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Clusterin (SGP-2) transient overexpression decreases proliferation rate of SV40-immortalized human prostate epithelial cells by slowing down cell cycle progression

Saverio Bettuzzi*,[1], Francesca Scorcioni[2], Serenella Astancolle[2], Pierpaola Davalli[2], Maurizio Scaltriti[2] and Arnaldo Corti[2]

[1]*Dipartimento di Medicina Sperimentale, Università di Parma, Via Volturno, 39–43100 Parma, Italy;* [2]*Dipartimento di Scienze Biomediche, Università di Modena e Reggio Emilia, Via 6, Campi 287–41100 Modena, Italy*

Clusterin is a highly conserved, widely distributed glycoprotein whose biological significance is still debated. Involved in many biological processes and disease states, clusterin is induced by cell injury and tissue regression, but is repressed during cell proliferation. We have previously reported that clusterin mRNA induction is associated with epithelial cell atrophy in the rat prostate and both clusterin transcript and protein accumulated in quiescent normal human skin fibroblasts. Here we show that transient clusterin overexpression, in SV40-immortalized human prostate epithelial cells (PNT2), resulted in increased accumulation of cells in the G_0/G_1 phases of the cell cycle, accompanied by slowdown of cell cycle progression and decrease of DNA synthesis. The activities of ornithine decarboxylase (ODC) and S-adenosylmethionine decarboxylase (AdoMetDC), and the level of histone H3 mRNA (markers of cell proliferation) concomitantly decreased, while Gas1 mRNA (a marker of cell quiescence) accumulated. Thus it appears that clusterin, by opposing the effect of SV40 on the proliferation rate of PNT2 cells, acts as an anti-oncogene in the prostate, suggesting a role for this gene in controlling proliferation of normal and transformed prostate epithelial cells.
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Clusterin is a heterodimeric-secreted glycoprotein consisting of two subunits, α and β, joined by a unique five-disulfide bond motif in the mature extra cellular glycosylated form (Lakins *et al*., 1998). It was so named because of its capability to elicit 'clustering' of several different cell types. In the rat ventral prostate undergoing tissue regression upon surgical or pharmacological androgen ablation, clusterin gene is dramatically induced (Bettuzzi *et al*., 1989; Astancolle *et al*., 2000; Buttyan *et al*., 1989). In the normal gland, clusterin is exclusively expressed in cuboidal (atrophic) epithelial cells committed to apoptosis (Bettuzzi *et al*., 1992; Marinelli *et al*., 1994). In the same organ, clusterin mRNA undergoes linear accumulation with aging (Bettuzzi *et al*., 1994; Marinelli *et al*., 1994). The human counterpart of the clusterin gene is present as a single copy in the human genome and localizes to chromosome 8 (8p21) (Purrello *et al*., 1991; Fink *et al*., 1993). Both clusterin mRNA and protein were found in most animal and human tissues, where this gene is under the control of a plethora of hormones. Although several synonyms/acronyms are still in use to name this gene/protein, among which are sulfated glycoprotein 2 (SGP-2) (Griswold *et al*., 1988), testosterone-repressed prostate message 2 (TRPM-2) (Buttyan *et al*., 1989), ApoJ (Burkey *et al*., 1991), SP-40,40 (Murphy *et al*., 1988), CLI (Jenne and Tschopp, 1989) and others, the name 'clusterin' has been proposed in accordance with an international consensus regarding terminology (Rosenberg and Silkensen, 1995). This highly conserved protein has been proposed as playing important roles in many biological processes (cell adhesion and cell–cell interactions, organogenesis, cell differentiation and transformation, membrane lipid remodelling and transport, reproduction, complement regulation, oxidative stress, senescence, etc.) and in many disease states (atherosclerosis, neurodegenerative and renal diseases, infertility, myocardial infarction, etc.), but most of the studies so far available are still confined to the descriptive level (Rosenberg and Silkensen, 1995). Among these investigations, the evidence for a strong association between clusterin and apoptosis (Pearse *et al*., 1992), together with the report of its up-regulation in cells surviving death (French *et al*., 1994; Koch Brandt and Morgans, 1996) and in mammalian senescence (Bettuzzi *et al*., 1994; Gonos *et al*., 1998; Marinelli *et al*., 1994), suggest a possible role for this gene in protecting cells from death (French *et al*., 1994; Koch Brandt and Morgans, 1996; Miyake *et al*., 2000; Viard *et al*., 1999). In a recent work, different forms of clusterin have been immunologically distinguished in apoptotic and death surviving cells (Lakins *et al*.,

*Correspondence: S Bettuzzi, Dipartimento di Medicina Sperimentale, Complesso Biotecnologico Integrato, Università di Parma, Via Volturno, 39–43100 Parma, Italy; E-mail: saverio.bettuzzi@unipr.it
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1998), suggesting that under the name 'clusterin' a family of different proteins, with different biological significance, are probably still awaiting for a better definition.

We previously reported that clusterin expression levels depend on the proliferation rate in normal human cells, being up-regulated in quiescent normal skin fibroblasts (Bettuzzi *et al*., 1999a) and down-regulated in PHA-stimulated lymphocytes (Grassilli *et al*., 1991). In view of our interest in investigating the possible involvement of clusterin in normal and pathological prostate gland proliferation and differentiation, we studied the changes in proliferation activity occurring in transiently transfected PNT2 cells, derived from normal human epithelial prostate cells by SV40-immortalization (Blanchere *et al*., 1998; Cussenot *et al*., 1991). PNT2 cells retain characteristics typical of well-differentiated cells with respect to morphological features, cytokeratins expression profile and doubling time (Cussenot *et al*., 1991).

Clusterin overexpression was driven in PNT2 cells by the LacSwitch II Inducible Mammalian Expression System (Stratagene, La Jolla, CA, USA), that includes the operator vector pCMVLac1 (the Lac repressor expressing plasmid) and the expression vector pOP-SVI-MCS. For that purpose, the full-length 1513 bp cDNA fragment coding for rat clusterin (Bettuzzi *et al*., 1989) was inserted into pOP-SVI-MCS to originate the pOP/SGP-2, a construct that drives clusterin overexpression under the control of the strong universal Rous Sarcoma Virus (RSV) promoter. Overall transfection efficiency in PNT2 cells was assessed by detecting the relative expression of the reporter gene EGFP (Enhanced Green Fluorescent Protein) by means of Confocal Laser Spectral Microscopy (CLSM) and comparing different transfection protocols (data not shown). Dosper reagent was chosen, because under these experimental conditions, nearly all cells appeared to be positive when observed by CLSM (data not shown). Although in most of the transfected cells the level of expression was rather low, no signs of cytotoxicity were evident. This protocol allowed us to study the possible biological effects of clusterin gene overexpression while meeting two important experimental conditions: (1) moderate/low level of transient gene overexpression, closer to physiological conditions in which clusterin is generally induced; (2) an overall transfection efficiency that was close to 100% of PNT2 cells, a condition implying no need for sorting out the positively transfected cells from the negative ones. Co-transfections with pCMVLac1, together with the mammalian expression vector pOPR13CAT carrying the chloramphenicol acetyltransferase (CAT) reporter gene, were performed to set out the conditions for gene repression. A co-transfection with 1 : 4 molar ratio (pCMVLac1 : pOPR13CAT) resulted in highly efficient repression of CAT activity. Administration of IPTG quickly induced CAT activity, starting as soon as 2 h and reaching a peak between 16 and 24 h (data not shown). Thus, for clusterin overexpression experiments, PNT2 cells were analysed within a time frame which starts from the beginning of the transfection, includes the IPTG-induction and extends to 36 h, a time interval required for completion of cell replication (PNT2 cells average doubling time: 35.9 ± 1.6 h) (Cussenot *et al*., 1991).

The vector pOP/SGP-2 allowed to introduce clusterin cDNA (Bettuzzi *et al*., 1989) in a repressed state into PNT2 cells. Northern hybridization (Figure 1a) and Western blot analysis (Figure 1b) were performed in control cells (cells transfected with the empty vector alone; Figure 1a, Co and 1b, Co) and in transiently

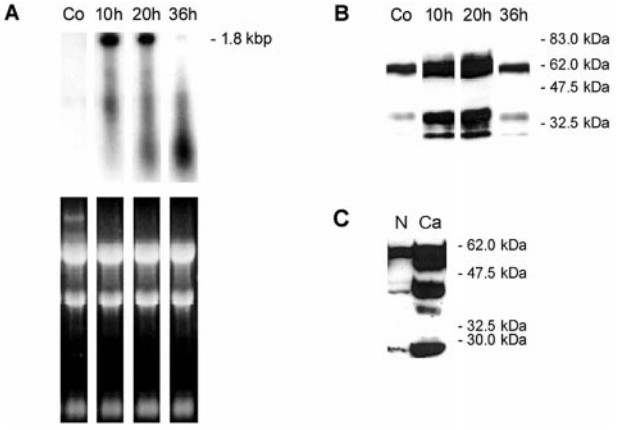

Figure 1 Northern and Western blot analyses for determination of the time-course of clusterin overexpression in transiently transfected PNT2 cells. For Northern blot analyses, total RNA was extracted and 10 μg aliquots were electrophoresed, blotted and then hybridized to the specific cDNA probes as previously described (Bettuzzi *et al*., 1994). Quantitation of the autoradiograms was obtained by densitometric scanning using a LKB Ultrascan XL densitometer. Western blot analyses were performed by chemiluminescence as previously described (Astancolle *et al*., 2000). Cells were harvested and directly lysed at 100°C for 10 min in SDS–PAGE loading buffer. The total protein extract, equivalent to 4×10^5 cells, was then loaded on each lane and resolved by electrophoresis on 10% polyacrylamide gel. Clusterin immunoreactive bands were detected using polyclonal anti-rat clusterin antibodies commecially available, obtained from rabbits immunized with a mixture of four synthetic peptides corresponding to the following regions: amino acids residues 1–17; 32–48; 52–72; 133–148 (Upstate Biotechnology, Lake Placid, NY, USA). (**a**) Northern hybridization analysis performed in: Co, controls (cells co-transfected with pCMVLac1 and pOP/SGP-2; no IPTG administration); 10, 20 and 36 h, clusterin overexpressing cells (cells co-transfected with pCMVLac1 and pOP/SGP-2; IPTG administrated for the time indicated). The induced 1.8-kbp transcript coding for clusterin was detected until up to 20 h of IPTG induction. Etidium bromide staining of the total RNA samples is also shown for comparison. (**b**) Western blot analysis of clusterin immunoreactive bands in PNT2 cell extracts obtained from controls and clusterin overexpressing cells as in (**a**): accumulation of clusterin immunoreactive protein forms was evident until up to 20 h after IPTG induction. (**c**) the pattern of immunoreactive forms of clusterin in normal (N) and 4-day castrated rat prostate (Ca) is also shown for comparison. The result was obtained with the same polyclonal anti-rat antibodies used for (**b**). Under these experimental conditions, the purchased antiserum exhibited cross-reactivity between human and rat clusterin (compare Figure 1b,c), recognizing different forms of clusterin protein. The same amount of total protein was loaded on each gel lane shown in panels **b** and **c**. Cross-reactivity was also previously observed in human prostate carcinoma specimens (Bettuzzi *et al*., 2000). The data shown are representative of four independent experiments

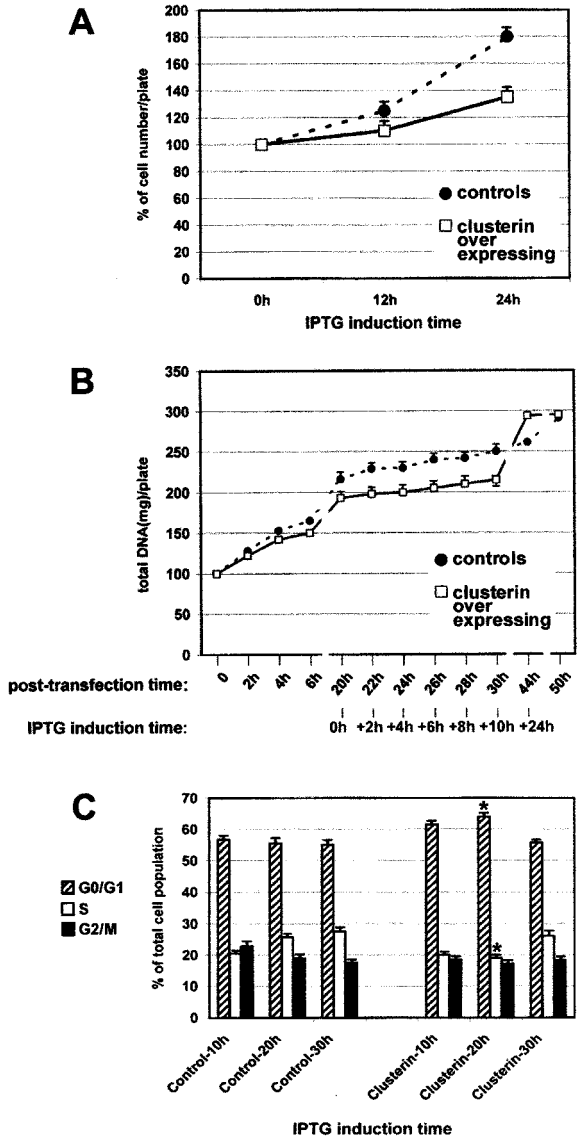

Figure 2 Effect of clusterin transient overexpression on cell proliferation, DNA synthesis and cell cycle progression of PNT2 cells. (**a**) growth of PNT2 cells at 12 and 24 h after IPTG induction of clusterin overexpression. Controls (as in Figure 1), dashed line; clusterin-overexpressing cells (as in Figure 1), solid line. The result is expressed as % of total cell number/plate (100% = 0 h) ± s.d. from four independent experiments. (**b**) total DNA amount (mg/plate) in controls (dashed line) and clusterin overexpressing cells (solid line) as determined at the indicated time after IPTG induction. Data are expressed as mg DNA/plate ± s.d. from four independent experiments. DNA determination was performed according to Labarca and Paigen (1980). (**c**) Histogram showing the relative percentages of PNT2 cells in the different phases of the cell cycle as assessed by FACS double parametric analysis after BrdU incorporation and anti-BrdU/FITC and PI staining. Two-parameter FACS analysis was as previously described (Scorcioni *et al*., 2001). Cells were routinely transfected in the presence of 10% FBS-containing growth medium using Dosper transfection reagent (from Roche Diagnostics Corp. Indianapolis IN, USA) Transfection experiments were performed by adding the operator vector pCMVLac1 plus pOP/SGP-2 plasmid (or the same amount of empty vector, on an equimolar basis, for controls) to each cell culture for the time indicated. Cells were transfected for 24 h, then 5 mM IPTG was added for the indicated time to all cell cultures to induce clusterin. Controls (cells transfected with pCMVLac1 and the empty expression vector pOPRsviMCS) were harvested 10 h (control-10 h), 20 h (control-20 h) and 30 h (control-30 h) after IPTG administration; clusterin overexpressing cells (cells transfected with pCMVLac1 and pOP/SGP-2) were also harvested 10 h (clusterin-10 h), 20 h (clusterin-20 h) and 30 h (clusterin-30 h) after IPTG induction. Hatched bars, G_0/G_1 phases; white bars, S phase; black bars, G_2/M phases. The result is the mean of cell population (% of total) ± s.d. obtained from six independent experiments. *t*-test(p.c.) analysis: (*) = $P < 0.05$ with respect to matched time-point controls

transfected cells after IPTG induction (Figure 1a, 10–36 h and Figure 1b, 10–36 h). For comparison, Western blot analysis using the same policlonal anti-rat clusterin Ab was also performed in parallel in ventral prostate from normal (Figure 1c, N) or 4-day castrated rat (Figure 1c, Ca). Cross-reactivity between human and rat clusterin is evident, resulting in a complex pattern both with human and rat extracts due to different clusterin protein forms consisting of the high molecular weight, not-glycosylated precursor (53 kDa), mature glycosylated forms (about 63–65 kDa) and subunits (in the range of 29–43 kDa). PNT2 control cells showed a very low amount of the clusterin transcript (that can only be detected by prolonged autoradiography) and basal levels of the protein. Accumulation of both clusterin mRNA and protein was evident in transiently transfected PNT2 cells at 10 h and 20 h after IPTG induction, confirming that clusterin overexpression was indeed achieved under these experimental conditions. However, clusterin transcript decreased markedly 36 h after IPTG induction, returning to basal level.

During transient overexpression of clusterin in PNT2 cells following IPTG-induction, the total number of cells/plate was lower (Figure 2a) and, consistent with that, the total amount of DNA/plate, after IPTG induction, was also lower in clusterin-overexpressing PNT2 cells with respect to controls (Figure 2b). Thus, reduced cell growth and DNA synthesis occurred during clusterin overexpression (compare Figure 1 and Figure 2). This was confirmed by Flow Cytometry (FACS) analysis of PNT2 cell proliferation. The number of cells in G_0/G_1 phases increased significantly at 10 and 20 h after IPTG administration with respect to controls, with a corresponding decrease in the number of cells in S phase and in G_2/M phases (Figure 2c). This effect disappeared at 30 h, when the proliferation pattern of clusterin-overexpressing cells became similar to controls (Figure 2c). The return to basal proliferation activity was concomitant with the drop in clusterin overexpression that we detected at 36 h after IPTG-induction (Figure 1), in coincidence with the average doubling time of PNT2 cells. This effect can be explained as a result of the 'dilution' of the expression plasmid, which is not duplicated, but is shared among the newly replicated cells, in concomitance with the outcome of intracellular plasmid-degradation processes.

Taken together, the above data suggest that clusterin overexpression could delay or slow down cell cycle

progression. To address this, we used FACS analysis to determine the time required for PNT2 cells to proceed from G_1 to G_2/M phases through S phase. Time-pulse BrdU incorporation into chromatin, by actively DNA-synthesizing cells, was obtained by exposing transfected, IPTG-induced cells to BrdU for 20 min, followed by repeated washing and growth in standard medium. Cells were then harvested and cell cycle progression was analysed by FACS at 2.5, 5.0 and 10.0 h after labelling (Figure 3). The progression through the cell cycle of control cells (Figure 3, b–e) was faster when compared to clusterin-overexpressing cells (Figure 3, f–h), showing that clusterin overexpression was indeed capable of slowing down cell cycle progression, extending the time interval between G_1 and G_2/M phases. Consistent with this interpretation of the data, the level of incorporation of BrdU was clearly lower in clusterin-overexpressing cells, as shown by comparing panels c and f (grey arrows) or d and h (black arrows) of Figure 3. This was confirmed by double fluorescence labelling and microscopy analysis of PNT2 transfected cells. In fact, Figure 4 shows that early IPTG-induced (12 h) individual PNT2 cells, exhibiting the highest levels of clusterin expression, fail to incorporate BrdU even after 12 h incubation in the presence of the analogue.

Since enhanced polyamine biosynthesis is generally associated with cell proliferation (Canellakis *et al*., 1989; Heby, 1981; Lowe, 1980; Luk and Casero, 1987; Pegg, 1988), we sought to determine whether ornithine decarboxylase (ODC) and S-adenosylmethionine decarboxylase (AdoMetDC) activities, the two rate-limiting enzymes of polyamine biosynthesis, were inhibited in clusterin-overexpressing PNT2 cells during IPTG induction (Figure 5). At 10, 20 and 25 h after IPTG induction, both ODC (Figure 5a) and AdoMetDC (Figure 5b) activities were remarkably lower with respect to controls, suggesting that clusterin overexpression reduced proliferation activity. Histone H3 mRNA (Plumb *et al*., 1983) is a specific marker of the S phase of the cell cycle (Konishi *et al*., 1996), while growth-arrest specific gene 1 (Gas1) is a marker (and an inducer) of cell quiescence in normal human cells (Del Sal *et al*., 1992, 1994). We previously used both molecular markers to assess cell proliferation activity in different experimental systems (Bettuzzi *et al*., 1999a,b, 2000). Northern blot analyses performed on clusterin-overexpressing PNT2 cells (Figure 5c) showed a decrease in the level of histone H3 mRNA and an accumulation of Gas1 mRNA during clusterin overexpression, revealing an expression pattern consistent with decreased cell proliferation activity.

In normal human skin fibroblasts, clusterin accumulation coincided with decreased proliferation rate, being maximally expressed in cells brought to quiescence by serum starvation. (Bettuzzi *et al*., 1999a). Contrariwise, in resting human lymphocytes, clusterin exhibited a dramatic down-regulation when cells were stimulated to grow by PHA treatment (Grassilli *et al*., 1991). Thus, clusterin was proposed to participate in cell growth-controlling events. Evidence of clusterin association to cells regressing to an atrophic state has

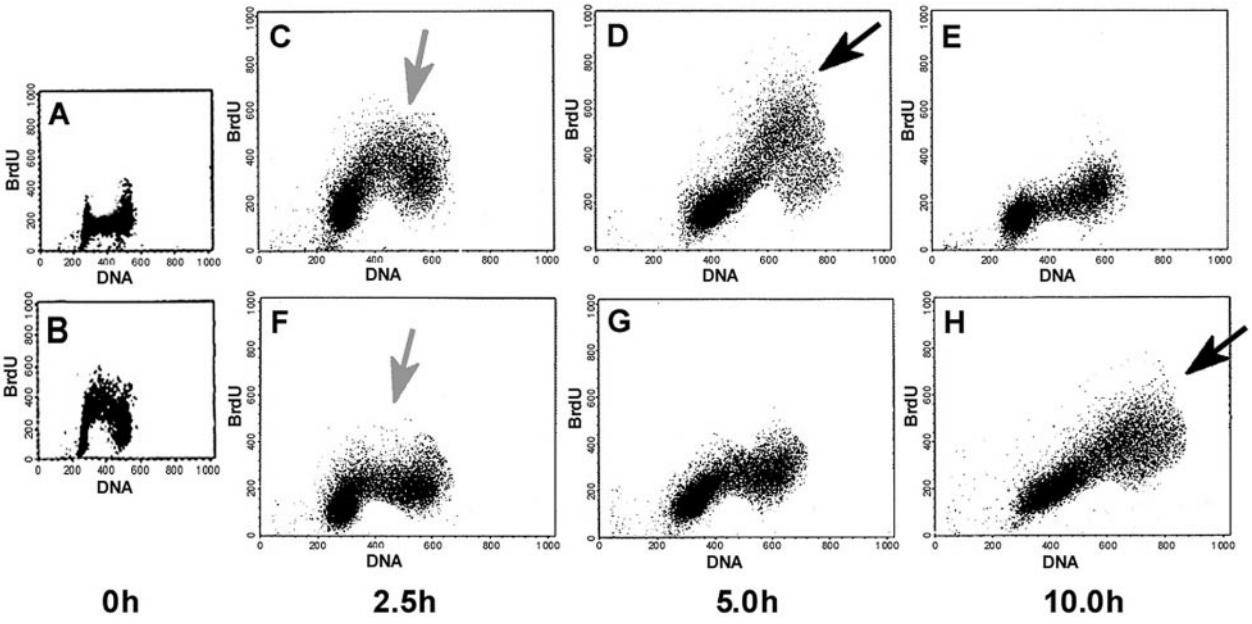

Figure 3 Slowdown of cell cycle progression during clusterin transient overexpression in PNT2 cells. Cells were subjected to the same experimental protocol as in Figure 2 (IPTG induction: 20 h). Time-course study of cell cycle progression was performed by exposing cells to 20 mM BrdU for 20 min. Then fresh media was added and cells were further incubated at 37°C. At the time indicated, cells were harvested and analysed by two-parameter FACS analysis. A, negative control (no administration of BrdU to cell cultures; not transfected cells); B, positive control (BrdU was administered to cell cultures; not transfected cells); C–E, cells co-transfected with pCMVLac1 and pOPRsviMCS (empty vector); F-H, cells co-transfected with pCMVLac1 and pOP/SGP-2 (clusterin-overexpressing PNT2 cells). A representative result out of three independent experiments is shown

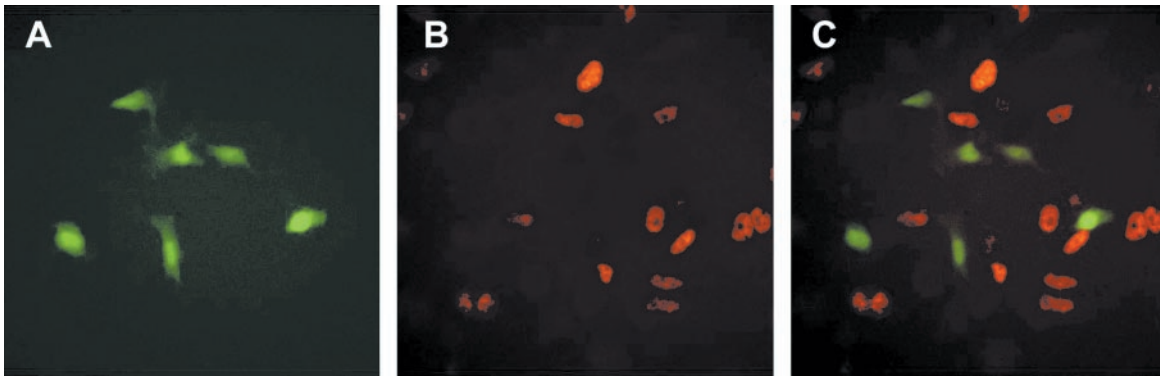

Figure 4 PNT2 cells showing highest levels of clusterin overexpression fail to incoporate BrdU. Double fluorescence immunocytochemistry was performed in PNT2 cells subjected to the same experimental protocol as in Figure 3. Transfected PNT2 cells were exposed to IPTG induction for 12 h in the presence of 20 mM BrdU, then fixed in 4% paraformaldehyde, permeabilized in 0.3% Triton X-100 in PBS for 15 min at room temperature, and subjected to double fluorescence immunocytochemistry using polyclonal anti-rat clusterin (as in Figure 1) and mouse IgG anti-BrdU (Dako, Denmark). Primary antibodies were evidenced by means of a mixture of appropriate secondary antibodies: anti-rabbit-Alexa fluor™ 488 -green fluorescence (Eugene-Leiden, The Netherlands) for clusterin, and anti-mouse-Alexa fluor™ 568 -red fluorescence (Eugene-Leiden, The Netherlands) for BrdU. Cells were then analysed by means of confocal fluorescence microscopy: red fluorescence shows BrdU immunostaining and green fluorecence shows clusterin expression. (**a**) early IPTG-induced, clusterin-overexpressing cells; (**b**) BrdU-incorporating cells; (**c**) merged image of pictures **a** and **b**. A representative result out of three independent experiments is shown

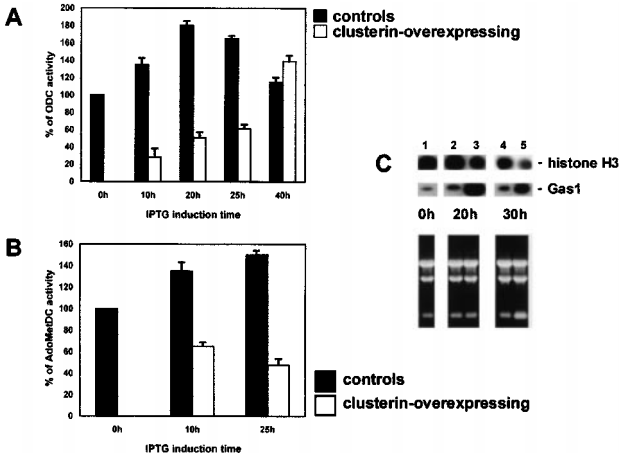

Figure 5 Changes in molecular markers of cell proliferation induced during clusterin overexpression: determination of ODC and AdoMetDC activities and Northern blot analysis of histone H3 and Gas1 mRNAs. ODC (**a**) and AdoMetDC (**b**) activities were determinated in PNT2 cells subjected to the same experimental protocol as in Figure 3 by measuring the release of $^{14}CO_2$ from [1-^{14}C]ornithine and S-adenosyl-L-[carboxy-^{14}C]methionine, respectively, by enzyme extracts (Bettuzzi *et al*., 1995). After IPTG induction, cells were harvested 10, 20, 25 and 40 h for ODC assay, 10 and 25 h for AdoMetDC assay. Black bars, controls; white bars, clusterin-overexpressing cells. The results are expressed as % of enzymatic activity of 0 h control (no IPTG administration, 0 h = 100%) ± s.d. from four independent experiments. (**c**) PNT2 cells, as in the case of **a** and **b**, were harvested 20 and 30 h after IPTG induction. Total RNA (10 μg/lane) was hybridized to specific histone H3 and Gas1 cDNA probes. 1, 2, 4: controls; 3 and 5: clusterin-overexpressing PNT2 cells. Etidium bromide staining of total RNA is also shown for comparison. A representative result out of three independent experiments is shown

been reported by Marinelli *et al*. (1994), and Huang *et al*. (1997), who showed that surgically induced spinal cord injury resulted in acute decrease of both serum testosterone level and prostate weight, accompanied by clusterin overexpression in the gland. At 2 weeks post-injury, prostate growth resumed, but clusterin levels remained elevated and this was accompanied by decrease in the height of the ventral prostate epithelial cells, suggestive of an atrophic state of these cells.

Increases in the levels of polyamines and activities of the two regulatory enzymes of their biosynthesis, ODC and AdoMetDC, occur during cell transformation induced by growth factors, carcinogens, viruses or oncogenes (Pegg, 1988). As a matter of fact, ODC activity was shown to be critical for cell transformation, and ODC gene was suggested to be a proto-oncogene (Auvinen *et al*., 1992); (Holtta *et al*., 1993). The levels of expression of these genes were significantly higher in human prostatic carcinoma (CaP) with respect to the benign counterpart, and correlated with the degree of malignancy while, under the same conditions, clusterin was down-regulated (Bettuzzi *et al*., 2000). Clusterin and ODC gene expression are inversely correlated in other experimental models (Bettuzzi *et al*., 1992; Grassilli *et al*., 1991) and this was confirmed by the data presented here on the concomitance of clusterin overexpression and decreased polyamine biosynthesis. This suggests the hypothesis that clusterin could directly affect the regulation of polyamine metabolism, thus decreasing cell proliferation rate and possibly interfering with cell transformation processes. In human prostate specimens clusterin down-regulation is an early event during CaP progression, in that it occurs not only in highly malignant cancer but also in well-differentiated tumours (Bettuzzi *et al*., 2000). This down-regulation could result, among other mechanisms, from a specific genetic alteration or loss. Indeed, clusterin gene is a single copy gene that maps to human chromosome 8 (Purrello *et al*., 1991) in 8p21 (Fink *et al*., 1993), a region which contains one of the most frequently

deleted loci observed in prostate cancer cells, and where it has been hypothesized that one (or more) tumour suppressor genes are located (Latil *et al*., 1994, 1996; Emmert Buck *et al*., 1995). Together with the present findings, this poses the question of whether clusterin could act as a tumour suppressor gene in prostate carcinoma. This takes on a particular meaning when considering our previous data showing a specific, dramatic and progressive accumulation of clusterin mRNA in the prostate of the ageing rat (Bettuzzi *et al*., 1994). This is due to the progressive extension, during ageing, of the cuboidal (atrophic) clusterin-expressing epithelium at the expense of the columnar one (not expressing clusterin) (Marinelli *et al*., 1994), an event that is not accompanied by increase in apoptotic activity. In fact, both DNA content and weight of the gland do not decrease upon ageing (Marinelli *et al*., 1994). During rat prostate ageing the epithelial cells tend to become atrophic, quiescent and long-lived; which may explain why, contrary to humans, age-related malignant transformation of the prostate is very unlikely to occur in this species. The data presented here suggest that clusterin overexpression could induce the quiescent state of the prostatic epithelial cells, exerting a role in preventing pathological proliferation of the secretory epithelia.

The biological effects obtained in human prostate epithelial cells immortalized by stable overexpression of SV40 large-T antigen (Cussenot *et al*., 1991) take on a particular meaning when considering that the expression of the SV40 early genes (T and t antigen, Tag), driven by the prostate specific promoter probasin in the autochthonous transgenic adenocarcinoma of the mouse prostate (TRAMP) model, spontaneously develops metastatic CaP, closely mimicking progressive forms of human disease (Greenberg *et al*., 1995). Our data suggest that clusterin gene could be considered a possible candidate for an important role in the control of normal and pathological cell proliferation, acting as an anti-oncogene in the prostate. This role would result from different functions played, through previously unknown mechanism(s), by the different alternative intracellular forms of the protein that have been reported within separate intracellular compartments (Lakins *et al*., 1998).

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