The clusterin paradigm in prostate and breast carcinogenesis

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Abstract

The biological functions of clusterin (CLU, also known as ApoJ, SGP2, TRPM-2, CLI) have been puzzling the researchers since its first discovery in the early 80’s. We know that CLU is a single 9-exons gene expressing three protein forms with different sub-cellular localisations and diverse biological functions. Despite the many reports from many research teams on CLU action and its relation to tumourigenesis, contradictions in the data and alternative hypothesis still exist. Understanding the role of CLU in tumourigenesis is complicated not only by the existence of different protein forms but also by the changes of tumours over time and the selection pressures imposed by treatments such as hormone ablation or chemotherapy. This review focuses on recent discoveries concerning the role of CLU in prostate and breast cancer onset and progression.

Although CLU acts primarily as a tumour suppressor in the early stages of carcinogenesis, consistent with its role in the involution of the prostate following castration, late stage cancer may overexpress CLU following chemotherapeutic drugs or hormonal ablation therapy. High expression of secreted or cytoplasmic CLU may represent a pro-survival stimulus because it confers increased resistance to killing by anti-cancer drugs or enhances tumour cell survival in specific niches.

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Introduction

Clusterin (CLU) is an enigmatic glycoprotein with a nearly ubiquitous tissue distribution, apparently involved in many biological processes such as sperm maturation, tissue differentiation, tissue remodelling, membrane recycling, lipid transportation, cell–cell or cell–substratum interaction, cell proliferation and cell death. CLU is also believed to be involved in many pathological states such as neurodegeneration, aging and cancer (Rosenberg & Silkensen 1995, Wilson & Easterbrook-Smith 2000, Trougakos & Gonos 2002, Shannan et al. 2006).

CLU was first isolated in ram rete testis fluid (Blaschuk et al. 1983, Fritz et al. 1983). After this first report, a wide range of tissues which can express this gene have been found (Fischer-Colbrie et al. 1984, Choi et al. 1989, de Silva et al. 1990a, James et al. 1991). Because of that, many different names have been given to CLU, often depending on the specific research issue. It was first isolated in humans by Jenne & Tschopp (1989) as complement cytolysis inhibitor (CLI), a protein which showed a high amino acid sequence homology (75.6%) to rat sulfated glycoprotein-2 (SGP-2), one of the most important glycoprotein produced by rat Sertoli cells (Collard & Griswold 1987) and suggested to play a role in sperm maturation. In the ventral prostate of rats, CLU mRNA was first identified as testosterone-repressed prostate message-2 (TRPM-2; Montpetit et al. 1986). In 1989, Bettuzzi et al. (1989), after complete cDNA cloning, sequencing and comparison, have discovered that TRPM-2 was completely homologue to full-length SGP-2 cDNA. Just an year before, Cheng et al. (1988) found that SGP-2 was identical to CLU, a serum protein involved in aggregation of heterologous erythrocytes. Thus, the same cDNA/protein was finally found to be implicated in important biological phenomena so different as erythrocyte aggregation,
CLU was found dysregulated in many types of cancer, including prostate and breast cancer. The CLU ‘paradox’ arises because a lot of data reported in scientific literature appear contradictory. The aim of this review is to give an answer to the main question: is CLU a positive or a negative modulator of mammalian tumourigenesis? We attempt to reconcile the experimental results, although apparently contradictory, from the different laboratories in order to synthesise the CLU ‘paradigm’ and render a complete picture of the role of CLU in tumourigenesis.

**CLU gene and transcripts**

In humans, the CLU gene maps on chromosome 8 in a region that is frequently deleted in prostate cancer (8p21-p12). It is a unique gene, organised into nine exons of different size, spanning a region of 17 877 bps.

Early studies of CLU gene products reported divergent 5′-untranslated regions (5′-UTR) in RNAs from rats (Collard & Griswold 1987, Wong et al. 1993), humans (Jenne & Tschopp 1989, Kirszbaum et al. 1989, O’Bryan et al. 1990) and mice (French et al. 1993, Jordan-Starck et al. 1994). Therefore, the possibility of alternative first exons for CLU transcription products in different mammalian species was already known at that time. Immediately after, Wong et al. (1994) characterised the human CLU gene and identified a unique exon 1 sequence according to the genomic databases available at that time. However, these authors admitted that the data obtained are insufficient to rule out the possibility of alternative exon 1 usage. Until 2006, a very simple view prevailed, and it was commonly accepted that a unique transcript of about 1.9 kb came from transcription of the CLU gene (de Silva et al. 1990a). Now, a recent GenBank update has earmarked two transcriptional isoforms of human CLU called isoform 1, NM_001831.2 and isoform 2, NM_203339.1, which are listed as RefSeq. These two transcripts are probably originated from two initiation start sites and are only produced in humans and chimpanzees (Cochrane et al. 2007). Consulting the Alternative Splicing Annotation Project (ASAP) database (Lee et al. 2003) for the CLU unigene cluster (Hs.75106) a third transcript, called isoform 11036, appears as one of the most probable mRNA variants (Andersen et al. 2007). Quite commonly in mammals, several genes produce distinct mRNA isoforms with heterogeneous 5′ ends. This can generate mRNAs that encode for the same protein, but have a different 5′-UTR. In some cases, when the translation start site exists within the first exon, it encodes for distinct protein with different, alternative or opposite biological activities. The CLU gene seems to be a paradigmatic example of such complex regulation. The three isoforms previously cited contain nine exons, eight introns and a terminal 5′-UTR. Each of these transcripts has a unique fragment of exon 1 and shares the remaining sequence from exon 2 to exon 9, as shown in Fig. 1. Actually, isoform 1 is a 5′ extended sequence end of the previously published mRNA identified by the GenBank accession code M64722. Interestingly, in the complete sequence of exon 1, previously reported in a truncated form, Open Reading Frame Finder (ORF Finder: a software tool freely available at the NCBI web site) showed the presence of an additional functional AUG, unknown before. This mRNA is predicted to produce a putative protein of 501 amino acids of a molecular weight of 57.8 kDa. According to a computational prediction of its sub-cellular localisation by the PSORT program (Horton et al. 2007), this putative CLU form has a prevalent cytoplasmic/nuclear localisation and may account for the existence of an intracellular form of CLU escaping the secretory pathway. Dissimilarly, isoform 2 has an alternative untranslated exon 1. The AUG of the main ORF is located in exon 2, in a region common to the three transcripts and immediately upstream of a functional endoplasmic reticulum (ER) localisation leader sequence. This mRNA is predicted to produce the protein of 449 amino acids targeted to secretion, which is the most extensively studied form of CLU (de Silva et al. 1990a,b, Burkey et al. 1991, Choi-Miura et al. 1992). All the transcripts described possess a third AUG that is again in frame with the others and localises on exon 3. Leskov et al. (2003) found an alternative mRNA in MCF-7 mammary
cancer cells in which the exon 1 of isoform 1 is directly joined to exon 3, therefore lacking exon 2, and consequently both the AUG and the ER localisation sequence contained in this exon. The authors found that this alternative mRNA is constitutively expressed in MCF-7 cells at very low levels, coding for a precursor protein called pnCLU, a putative nuclear pro-death form. Since its discovery, its existence has never been confirmed later in other cell lines or tissues (Andersen et al. 2007, Cochrane et al. 2007, Schepeler et al. 2007), and it is not one of the most probable splicing variant according to the ASAP database. Therefore, it should be seriously considered that this transcript is either very specific to MCF7 cells (for reasons still unknown) or an experimental artefact.

The accumulation of evidence now confirms the existence of the previously described CLU transcripts (i.e. isoform 1, isoform 2 and isoform 11036), rendering the idea that the human CLU gene produces a unique transcript obsolete. Cochrane et al. (2007) found that isoform 1 and isoform 2 are concomitantly expressed in prostate cancer cell lines, but are differentially regulated by androgens. Isoform 1 is down-regulated by androgens, whereas isoform 2 is up-regulated by androgens and its level increases during the progression to androgen-independent prostate cancer in LNCaP xenograft tumours. Recently, Andersen et al. (2007) and Schepeler et al. (2007) have confirmed that all the three CLU mRNA variants, i.e. isoform 1, isoform 2 and isoform 11036, are expressed in various cell types including prostate and breast cancer cell lines. The same research team found that different signalling pathways affect CLU mRNA isoforms production. In particular, they found that the Wnt signalling pathway, via TCF1, specifically regulates the expression levels of isoform 1 but not those of isoform 2 (Schepeler et al. 2007).

CLU protein forms

It is now clear that CLU encodes more than one mRNA and that several protein forms derive from the unique gene, but what still remains to be defined is how each transcript, discussed above, relates to the diverse CLU forms observed at the protein level.

Recent information about the pathway of CLU degradation has given knowledge about the basic metabolism of this protein in the cell. In prostate cancer PC3 cells, the half-life of CLU is <2 h. Rapid degradation occurs through the proteasome. Rapid degradation may be the main way prostate cancer cells avoid accumulation of CLU and escape apoptotic death (Rizzi et al. 2009a).

Secreted CLU

The most extensively studied form of CLU is the secreted form (sCLU), a 75–80 kDa glycosylated α-β-heterodimer present in almost all physiological fluids, consisting of two chains of about 40 kDa each, linked by five disulphide bonds (de Silva et al. 1990a). sCLU translation starts from the AUG located in the second exon and produces a precursor protein of 49 kDa. This protein is directed to the ER by a leader signalling sequence. Then the protein is transported to the Golgi, heavily glycosylated and cleaved, generating two chains tightly bound by five disulphide bridges (Kirschbaum et al. 1992). The partially glycosylated, uncleaved protein is detectable as a 60 kDa band by SDS-PAGE. There is now substantial evidence that sCLU has a chaperone action like that of the small heat shock proteins and is one of the first described extracellular chaperones (Kirschbaum et al. 1989, Humphreys et al. 1999, Poon et al. 2000, Wilson & Easterbrook-Smith 2000, Lakins et al. 2002).

Sequence analysis of sCLU reveals several motifs that may be important for its chaperone action. Amphipathic α-helices located at the N-terminus of the α-subunit and at both the N- and C-termini of the β-subunit possess hydrophobic surfaces that are believed to mediate the binding of CLU to hydrophobic ligands (Law & Griswold 1994). It has been hypothesised that CLU has a flexible binding site (Bailey et al. 2001) due to the combination of an intrinsically disordered region and amphipathic α-helices ordered structures as shown in Fig. 2.

Intracellular CLU (nCLU and cCLU)

In their pioneering work, Reddy et al. presented the first experimental evidence showing that, in addition to the secreted form of the protein, a nuclear form of CLU (nCLU) exists. The intracellular form of the protein was found to be induced in response to treatment with

![Figure 2 Location of ordered and disordered regions in the CLU protein.](Image 300x73 to 509x179)
transforming growth factor β (TGF-β) of epithelial cell lines (Reddy et al. 1996). A few years later Yang et al. (2000) identified a 55 kDa nuclear form of CLU induced by ionizing radiation (IR) in MCF-7:WS8 breast cancer cell line. Other authors later confirmed that a shorter uncleaved, unglycosylated CLU protein of 45–55 kDa is usually detected concomitantly with the immunohistochemical detection of CLU inside the nucleus (Leskov et al. 2003, Caccamo et al. 2005). The origin of the cytoplasmic (cCLU) and nCLU proteins is currently the subject of intensive research in cancer.

Various mechanisms have been proposed to explain the presence of CLU in the nuclear and cytosolic compartments including: 1) alternative initiation of transcription in epithelial cell lines stimulated with TGF-β (Reddy et al. 1996); 2) alternative splicing in MCF-7 cells exposed to IR (Leskov et al. 2003); 3) alternative translation in prostate cancer cells (Moretti et al. 2007); 4) retrotranslocation from the Golgi apparatus to the cytosol via an ERAD-like pathway in stressed cells (Nizard et al. 2007); 5) re-internalisation of sCLU from the extracellular milieu into the cytosol (Kang et al. 2005). In addition, we found that removal of the leader sequence from sCLU cDNA led to the production of a 49 kDa protein band in epithelial prostate cell lines (PC3 and PNT1A). This molecular weight corresponds to the expected size of the non-glycosylated and uncleaved protein form (Scaltriti et al. 2004b).

It is important to note that none of these studies have isolated and sequenced the intracellular forms, therefore it is not known if CLU proteins of different mass result from alternative splicing or transcription initiation or simply represent species at different stages of maturation (e.g. cleaved or uncleaved, at different stages of glycosylation). At the moment we do not have definitive experimental data illustrating which structural characteristics and domains are specific for each different protein product. We also do not know exactly which domains are necessary for nuclear targeting, since at least three nuclear localisation signal, present in the human CLU amino acid sequence, have been identified but found not to be necessary for nuclear targeting (Scaltriti et al. 2004b). So, while we have a rough idea about the structure of sCLU, we still do not know how nCLU looks like. Nevertheless, it seems reasonable that the biological role of CLU outside and inside the cell is different.

It is now generally accepted that expression of nCLU is associated with cell death, as demonstrated in both prostate (Caccamo et al. 2003, 2005, Moretti et al. 2007) and breast cancer cell lines (Yang et al. 2000, Leskov et al. 2001). It has been proposed that nCLU binds the Ku70/Ku80 complex, a nuclear DNA end binding heterodimer, interfering with DNA double-strand breaks repair systems (Yang et al. 2000).

**Regulation of CLU gene expression by oncogenes, oncoproteins and epigenetic factors**

CLU is the prototypical multifunctional gene: regulating apoptosis, cell–cell interactions, protein stability, cell signalling, proliferation and, finally, transformation. In spite of the multiple functions that have been ascribed to CLU, its genetic inactivation in mice is well-tolerated and CLU knockout (CluKo) mice develop and live normally (McLaughlin et al. 2000).

CLU expression is regulated by many cis-acting elements and trans-factors which may be responsible for the complex tissue-specific control of the gene (Michel et al. 1997). Trans-factors that have been shown to interact with the CLU promoter and regulate its activity include: Egr-1 (Criswell et al. 2005), members of the AP-1 complex (Jin & Howe 1999), HSF1/2 (Loison et al. 2006) and B-Myb (Cervellera et al. 2000).

**CLU expression and growth factors signalling**

TGF-β positively modulates CLU expression via activation of an AP-1 site in the mammalian CLU promoter through the removal of the trans-repression effect of c-Fos by TGF-β (Jin & Howe 1999). More recently, it has been demonstrated that exposure of keratinocyte cells to vanadium induces apoptosis, c-Fos expression and a switch from sCLU to nCLU (Markopoulou et al. 2009). CLU is up-regulated twice during mouse mammary gland development: the first time at the end of pregnancy and the second time at the beginning of the involution. Therefore, it represents a marker of mammary epithelial cell differentiation. The second and most dramatic up-regulation of CLU coincides with the potent induction of TGF-β1, dependent on β1-integrin ligand-binding activity (Ithana et al. 2007).

IR activates TGF-β signalling in many cell types and tissues, including epithelial cells. Direct transcriptional activation of the CLU gene as a result of an activated TGF-β1 pathway may result in production of both sCLU and nCLU in MCF-7 breast cancer cells (Yang et al. 2000, Klokov et al. 2004). Ionizing irradiation activates a signalling pathway that emanates from two growth factors receptors: epidermal growth factor receptor (EGFR) and insulin-like growth factor
receptor (IGFR) (Yang et al. 2000). It was subsequently demonstrated that IGFR, but not EGFR, mediates the induction of sCLU in response to irradiation (Criswell et al. 2005). In this context, sCLU is induced as a protective response to damaging stress since knockdown of CLU by RNA interference accelerates cell death (Criswell et al. 2005).

CLU expression and oncogenes

Several studies show that CLU is regulated by oncogenes and oncoproteins. The first evidence that CLU expression is modulated by oncogenic activity was published in 1989, which reported that a thermally inducible gene, orthologue of rat CLU, was activated by retroviral oncogenes such as v-src, v-fps and v-mil (Michel et al. 1989). Induction by the oncogenic kinases was dependent on the AP-1 binding site.

Klock et al. published the first study demonstrating the role of two classical proto-oncogenes, namely c-Myc and H-Ras in regulating CLU expression. It was reported that overexpression of H-Ras, but not of c-Myc, caused repression of CLU expression at the mRNA level in the rat embryo fibroblast cell line Rat-1 (Klock et al. 1998). CLU is up-regulated when H-Ras is down-regulated (Kyprianou et al. 1991) in human MCF-7 breast cancer cells. Analysis of gene expression in rat fibroblasts transformed with activated H-Ras confirmed that CLU is one of the most significantly down-regulated genes (Lund et al. 2006).

Although it was initially thought that c-Myc could not regulate the expression of CLU (Klock et al. 1998), the group lead by Andrei Thomas-Tikhonenko et al. (2004) reported that ectopic levels of c-Myc could strongly repress the expression of CLU in murine colonocytes or human keratinocytes, and forced over-expression of CLU could inhibit, at least partially, c-Myc-dependent tumorigenesis. It has been recently shown that also N-Myc, similar to c-Myc, is a negative regulator of CLU (Chayka et al. 2009).

The mechanisms of repression by Myc family members appear to be complex and are still a matter of active investigation. On the other hand, recent studies suggest that Ras-mediated silencing of CLU is epigenetic (Lund et al. 2006). Ras induces deacetylation of the CLU promoter followed by methylation of a CpG island placed 14.5 kb upstream of the transcription start site (Lund et al. 2006).

A plausible hypothesis to explain the fact that oncogenes generally down-regulate CLU is that suppression of CLU expression is required for oncogene-dependent transformation.

Epigenetic regulation of CLU expression in cancer

Other research groups have observed epigenetic silencing of CLU in transformed cells and cancer tissues. CLU is methylated in the transgenic adenocarcinoma of mouse prostate (TRAMP)-C2 murine prostate cancer cell line, as well as in the human prostate cancer cell line LNCaP. CLU expression is significantly reduced in untreated and hormone-refractory human prostate carcinomas with respect to normal tissue (Rauhala et al. 2008). At the moment, we do not have experimental evidence of this mechanism in breast cancer cell lines, but further support of this hypothesis comes from the work by Nuutinen et al. (2005), who show that CLU transcription is silenced by gene methylation and deacetylation in human neuroblastoma and neuronal cell lines. A similar mechanism of epigenetic regulation has also been described in tumour-conditioned endothelial cells (Hellebrekers et al. 2007). In this system, CLU is significantly down-regulated through histone H3 deacetylation and loss of H3 lysine-4 methylation. Proliferation of endothelial cells was significantly induced upon down-regulation of CLU, indicating that this gene inhibits endothelial cell growth. Finally, three-dimensional sprouting of endothelial cell spheroids in a collagen gel was significantly increased by down-regulation of CLU, suggesting that CLU expression is inversely related with neoangiogenesis and cells sprouting.

CLU and NF-κB signalling

Nuclear factor κB (NF-κB) is a multifunctional transcription factor that has a central importance in immunity and cancer. NF-κB is activated in response to external stimuli, (i.e. engagement of the tumour necrosis factor receptor by its ligand), by the IκK kinases α, β and γ, which phosphorylate the inhibitors of NF-κB (IκBs), liberating a transcriptionally active NF-κB molecule. The first evidence that NF-κB regulates CLU expression was provided by a study aimed to systematically analyse and isolate all NF-κB target genes in mouse embryo fibroblasts. Among the plethora of genes activated by NF-κB, CLU was one of the most highly regulated genes (Li et al. 2002). Interestingly, knockout of any one of the three IκKs resulted in lack of activation of CLU, suggesting that its activation is dependent on the whole NF-κB signalosome.

But the effects of NF-κB signalling on CLU expression are more complex, and interestingly it was later shown that CLU regulates NF-κB activity in a negative manner by stabilising the inhibitor IκBs
(Santilli et al. 2003, Devauchelle et al. 2006, Savkovic et al. 2007, Takase et al. 2008a,b). By western blot analysis, we have observed an increased expression level of IκBα and a reduced expression of NF-κB also in PNT1A human immortalised prostate epithelial cells stably overexpressing CLU compared with control cells transfected with empty vector (Bettuzzi et al. 2009). Confocal microscopy has confirmed that CLU over-expression has caused cytoplasmatic accumulation of p65. All these evidences lead to the hypothesis that CLU participates in a negative loop in which transcriptional activation of CLU is evoked to dampen NF-κB activity.

Notably, key pro-survival/proliferation NF-κB target genes, such as p-AKT, cyclin D1 and BCL2, were down-regulated in the presence of exogenous CLU. In contrast, expression of the growth suppressor p21 was increased and a robust slow-down of the cell cycle was detected in CLU-transfected PNT1A cells (Bettuzzi et al. 2009).

CLU expression in prostate and breast cancer

It is now evident that changes in CLU expression are important events in cancer development, but the specific role of CLU in tumourigenesis is still a matter of debate. In different kinds of cancers, CLU has been reported to be up- or down-regulated (Redondo et al. 2000, Xie et al. 2002, 2005, Zhang et al. 2003, Chen et al. 2004, Pucci et al. 2004). We have found that CLU mRNA and protein products are down-regulated in both low-grade and high-grade prostate cancer (Bettuzzi et al. 2000, Scaltriti et al. 2004a, Rizzi et al. 2008). The study was conducted in surgical prostate specimens from patients affected by prostatic adenocarcinoma graded from 1 to 5 (Scaltriti et al. 2004a). CLU was down-regulated in tumour samples in comparison with benign-matched tissues. In benign tissue, both epithelial and stromal cells express CLU, but the staining is stronger in the stromal compartment. In low-grade prostate cancer, CLU is colocalised with GAS-1 (a marker of cell quiescence) in the stromal compartment and accumulates in the basal lamina. In high-grade tumours, CLU stains the remnants of stromal matrix, while epithelial cancer cells were rarely positive. When seen in these cells, CLU staining is confined to the cytoplasm. It might be hypothesised that extracellular CLU protein secreted by stromal cells is involved in tissue remodelling processes during stromal compartment involution due to cancer progression. Others confirmed the intense staining for CLU in stromal cells, and found that only CLU staining of stroma was associated with prostate cancer recurrence (Pins et al. 2004). Interestingly, some years later Tomlins et al. (2007) used laser micro-dissected samples and found that CLU gene is differentially expressed in the stroma and in the epithelium, being more expressed in the stromal compartment. These results showing high-CLU distribution in the stromal compartment associated to down-regulation in the tumour cells disagree with earlier data reported by July et al. (2002). In their work, the authors show that CLU expression is limited to the epithelial compartment, being significantly higher in prostate cancer of patients who received neoadjuvant hormone therapy and suggesting that CLU could play an important role in the onset of castration-resistant disease. This discrepancy may be explained by the existence of different CLU forms. The possibility that they may undergo specific changes of expression during the different phases of neoplastic transformation must also be considered. Furthermore, sub-cellular localisation of CLU might also be an important key to define its biological functions. At the moment, we cannot rule out the possibility that CLU might act as a negative tumour modulator in the early stage of prostate cancer, while being recruited as a positive tumour growth modulator in the more advanced stages. This may very likely happen during the onset of androgen and chemotherapy resistance.

Oncomine is a public database containing a large collection of gene expression experiments on human cancer (Rhodes et al. 2004). We have interrogated this database to understand whether CLU is up- or down-regulated in prostate cancer (PCa). The meta-analysis of available data showed that CLU mRNA is differentially expressed in cancer tissue compared with normal prostate. In 14 out of 15 independent studies comparing benign tissue to prostate cancer, CLU was found significantly down-regulated (the results of 12 studies with the most significant results ($P<0.0001$) are shown in Fig. 3A).

Remarkably, CLU expression is inversely proportional to the grade and/or metastatic stage of PCa in eight out of eight studies (the result of one study is shown in Fig. 3B; the complete set of experiments can be seen at www.oncomine.org).

Redondo et al. (2000) studied CLU expression in breast cancer. They studied 114 formalin-fixed/paraffin-embedded invasive breast carcinoma samples and 40 normal glandular epithelia controls. They used a non-commercially available antibody anti-human CLU for this study; the specificity of this antibody was previously evaluated in western blot analysis but not in immunohistochemistry analysis (IHC) by the researchers who developed the antibody (Murphy et al. 1988).
CLU expression. A more recent study has evaluated between CLU immunostaining and cancer stage or the other hand, they did not find any correlation progesterone and oestrogen receptors expression. On and a negative correlation with the status of between the extent of the reaction and the tumour size, for CLU. The authors also found a positive correlation and 53% of tumour samples showed a positive staining shown: 1, LaPointe et al. (2001); 6, Dhanasekaran et al. (2002); 8, Singh et al. (2005); 10, Luo et al. (2007); 11, Varambally et al. (2005); 12, Holzbeierlein et al. (2004). Dark grey boxes, CLU expression in normal tissues; medium grey boxes, CLU expression in cancer tissues. (B) CLU expression is inversely proportional to the Gleason grade and metastatic stage of prostate cancer. The box plot representation is from the study by Vanaja et al. (2003).

CLU expression was scored positive when more than 10% of tumour cells showed staining. The expression of CLU in normal epithelial cells was always negative and 53% of tumour samples showed a positive staining for CLU. The authors also found a positive correlation between the extent of the reaction and the tumour size, and a negative correlation with the status of progesterone and oestrogen receptors expression. On the other hand, they did not find any correlation between CLU immunostaining and cancer stage or prognosis. A more recent study has evaluated CLU expression in 141 breast cancer samples using a commercially available antibody (Kruger et al. 2007). As in the study by Redondo et al., CLU staining was considered positive when found in at least 10% of the cancer cells. Normal tissue was not included in the experimental design of this work, so this study does not really answer the basic question whether CLU is up- or down-regulated in breast cancer. Positive CLU staining was found in 26% of the tumours analysed. Significant correlation was found with histological tumour grade. It must be stated that the same data also show that the remaining 74% of tumour samples were negative for CLU expression. Therefore, further studies are required to understand whether CLU is expressed only in some specific sub-sets of breast cancer, or whether adjuvant radiotherapy has significantly affected CLU expression in primary tumours. This is predictable since X-ray irradiation potently enhances CLU expression in tissues (Yang et al. 2000, Klokov et al. 2004).

We interrogated the Oncomine database again to explore whether CLU mRNA is up- or down-regulated in breast cancer. Nine studies show that CLU is differentially expressed in breast cancer samples versus normal/benign tissues. In all of these studies, CLU mRNA is down-regulated in cancer tissue with respect to normal/benign tissue (Fig. 4A). As in the case of prostate cancer, CLU is down-regulated as a function of the tumour grade, being more repressed in poorly differentiated tumours (Fig. 4B).

**Hormone-refractory and metastatic cancers: the challenge of CLU antisense therapy**

Localised prostate disease is generally managed by surgery or local radiation therapy. About one third of the patients treated with conventional clinical protocols will develop metastases and undergo androgen ablation therapy. Unfortunately, too often the disease progresses towards the resistant phenotype turning to a hormone-independent state, also called castration-resistant prostate cancer (CRPC). CRPC is unresponsive to further hormonal therapy and prognosis is very poor (median survival is ~1 year). Breast cancer is commonly treated by various combinations of surgery, radiation therapy, chemotherapy and hormone therapy. In general, at the beginning of therapy, systemic agents are active, being effective in 90% of primary breast cancers and 50% of metastases. However, after a variable period of time, progression occurs. At that point, resistance to therapy is not only common, but unfortunately expected (Gonzalez-Angulo et al. 2007).
CLU expression is down-regulated in breast cancer. (A) Meta-analysis of available microarrays data on the Oncomine website. CLU mRNA is significantly down-regulated in breast cancer tissue compared with normal breast. Box plot analysis of 9 studies and associated references are shown: 1, Richardson et al. (2006); 2, Finak et al. (2008); 3, Radvanyi et al. (2005); 4, Sorlie et al. (2001); 5, Turashvili et al. (2007) (lobular cancer); 6 and 7, Perou et al. (2000) (lobular cancer, ductal cancer); 8, Karnoub et al. (2007); 9, Turashvili et al. (2007) (ductal cancer). Dark grey boxes, CLU expression in benign breast; medium grey boxes, CLU expression in breast cancer. (B) CLU expression is inversely proportional to the differentiation grade of breast tumour. The box plot representation is from the study by Sorlie et al. (2001). Well-differentiated breast cancer, grade 1; moderately differentiated breast cancer, grade 2; poorly differentiated cancer, grade 3.

New experimental therapies such as gene therapy, immunotherapy and inhibition of specific cell signalling pathways are currently being investigated to overcome the poor efficacy of current treatments.

Despite the fact that that CLU is down-regulated in the majority of naïve cancer cells (Figs 3A, B and 4A, B), its expression was found up-regulated in prostate and breast cancer cells resistant to conventional chemotherapy or hormonal therapy (Miyake et al. 2000b,c, Mallory et al. 2005, Ranney et al. 2007). Cappelletti et al. found that CLU is up-regulated in a sub-set of patients bearing breast cancer not responding to the neoadjuvant anti-oestrogen therapy with tamoxifen. They suggested that this gene might be directly or indirectly involved in the mechanism of resistance to anti-oestrogen (Cappelletti et al. 2008). Others suggested that CLU is involved in the acquisition of chemoresistance by mediating inhibition of TRAIL-triggered apoptosis in docetaxel-resistant PC3 prostate cancer cells (Sallman et al. 2007). Other findings support the hypothesis that silencing CLU expression can enhance the cytotoxicity of chemotherapeutic agents (Trougakos et al. 2004, Redondo et al. 2007, Sowery et al. 2008), as well as IR (Zellweger et al. 2002, Criswell et al. 2005) or androgen ablation therapy (Gleave & Miyake 2005, Toffanin et al. 2008). Antisense oligonucleotide (ASO) directed against CLU mRNA has been developed and approved for clinical trials (Chi et al. 2005). The CLU ASO is called OGX-011: a 21-mer modified ASO directed against the translation start site located in exon 2. Preclinical data indicate that OGX-011, when given alone, reduced IC50 in PC3 cells treated with conventional chemotherapeutic drugs, but had no effect on the growth of established PC3 tumours (Miyake et al. 2000a). Treatment of MCF-7 cells with OGX-011 or siRNA against CLU enhanced chemosensitivity to paclitaxel (So et al. 2005). Therefore, CLU has been proposed as an interesting target in CRPC and metastatic breast cancer.

The phase I trial, having a unique pharmacodynamic endpoint, has been completed. The study demonstrated that active concentration of OGX-011 reached prostate cancer tissues and inhibited CLU expression with tolerable side effects (Chi et al. 2005, 2008). A phase II clinical trial in men with high-risk localised PCa approved by NCI is currently ongoing. The purpose of this trial is to assess the effects of combined therapy with androgen ablation and OGX-011 given prior to radical prostatectomy in men with localised PCa characterised by high-risk features.

Despite the promising results obtained in vitro in MCF-7 breast cancer cells and in vivo in athymic mice bearing MCF-7 tumours (So et al. 2005), the results of phase II clinical trials recently published are disappointing (Chia et al. 2009). The primary objective of this phase II trial was to assess both safety and efficacy of the combination of OGX-011 and docetaxel for metastatic breast cancer. The clinical goal was to
reach a response rate in the group treated with the combination of the two agents ≥55%. The combination was found well tolerated, but although serum CLU decreased on treatment, there was no relationship observed between the amount of decrease and response. The detected response rate was actually equal to 35% similar indeed to that expected from the single agent docetaxel. On the basis of these results, the trial did not meet the criteria to proceed to the second stage of accrual (Chia et al. 2009).

Chemoprevention of prostate cancer: a role for CLU in the mechanism of action of green tea catechins

Chemoprevention is defined as pharmacological intervention with natural or synthetic compounds to prevent, inhibit, delay or reverse carcinogenesis (Sporn et al. 1976). An expanded definition of cancer chemoprevention includes compounds that block neoplastic inception as well as reversing the progression of transformed cells before the appearance of clinically relevant malignant lesions (William et al. 2009).

Prostate cancer represents an ideal target for chemoprevention, because of its high incidence and long latency period before the development of clinically evident disease. Epidemiological and case control studies provide support for the chemopreventive effect of bioactive compounds, such as catechins, extracted from green tea (Jian et al. 2004). The possible anti-cancer activity of green tea catechins (GTCs) may be explained by a number of different mechanisms extensively reviewed by Khan et al. (2006). Epigallocatechin-3-gallate (EGCG), the most abundant catechin contained in green tea, potently inhibited the growth of both SV40-immortalised PNT1A and metastatic PC3 cells, while normal human prostatic epithelial cells were not significantly affected (Caporali et al. 2004). In immortalised and cancer cell lines treated with EGCG, CLU protein was increased, accompanied by cell death markers such as caspase 9, while CLU protein remained undetectable before and after treatment of primary cell cultures at the same doses (Caporali et al. 2004). This result is remarkable, since EGCG and catechins generally inhibit gene expression and protein activity, interfering with the transcription process probably by direct binding. CLU seems to be one of the few genes up-regulated by GTCs.

The TRAMP model of prostate carcinogenesis was developed as an important tool for understanding the progression of PCa (Greenberg et al. 1995). TRAMP mice display in situ and invasive PCa, mimicking the whole spectrum of human prostate cancer progression from prostatic intraepithelial neoplasia (PIN) to androgen-independent disease (Kaplan-Lefko et al. 2003). TRAMP mice express the SV-40 T/t antigens under the control of the prostate-specific, androgen-dependent minimal rat probasin promoter. Development of prostate cancer in TRAMP mice is thus SV40-driven and age-related. Gupta was first to report GTC effect on TRAMP (Gupta et al. 2001).

We found that CLU expression is down-regulated during prostate cancer progression in the TRAMP mouse model (Caporali et al. 2004). Oral administration of 0.3% GTCs in drinking water to male TRAMP mice reduced PCa onset from 100 to 20% without any evidence of adverse events. Animals responding to GTCs displayed recovery of CLU expression followed by reactivation of caspase 9 expression, while those refractory to GTCs, i.e. tumour-bearing mice, did not express either CLU or caspase 9. Both CLU and caspase 9 expressions were determined by IHC as well as western blots of pooled prostates (Caporali et al. 2004, Scaltriti et al. 2006).

Based upon these preclinical findings, we conducted a proof-of-principle trial to assess the potential efficacy of GTCs in the prevention of PCa in 60 patients bearing HGPIN, a premalignant lesion associated with increased risk of coexistent PCa (Bettuzzi et al. 2006). The primary endpoint of the study was to determine the impact of GTCs administration on prevalence/progression of PCa. Following one year of treatment, only 1/30 (3%) of patients who received the green tea polyphenols were diagnosed with cancer compared with 9/30 (30%) of the placebo group. In a recent follow-up of the patients in this study (Brausi et al. 2008), a Kaplan–Meier analysis showed 90% of GTCs-treated patients remained free of cancer diagnosis after 3 years versus 50% of placebo patients. Preliminary data from our laboratory confirm that CLU is up-regulated in biopsies from GTCs-treated subjects, while it remains unchanged in placebo-treated specimens (Rizzi et al. 2009b). Further work will be necessary in order to understand the role of CLU in GTCs-mediated chemoprevention.

New insights of CLU as tumour modulator: is CLU a new tumour suppressor gene?

To investigate whether genetic inactivation of CLU in vivo causes phenotypic alterations in the prostate epithelium, we carried out the morphological inspection of the prostate of mice in which CLU had been genetically inactivated. CluKo mice do not show overt
defects, despite being more prone to cardiac auto-immunity (McLaughlin et al. 2000). Surprisingly, we found advanced PIN or differentiated carcinoma in 100 and 87% of mice with homozygous or heterozygous deletion of CLU. Wild-type siblings did not show any cancer lesions. We found a higher expression of the proliferation marker Ki67 (i.e. higher proliferation index) in the normal portion of prostatic tissue of homo- and heterozygous CluKo mice with regard to wild-type controls (Bettuzzi et al. 2009). We also found that p65 NF-κB staining was much more intense in the normal portion of the prostate or in low-grade PIN lesions of CluKo mice compared with wild-type controls. NF-κB plays an important role in the onset of mammalian prostate cancer, being required for prostate cancer cell proliferation and survival (Shukla et al. 2004, 2005, Suh & Rabson 2004).

To further assess whether prostate tumourigenesis is affected by the loss of CLU expression, we crossed TRAMP mice with CluKo mice. Inactivation of one or both CLU alleles in TRAMP mice led to more advanced invasive disease. Tumour spreading and homing were enhanced in TRAMP/CluKo mice, metastases occurred earlier in ectopic sites and also the survival was decreased; 30% of the TRAMP–CLU(−/−) mice died by 28 weeks versus none of the TRAMP only mice, (P < 0.01; Bettuzzi et al. 2009).

Thus, cancer progression is certainly not suppressed or delayed by absence of CLU but, on the contrary, loss of CLU significantly favours the rapid onset of tumour growth. These findings are corroborated by previous results indicating that CluKo mice are also more susceptible than normal mice to chemically induced skin tumourigenesis, suggesting that CLU might be a negative modulator of epithelial cell transformation (Thomas-Tikhonenko et al. 2004).

CLU was also found to act as negative tumour modulator in neuroblastoma. It has been recently proved that the aggressiveness of neuroblastomas arising in MYCN-transgenic mice has significantly increased after deletion of the CLU gene (Chayka et al. 2009). Similar to the effects observed in TRAMP mice, depletion of CLU in MYCN-transgenic mice caused activation of NF-κB signalling and epithelial-to-mesenchymal transition. Thus, inhibiting NF-κB and maintaining an epithelial phenotype are likely to be critical features of the CLU tumour suppressive functions (Chayka et al. 2009).

**Figure 5** CLU and tumourigenesis. CLU basal level of expression in benign cells is low and confined to sCLU production (1). Following induction of apoptosis by different apoptotic stimuli (2) epithelial cells would switch to production of nCLU (3) which, in turn, would induce programmed cell death mainly through anoikis-driven apoptosis (4). Genetic lesions, which occur during early phases of tumourigenesis, may lead to acquisition of survival advantages resulting in clonal selection (5). Under these conditions, blocking CLU from entering the nucleus would be a key step in the transformation process (6). At this stage, the selection of cells tolerating high levels of cytoplasmic CLU (cCLU) would signal acquisition of a resistant phenotype. This event is associated with impaired mitosis (7), as demonstrated by the production of polynucleated cells (8). A potential new anti-cancer therapy could be based on the re-targeting of CLU in the nuclei to cause commitment of resistant cells to death.
Conclusions

Many research teams have produced a great amount of literature and important data on CLU action and tumourigenesis. Nevertheless, contradictions and alternative hypothesis still exist due to different experimental models or tools and to different interpretations. We should now be able to synthesise this enormous amount of data into a better understanding of CLU action.

Is CLU a positive or a negative modulator of mammalian tumourigenesis? Taking the prostate as the paradigm, we know that CLU is the most powerful overexpressed gene during apoptosis-driven rat prostate regression induced by castration or pharmacological androgen ablation (Bettuzzi et al. 1989, Astancolle et al. 2000). If CLU exerts a pro-survival action, why is apoptosis induced and why does the prostate gland shrink concomitantly with CLU overexpression?

Understanding the role of CLU in tumourigenesis is complicated by the different protein forms of CLU. It is also complicated by the time-course of the disease and the selection pressures imposed on the cancer by treatments such as hormone ablation or chemotherapy. CLU is down-regulated in the majority of naïve cancers at the mRNA level, according to Oncomine data. However, up-regulation of CLU is possible in some cancer cells, especially after adjuvant hormonal therapy. Scaltriti et al. (2004a) described that the vast majority of prostate cancer cells were devoid of CLU, but a few nests of morphologically undistinguishable cancer cells had a strong positive signal for CLU. Are these cells already androgen-independent, escaping CLU control?

Scaltriti et al. (2004b) and Moretti et al. (2007) have investigated CLU action by comparing immortalised PNT1A cells (mimicking the early stages of transformation) to metastatic, androgen-independent PC3 cells. In both cell types, nuclear localisation of CLU inhibits cell proliferation, causes cell death and also inhibits cell migration/invasion by interaction with α-actinin. Interestingly, following treatment by CLU overexpression through transient transfection, stable cell clones overexpressing CLU emerged which do not show any nuclear localisation of CLU. These cells acquire resistance to apoptosis and tolerate high intracellular cytoplasmic levels of CLU. Thus, does acquisition of resistance to apoptosis require expression of CLU for survival? How important is the inhibition of CLU entering the nucleus?

Is it now possible to reconcile all the experimental results and contributions from different laboratories on the role of CLU in tumourigenesis, even when apparently contradictory? We believe this synthesis is possible and our hypothesis is provided in Fig. 5. Just like many other typical tumour suppressor genes, early-stage-associated events related to physiological action must be distinguished from late-stage-associated ones, when the tumour suppressive factor is inactivated or acquired improper activity. For instance, it has been recently shown that pRb, a paradigmatic tumour suppressor gene, is amplified and plays a promoting role in late-stage colon cancer by suppressing E2F1 and enhancing cell survival by activating the Wnt pathway (Morris et al. 2008).

Figure 5 starts from the general observation that, under physiological conditions, CLU basal level of expression in benign cells is low and confined to sCLU production (point 1). Following induction of apoptosis by different apoptotic stimuli (point 2), epithelial cells would switch to production of nCLU (point 3) which, in turn, would induce programmed-cell death, mainly through anoikis-driven apoptosis (point 4). The process of tumourigenesis is characterised by early genetic lesions, some of which may lead to acquisition of survival advantages, resulting in clonal selection (point 5). Under these conditions, blocking CLU from entering the nucleus would be a key step in the transformation process (point 6). At this stage, the detection of cells tolerating high levels of CLU by intense cytoplasm staining not associated to apoptosis would signal acquisition of a resistant phenotype. This resistant phenotype can continue cell transformation, leading to late-stages associated with impaired mitosis (point 7). In fact, improper mitotic spindle assembling would cause further genomic instability (point 8), as demonstrated by the production of polynucleated cells (Scaltriti et al. 2004b). At points 6–8, a potential new anti-cancer therapy would consist in re-targeting of CLU to the nucleus to commit resistant cells to death.

The hypothesis that CLU may act in vivo as a tumour suppressor gene is supported by a recent study which reports increased penetrance of metastatic neuroblastoma in mice when one or both CLU alleles are deleted (Chayka et al. 2009). These results confirmed our results with CluKo in the TRAMP murine model of prostate cancer (Bettuzzi et al. 2009). Consistent with the hypothesis that CLU is a negative modulator of tumour growth in mammals, we found that its expression is down-regulated in PCa (Bettuzzi et al. 2000, Scaltriti et al. 2004a, Rizzi et al. 2008), while its expression is restored in TRAMP mice responding to chemoprevention with GTCs (Caporali et al. 2004, Scaltriti et al. 2006).

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No mutations of \textit{CLU} have been found yet in human cancer, so it is likely that the mechanism of inactivation is epigenetic. This hypothesis is supported by the frequent observation of CpG island methylation or histone deacetylation in the proximity of the \textit{CLU} gene in different cancers, including PCa (Lund \textit{et al.} 2006, Hellebrekers \textit{et al.} 2007, Suuronen \textit{et al.} 2007, Rauhala \textit{et al.} 2008).

How can we reconcile this view with previous findings suggesting that \textit{CLU} is a pro-survival oncogene? The answer to this important issue probably lies in the fact that the process of tumourigenesis often exploits cellular genes, including tumour suppressor genes, for its own purposes. In a similar manner, we hypothesise that \textit{CLU} can lead a double life: on the one hand, it suppresses tumourigenesis and metastatic spread by inhibiting NF-κB activity; on the other hand, highly malignant cells can reactivate \textit{CLU} expression suppressing NF-κB and survive since a pro-apoptotic NF-κB signalling is often involved in replication stress induced by chemotherapeutic drugs.

\textbf{Declaration of interest}

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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