transferred to nylon membranes and hybridized with clusterin or fibronectin cDNA probes as described in Material and Methods. To correct for loading the blots were stripped and re-probed with a cDNA probe specific for 18S rRNA.
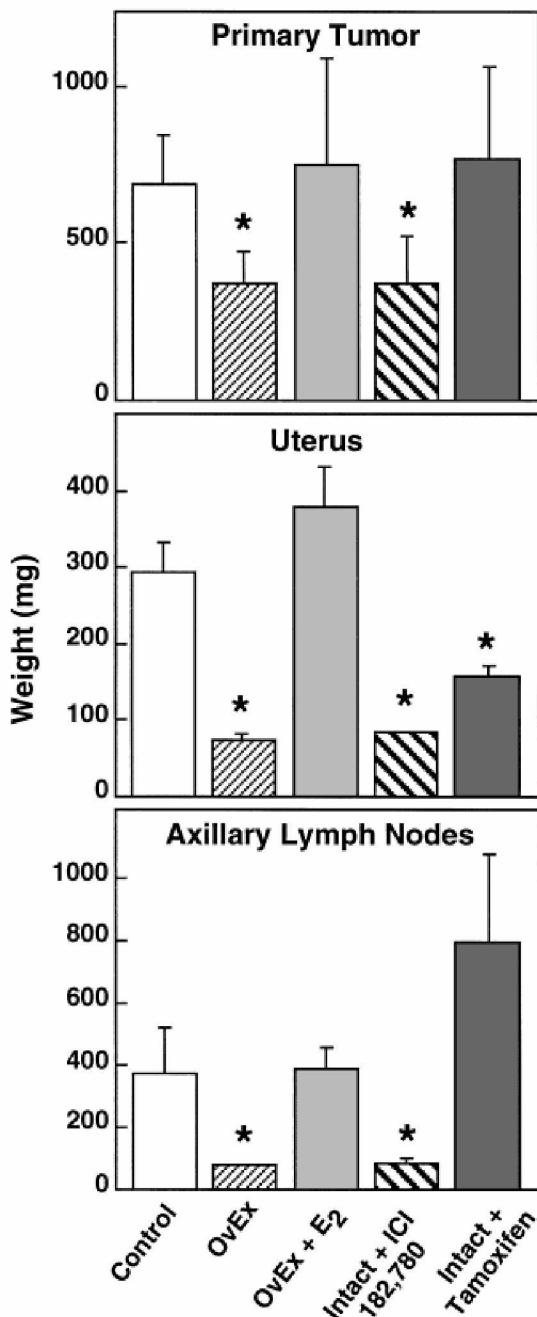
FIGURE 2 – Effect of hormone deprivation and anti-hormone treatment on primary and metastatic tumor growth of RUCA-I cells. RUCA-I cells (10^6) were injected s.c. into the right flank of intact syngeneic female DA/Han rats, ovariectomized DA/Han rats, ovariectomized DA/Han rats substituted with estradiol-undecylate, intact DA/Han rats treated with ICI 182,780 or intact DA/Han rats treated with tamoxifen. Panel a: Primary tumor weight (mean ± SD, n = 4); Panel b: Weight of the normal uterus (mean ± SD, n = 4); Panel c: Weight of ipsilateral lymph nodes (mean ± SD, n = 4). Stars above individual bars indicate values that differ significantly from the control at $p < 0.01$. The wet weight of axillary lymph nodes in animals treated with tamoxifen differed significantly from controls at $p < 0.05$.

nodes, promoting an axillary weight almost twice as high as that measured in untreated control animals ($p < 0.05$) (Fig. 2c).

The steady state clusterin mRNA level in the tumor tissue correlates with tumor weight at the end of the experiment, with high levels of clusterin expression in the tumors excised from untreated controls animals, from ovariectomized animals adminis-

tered estradiol, and from intact animals administered tamoxifen (Fig. 3a). Almost undetectable levels of clusterin expression were found in tumors grown in ovariectomized animals and in intact animals given ICI 182,780. The effects of estrogens on the steady state levels of complement C3 mRNA in the primary tumors was considerably less pronounced than the effects on clusterin (Fig. 3a). In this, and other Northern analyses of clusterin mRNA levels in RUCA-I cells grown *in vivo* and in normal uteri, the clusterin cDNA probes hybridized to a sequence of between 3.5—4.0 kb that showed the same expression pattern *in vivo* as clusterin. This sequence was not detected in Northern analyses of clusterin mRNA from RUCA-I cells grown *in vitro*. The identity of this sequence, and its relationship to clusterin mRNA have not been determined.

The regulation of clusterin steady state mRNA levels in lymphogenous metastases of RUCA-I exhibited the same expression pattern as that seen in the primary tumor. Clusterin mRNA was significantly expressed in axillary lymph nodes of untreated control animals, ovariectomized animals that were substituted with estradiol and intact animals treated with tamoxifen (Fig. 3b). Clusterin mRNA expression was barely detectable in lymph nodes of ovariectomized animals and were undetectable following treatment of intact animals with ICI 182,780 (Fig. 3b).
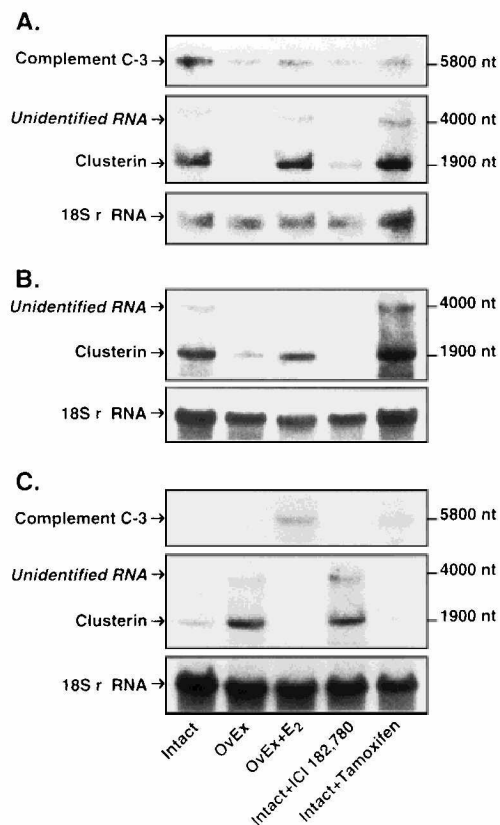

FIGURE 3 – Steady state mRNA levels of estrogen-sensitive genes in primary tumors, axillary lymph nodes and normal uterus. RUCA-I cells (10^6) were injected s.c. into the right flank of intact syngeneic female DA/Han rats, ovariectomized DA/Han rats, ovariectomized DA/Han rats substituted with estradiol-undecylate, intact DA/Han rats treated with ICI 182,780 or intact DA/Han rats treated with tamoxifen. RNA was extracted, electrophoresed on formaldehyde gels, transferred to nylon membranes and hybridized with cDNA probes specific for clusterin, or complement C3. To correct for loading the blots were stripped and re-probed with a probe specific for 18S rRNA. Panel a: steady state levels of clusterin and complement C3 mRNAs in primary tumors; Panel b: steady state level of clusterin mRNA in lymph node metastases; Panel c: steady state level of clusterin and complement C3 mRNAs in uterus.

In marked contrast, the effects of estrogens on clusterin mRNA expression in the uteri of host animals was very different from that seen in the primary tumors derived from RUCA-I cells or their lymph nodes metastases. In the normal uterus, estradiol suppressed clusterin expression, whereas clusterin mRNA levels were significantly induced after ovariectomy or treatment with the pure anti-estrogen ICI 182,780 (Fig. 3c). Tamoxifen, which has both agonistic and antagonistic effects, also suppressed clusterin expression. This conspicuous inversion of estrogenic regulation of gene expression in normal and tumorigenic tissue was not detected for complement C3 expression, which exhibited increased steady state mRNA levels in ovariectomized animals supplemented with estradiol and in intact animals treated with tamoxifen (Fig. 3c).

DISCUSSION

Our data demonstrate that clusterin is an estrogen-regulated gene in RUCA-I, rat endometrial adenocarcinoma cells, both *in vitro* and *in vivo*. Furthermore, the expression of clusterin mRNA *in vitro* appears to accurately reflect the expression of the gene in primary and metastatic tumors derived from RUCA-I cells *in vivo*. Although the estrus state of the control animals was not analyzed, there appears to be a marked switch in the regulation of clusterin by estrogens during the process of endometrial carcinogenesis, since the expression of clusterin in the normal uterus is repressed by estrogens, but is upregulated in RUCA-I primary adenocarcinomas and in lymph node metastases. In this respect, clusterin is the first gene described which has diametrically opposed hormonal control in a normal tissue and in tumors derived from that tissue. As such, clusterin may represent a valuable marker gene in the search for transcription factors that contribute to the malignant transformation of the endometrium and possibly of other hormone-dependent tumors, such as the prostate and breast. In this context, it would be interesting to determine how clusterin expression is modulated during the estrus cycle in normal uterine tissue and in malignant tissue.

The regulation of clusterin steady state mRNA levels by estrogens is particularly interesting since no estrogen response elements have been identified in the rat or human clusterin promoter (Wong *et al.*, 1993). While there are 8 half site androgen/glucocorticoid responsive elements within the first intron of the structural gene, it is unlikely that they are used by the estrogen receptor. It is more likely that estrogens regulate clusterin expression through the 2 functional AP-1 sites located in the proximal promoter, since it is well established that estrogen rapidly induces the transcription of a number of immediate response genes including members of the *fos* and *jun* families (Webb *et al.*, 1993; Bigsby and Li, 1994) through the estrogen response elements in the promoters of these genes (Hyder *et al.*, 1995). Since this mode of transcriptional regulation of the clusterin gene would require activation of the early response genes, clusterin expression most likely represents a relatively late response in this context. This may explain the relatively long time lag (approximately 15—20 hr) before estradiol induction of clusterin expression in RUCA-1 is measurable *in vitro*. Whether the estrogen receptor in RUCA-I cells exerts its action via AP-1 sites or there is an unidentified estrogen response element further upstream in the promoter, would require further investigation, particularly since estrogen action in a target cell apparently requires selective activation of different response elements.

Clusterin has been identified as a gene that is expressed during the involution of glandular tissues such as the breast and prostate by apoptosis (Tenniswood *et al.*, 1992). In these tissues, clusterin expression is usually transient since the gene is expressed in epithelial cells that die by apoptosis as the tissue regresses. In the rodent after hormone ablation, uterine regression is completed within 3—5 days (Moulton, 1994). Clusterin mRNA expression persists in endometrial tumors derived from RUCA-I cells for at least 30 days following ovariectomy or treatment with the anti-estrogen ICI 182,780, suggesting that it is unlikely to be associated solely with the apoptotic processes that is initially induced by these hormonal manipulations, and probably serves a cytoprotectant role, by mediating the clearance of redundant membranes and other cytolytic debris (Jenne and Tschopp, 1992).

Clusterin is also known to inhibit complement mediated cytolysis (O'Bryan *et al.*, 1990; Jenne and Tschopp, 1992). The glandular surface of the normal rat uterus represents an interface of the body with the external surroundings and the regulation of mucosal immunity in the female reproductive tract via the endocrine system is well established (Wira and Stern, 1992). In response to estradiol, polymeric IgA and the cytoplasmic portion of its receptor, secretory component, as well as IgG, enter uterine secretions, as part of the immune defense system (Sullivan *et al.*, 1983). Repression of clusterin expression, coupled with the upregulation of individual components of the complement cascade by gonadal steroids, probably ensures a competent immune defence system (Sullivan *et al.*, 1983). Conversely, tumors derived from the RUCA-I cells are highly metastatic (Vollmer and Schneider, 1997), and should be subject to immune surveillance while entering the circulation or invading other tissue compartments. In this context, clusterin mediated inhibition of complement-induced cytolysis probably protects the RUCA-I adenocarcinoma cells from complement-mediated lysis and contributes to the highly metastatic phenotype of these cells.

In summary, we have shown that regulation of the steady state mRNA level of clusterin in the rat uterus and in rat endometrial adenocarcinoma is controlled by estradiol, but is diametrically reversed in normal and malignant tissues, exhibiting estrogen repression of expression in normal tissue and inducible expression in tumor cells both *in vivo* and *in vitro*. Clusterin may therefore represent a target gene that can be used to monitor changes in the transcription factors and signal transduction pathways that are responsible for the progression from normal to malignant to metastatic tissue.

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