REVIEW

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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*. 1990*a*, 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 an 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,

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complement activity, sperm maturation and prostate gland involution driven by androgen depletion. This protein is now officially called CLU according to a recent international consensus agreement.

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*. 1990*a*). 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*. 1990*a*,*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

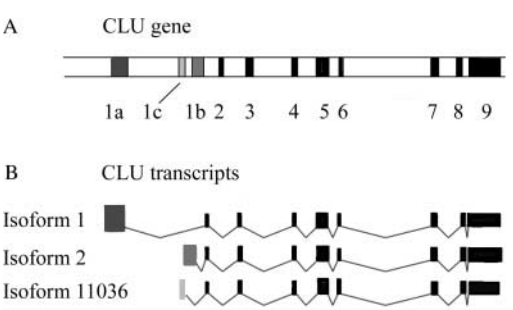

Figure 1 Structure of *CLU* gene and transcription products. (A) *CLU* gene maps on chromosome 8. The position of exons and introns on the gene is shown. Black bands: exons, white bands: introns. (B) Exonic structure of *CLU* mRNA variants. Dark grey exon, exon 1a of isoform 1; medium grey exon, exon 1b of isoform 2; light grey exon, exon 1c of isoform 11036.

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*. 2009*a*).

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*. 1990*a*). 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 (Kirszbaum *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 (Kirszbaum *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

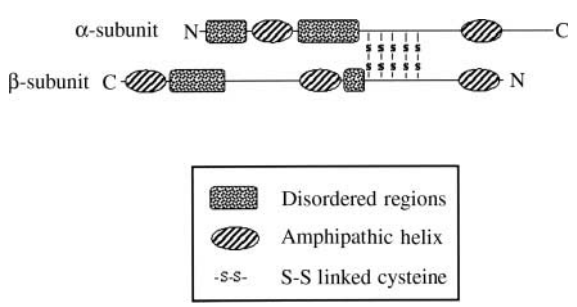

Figure 2 Location of ordered and disordered regions in the CLU protein.

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*. 2004*b*).

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*. 2004*b*). 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 (Itahana *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 overexpression of *CLU* could inhibit, at least partially, *c-Myc*-dependent tumourigenesis. 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*. 2008*a*,*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* overexpression has caused cytoplasmic 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*. 2004*a*, 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*. 2004*a*). *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 microdissected 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).

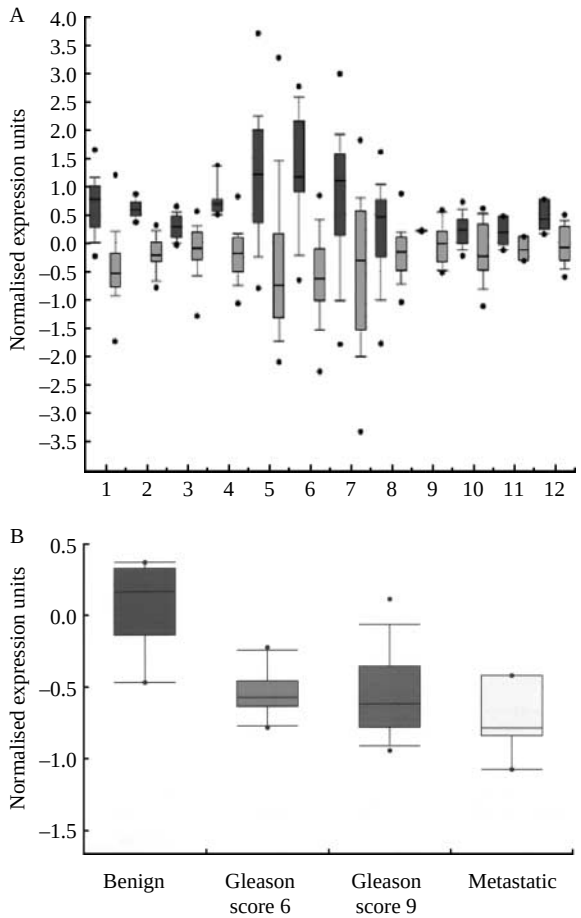

Figure 3 *CLU* expression is down-regulated in prostate cancer. (A) Meta-analysis of available microarrays data on the Oncomine website. *CLU* mRNA is significantly down-regulated in prostate cancer tissue compared with normal prostate. Box plot analysis of 12 studies and associated references are shown: 1, LaPointe *et al.* (2004); 2, Welsh *et al.* (2001); 3, Yu *et al.* (2004); 4, Vanaja *et al.* (2003); 5, Dhanasekaran *et al.* (2001); 6, Dhanasekaran *et al.* (2005); 7, Tomlins *et al.* (2007); 8, Singh *et al.* (2002); 9, LaTulippe *et al.* (2002); 10, Luo *et al.* (2002); 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).

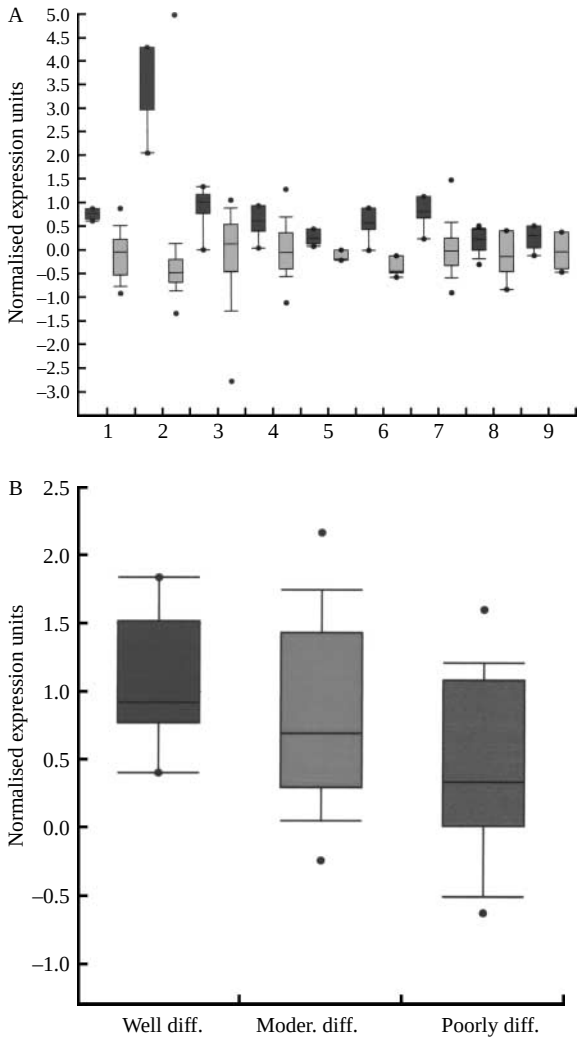

Figure 4 *CLU* expression is down-regulated in breast cancer. (A) Meta-analysis of available microarrays data on the Oncomine website. *CLU* mRNA is significantly down-regulated in breast cancer tissue compared with normal breast. Box plot analysis of 9 studies and associated references are shown: 1, Richardson *et al.* (2006); 2, Finak *et al.* (2008); 3, Radvanyi *et al.* (2005); 4, Sørlie *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 Sørlie *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.* 2000*b*,*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 tor-emifene. 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 chemo-therapeutic 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 IC_{50} in PC3 cells treated with conventional chemotherapeutic drugs, but had no effect on the growth of established PC3 tumours (Miyake *et al.* 2000*a*). Treatment of MCF-7 cells with OGX-011 or siRNA against CLU enhanced chemo-sensitivity 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 dis-appointing (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 $\geq$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). Epigallocathechin-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*. 2009*b*). 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 autoimmunity (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).

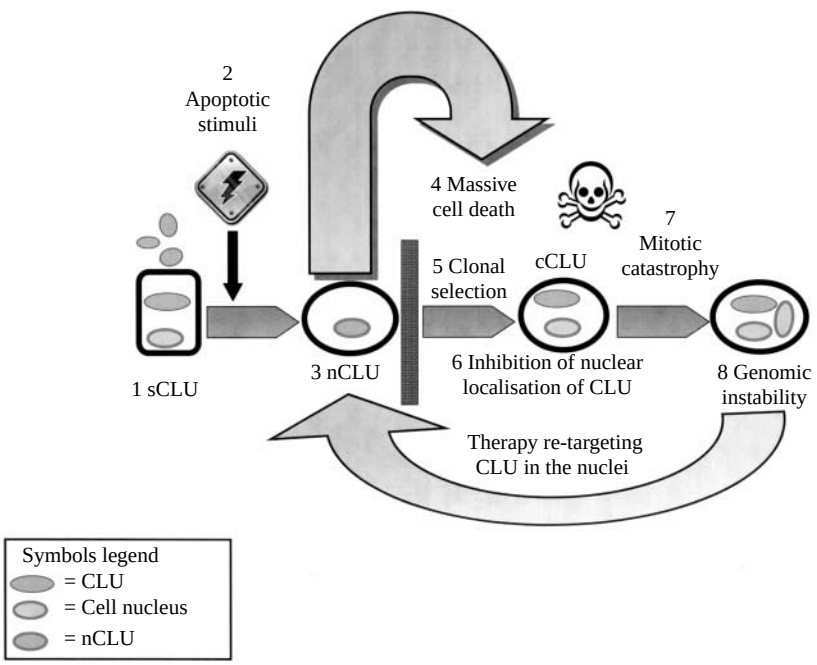

Figure 5 *CLU* and tumourigenesis. *CLU* basal level of expression in benign cells is low and confined to sCLU production (1). Following induction of apoptosis by different apoptotic stimuli (2) epithelial cells would switch to production of nCLU (3) which, in turn, would induce programmed cell death 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*. (2004*a*) 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*. (2004*b*) 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*. 2004*b*). 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 mammalians, we found that its expression is down-regulated in PCa (Bettuzzi *et al*. 2000, Scaltriti *et al*. 2004*a*, 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).

No mutations of *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 *CLU* gene in different cancers, including PCa (Lund *et al*. 2006, Hellebrekers *et al*. 2007, Suuronen *et al*. 2007, Rauhala *et al*. 2008).

How can we reconcile this view with previous findings suggesting that *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 *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 *CLU* expression suppressing NF-κB and survive since a pro-apoptotic NF-κB signalling is often involved in replication stress induced by chemotherapeutic drugs.

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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