Regulation of CLU Gene Expression by Oncogenes and Epigenetic Factors: Implications for Tumorigenesis

Arturo Sala,[*] Saverio Bettuzzi,[†] Sabina Pucci,[‡] Olesya Chayka,[*] Michael Dews,[§] and Andrei Thomas-Tikhonenko[§]

[*]*Molecular Haematology and Cancer Biology Unit, Institute of Child Health, University College London, United Kingdom*
[†]*Dipartimento di Medicina Sperimentale, Sezione di Biochimica, Biochimica Clinica e Biochimica dell'Esercizio Fisico, Università di Parma, Via Volturno 39-43100 Parma and Istituto Nazionale Biostrutture e Biosistemi (I.N.B.B.), Rome, Italy*
[‡]*Department of Biopathology, Institute of Anatomic Pathology, University of Rome "Tor Vergata," Rome, Italy*
[§]*Department of Pathology and Laboratory Medicine, University of Pennsylvania and Children's Hospital of Philadelphia, Philadelphia, Pennsylvania 19104, USA*

In no other field has the function of clusterin (CLU) been more controversial than in cancer genetics. After more than 20 years of research, there is still uncertainty with regard to the role of CLU in human cancers. Some investigators believe CLU to be an oncogene, others—an inhibitor of tumorigenesis. However, owing to the recent efforts of several laboratories, the role of CLU in important cellular processes like proliferation, apoptosis, differentiation, and transformation is beginning to emerge. The "enigmatic" CLU is becoming less so. In this chapter, we will review the work of research teams interested in understanding how CLU is regulated by oncogenic signaling. We will discuss how and under what circumstances oncogenes and epigenetic factors modify CLU expression, with important consequences for mammalian tumorigenesis. © 2009 Elsevier Inc.

Advances in CANCER RESEARCH

0065-230X/09 $35.00
DOI: 10.1016/S0065-230X(09)05007-6

I. INTRODUCTION

The role of clusterin (CLU) in cancer has been the matter of debate for many years. There are many reports, mainly based on studies with cancer cell lines, indicating that CLU is involved in promotion of tumorigenesis and in conferring resistance to chemotherapeutic drugs (Chi *et al.*, 2008; Chung *et al.*, 2004; Miyake *et al.*, 2003; Sallman *et al.*, 2007). However, more recent studies using mouse models of neuroblastoma and prostate cancer have established that an important function of CLU is to restrict tumor development (Bettuzzi *et al.*, in press; Chayka *et al.*, 2009). While some of the contrasting results observed so far could be explained by the use of different types of cell lines, reagents or procedures, we suggest here that CLU, lying at the crossroad of life and death, is at the same time an oncogene *and* a tumour suppressor gene. This concept will be developed and clarified in the course of this review.

The categorization of genes in strict functional classes clearly does not reflect the complexity of biological systems. The boundaries dividing gene functions are becoming blurred and to classify genes as oncogenes or tumor suppressors is, in the light of the more recent literature, an anachronism. Classical tumor suppressor genes like pRb, PML, and p21WAF-Cip are now found to promote human cancer in specific contexts (Cote *et al.*, 1998; Ito *et al.*, 2008; Morris *et al.*, 2008; Viale *et al.*, 2009). Conversely, protooncogenes like E2F1 and MYB have been shown to restrict tumor growth or promote the maintenance of normal cell division (Morris *et al.*, 2008; Pierce *et al.*, 1999; Tarasov *et al.*, 2008). Additionally, it is useful to keep in mind that association ("post hoc") does not imply causation ("propter hoc"). Just because a gene is overexpressed or underexpressed in certain tumors, one cannot conclude that it is driving or inhibiting neoplastic growth, respectively. Instead, its deregulation might be a defense mechanism that the host employs to maintain tissue homeostasis. Research on CLU could well be a case study in cancer gene complexity.

CLU is the prototypical multifunctional gene: it was found to regulate apoptosis, cell–cell interactions, protein stability, cell signaling, 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 animals develop and live normally (McLaughlin *et al.*, 2000). Since CLU expression in mammalian cells is highly modulated by certain pathological processes or exposure to physical and chemical agents, it is tempting to speculate that CLU is mainly required to respond to exogenous or endogenous stress signals. Indeed, CLU knockout mice are more susceptible than

wild-type mice to experimentally induced autoimmune diseases, and fibroblasts derived from CLU knockout mice are more sensitive to thermal injury (McLaughlin *et al.*, 2000; Santilli *et al.*, 2005). In cancer, expression of CLU has been shown to be either up- or downmodulated, although the data available on the Oncomine Web site, which represents a very large and growing collection of cDNA microarray experiments, shows that in the most cancer types CLU is downregulated (Fig. 1). It is still unclear whether the opposing observations published in the literature are caused by technical reasons—that is, use of different antibodies, cell lines, patients, etc.—or they reflect the fact that CLU can be a tumor suppressor and promoter, at the same time, depending on the specific biology of the disease and its phase of progression.

As we try to avoid the dualistic, Cartesian classification of cancer genes into oncogenes and tumor suppressors, we submit that a gene can inhibit tumor growth under untreated conditions (i.e., be "pro host") but at the same time act "antipatient" by rendering tumors resistant to chemo-, radio-, or biological therapy. Conversely, there are well-recognized examples when the oncogene initiates or even promotes tumor growth (or acts "antihost") but at the same time confers chemosensitivity (or acts "propatient"). While these distinctions have been largely absent from the CLU literature, for other cancer genes these complexities are well appreciated. The c-Myc protooncogene is a case in point.

At the cellular level many putative Myc target genes pertain to cell proliferation. Among them are ornithine decarboxylase, cyclins A and D2, cdc25A, cdk4, Id2, and telomerase. In addition, entry into the cell cycle is facilitated by repression of several genes, such as assorted cdk inhibitors, gadd45, etc. (reviewed recently in Meyer and Penn, 2008). Consistent with these observations, activation of Myc forces quiescent fibroblasts to reenter the cell cycle (Eilers *et al.*, 1989), and rodent fibroblasts with targeted disruption of Myc are severely deficient in cell proliferation (Mateyak *et al.*, 1997). Furthermore, at least in mice decreased expression of Myc results in hypoplasia (Trumpp *et al.*, 2001). Conversely, when overexpressed in many transgenic settings, c-Myc initiates tumor growth with very high penetrance (Morgenbesser and DePinho, 1994). Furthermore, c-Myc is overexpressed in a variety of spontaneous human cancers, making it a classical oncogene in the view of both mouse and human geneticists.

This "antihost" properties of Myc are balanced to some extent by the potentially "propatient" propensity of Myc to induce apoptosis (reviewed in Evan and Vousden, 2001), both intrinsic and extrinsic. To accomplish the former, Myc activates p53 via the ARF pathway (Zindy *et al.*, 1998). In

Fig. 1 CLU expression in primary tumors. Expression of CLU mRNA in Affymetrix experiments as represented on the Oncomine Web site (www.Oncomine.org). Shades of blue color indicate underexpression, whereas shades of red indicate overexpression. The intensity of the color is proportional to the statistical significance of the difference. Numbers indicate how many independent experiments show a significant difference in each tissue. Note that in the "Cancer vs. Normal" column (green rectangle) all the transformed tissues, with the exception of brain, show significant downregulation of CLU with respect to the corresponding normal samples.

addition, Myc appears to potentiate the extrinsic pathway triggered by ligation of a death receptor. Indeed, Myc has been shown to participate in apoptosis induced by Fas/CD95 ligand (Hueber *et al.*, 1997), TNF-α (Klefstrom *et al.*, 1994), and TRAIL (Ricci *et al.*, 2004).

To the extent that cytotoxic drugs inflict DNA damage and activate the intrinsic apoptotic pathway, one might predict that deregulation of Myc would be associated with chemosensitivity. Indeed, as exemplified by studies on human colon carcinoma, low-level Myc amplification (combined with wild-type p53 expression) increases susceptibility to 5-fluorouracil *in vivo* (Arango *et al.*, 2001). Similarly, human Burkitt's lymphomas with Myc overexpression appear to be intrinsically sensitive to TRAIL in the clinical setting (reviewed in Finnberg and El-Deiry, 2008).

The CLU situation has many interesting parallels with the Myc story. Not only is CLU downregulated by c-Myc but it has been reported to mediate TRAIL resistance in prostate cancer cells (Sallman *et al.*, 2007). Thus, it is tempting to extend this parallel and propose that CLU is to Myc as yin is to yang. According to this framework, CLU inherently inhibits cell proliferation and neoplastic growth (i.e., acts "prohost") but confers resistance to therapy (i.e., acts "antipatient")—hence its overexpression under certain conditions. Taking this view helps make sense of some famously contradictory data.

For example, the group lead by Martin Gleave has published a number of studies which suggest that expression of CLU is enhanced in human prostate cancer and antisense oligonucleotides targeting CLU expression inhibit prostate tumorigenesis *in vivo* and *in vitro* (Chi *et al.*, 2008; Miyake *et al.*, 2005). These results are contrasted by the work of the Bettuzzi group, which showed reduced expression of CLU during mouse and human prostate cancer progression (Caporali *et al.*, 2004; Scaltriti *et al.*, 2004b). The analysis of several gene expression studies available in the Oncomine database shows that there is a significant downregulation of the CLU mRNA in almost all types of cancer, as compared to matched normal tissue controls, corroborating the hypothesis that CLU expression is generally silenced in primary (i.e., frequently untreated) human cancers (Fig. 1).

As a further indication that CLU expression might be inactivated in mammalian tumorigenesis, CLU KO mice are more prone than wild-type counterparts to oncogene-induced tumorigenesis (Chayka *et al.*, 2009; Thomas-Tikhonenko *et al.*, 2004). This introduces the theme of this assay, namely, how CLU is regulated by oncogenic signaling and what role CLU plays in inhibiting mammalian cell transformation, as opposed to potentially promoting chemoresistance.

II. REGULATION OF CLU EXPRESSION BY TRANSFORMING ONCOGENES: EARLY EVIDENCE

Early reports demonstrated that expression of CLU is modulated during cell transformation. CLU expression was reported to be increased in gliomas compared to normal brain tissue (Danik *et al.*, 1991). In 1993, the group of

Michael Sporn observed that expression of CLU was increased after malignant transformation of the rat prostate caused by chemical carcinogenesis (Kadomatsu *et al.*, 1993). It should be noted that only the mRNA expression was detected in these early studies, leaving open the question of whether CLU protein was also upregulated. Further evidence that CLU might be important in human tumorigenesis originates from the observation that CLU expression is often modulated during apoptosis. A theory was elaborated suggesting that CLU is secreted during injury as a survival response in the face of apoptosis (Koch-Brandt and Morgans, 1996). Consequently, some research groups started to investigate whether oncogenic transcription factors could induce CLU, facilitating cell survival, transformation, and/or resistance to chemotherapeutic drug killing.

The first evidence that CLU expression is modulated by oncogenic activity was published in 1989 when it was first reported that a thermally inducible gene, called T64, was activated in avian cells by retroviral oncogenes with protein kinase activity such as v-src, v-fps, and v-mil (Michel *et al.*, 1989). Sequencing of T64 revealed that it was the avian orthologue of rat CLU. Subsequent investigations revealed that induction by the oncogenic kinases was dependent on the AP-1 binding site present in close proximity to the CLU transcription start site. Similarly, Herault *et al.* (1992) found that the gene most strongly overexpressed upon Rous sarcoma virus infection in quail neuroretina cells was CLU. Mutation of the TGACTCA motif in the CLU promoter abolished CAT activity of the reporter suggesting that the AP-1 binding site was required for induction by Src.

The role of AP-1 (a complex containing the Jun and Fos oncoproteins) in regulating CLU expression was confirmed later on in other contexts. For example, it was shown that TGF-β positively modulates CLU expression via activation of an AP-1 site in the mammalian CLU promoter (Jin and Howe, 1999). In this work, the authors proposed that the mechanism of activation is the removal of the *trans*-repression effect of c-Fos by TGF-β. In another study, exposure of HaCaT keratinocyte cells to vanadium was shown to induce apoptosis, c-Fos expression, and a switch from secreted to nuclear CLU (Markopoulou *et al.*, 2009). Ectopic expression of c-fos also induced apoptosis and nuclear CLU expression in HaCaT cells. The authors inferred that c-Fos controls the ratio of cytoplasmic versus nuclear fraction of CLU. However, it has not been resolved whether c-fos directly regulates the levels of the different CLU protein isoforms, or apoptosis resulting from c-Fos overexpression is actually causing the isoform switch.

Claudia Koch-Brandt's group was one of the first to study the role of two classical protooncogenes, namely c-MYC and Ha-RAS, in regulation of CLU expression. It was reported that overexpression of Ha-RAS, but not of c-MYC, in the rat embryo fibroblast cell line Rat-1 caused repression of

CLU expression at the mRNA level (Klock *et al.*, 1998). There had been no attempt to understand the mechanism by which Ha-RAS was causing the inhibitory effect, but this was clarified in subsequent studies that will be discussed later. This early evidence linking the activity of protooncogenes to CLU expression and the emerging role of CLU as a modulator of apoptosis prompted many other groups to study the relationship between oncogenic transcription factors and CLU.

III. REGULATION OF CLU EXPRESSION BY PROTOONCOGENIC TRANSCRIPTION FACTORS

Transcription factors are the essential molecular tools, with which the cell is able to respond to changing environmental conditions, stress, differentiating stimuli, or proliferative cues. Transcription factors can be tissue-specific or ubiquitously expressed, and oncogenic versions can be found in both categories. In the following sections, we will discuss in detail which oncogenic transcription factors have been found to regulate CLU and the biological consequences of its deregulation.

A. MYC

MYC is a small family of transcription factors composed of the prototype member, c-MYC, the neuronal-specific MYCN and the less-studied L-MYC. C-MYC is a major player in human tumorigenesis and its function in human cancer has been discussed in detail in many reviews (see references above and also (Lutz *et al.*, 2002; Vita and Henriksson, 2006; Yaylim-Eraltan *et al.*, 2008). 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 reported that ectopic levels of c-MYC could strongly repress the expression of CLU in murine colonocytes or human keratinocytes. One of the most interesting observations in this paper is that forced overexpression of CLU could inhibit, at least in part, c-MYC-dependent tumorigenesis. Indeed, CLU could attenuate proliferation of colonocytes transformed by c-MYC, and mice with a disrupted CLU gene were more prone to develop papillomas as a consequence of exposure to carcinogens (Thomas-Tikhonenko *et al.*, 2004). The concept that CLU could behave as an inhibitor of cell proliferation was not without precedent. Bettuzzi *et al.* (2002) showed that forced overexpression of CLU induced cell-cycle arrest of human prostate cells *in vitro*.

Neuronal MYC (MYCN) is also a negative regulator of CLU. It has been recently shown (Chayka *et al.*, 2009) that CLU is downregulated in the pediatric cancer neuroblastoma. Neuroblastoma is characterized by the amplification of MYCN, which is necessary and sufficient to induce transformation of embryonal sympathetic cells into malignant neuroblasts. In tumors with amplified MYCN, CLU is strongly downregulated and MYCN appears to induce CLU downregulation at least in part through transcriptional induction of the six-microRNA cluster miR-17-92 (composed of miR-17, -18, -19a/b, -20, and -92) (Dews *et al.*, 2006; O'Donnell *et al.*, 2005). These and other microRNAs are short noncoding RNAs that can specifically decrease protein output by decreasing translation and/or by mRNA destabilization (Mendell, 2008). In Chayka *et al.* it was demonstrated that the MYCN-induced miR-17-92 cluster downregulates CLU expression in neuroblastoma cells. The MYCN–CLU axis is functionally important, since mice with a disrupted CLU gene are more prone to the formation of neuroblastomas induced by transgenic expression of MYCN, thus suggesting that CLU is a repressor of MYCN tumorigenesis (Chayka *et al.*, 2009). A still unpublished study is yielding evidence suggesting that MYCN can also directly repress transcription of CLU through an E-box in the CLU 5′-flanking region which is conserved in different mammalian species.

More careful examination of the connection between miR-17-92 cluster members and CLU has revealed several surprises. While the Miranda algorithm (John *et al.*, 2004) predicts binding sites for several members of the miR-17-92 cluster within the 3′-UTR of human CLU, these predictions could not be confirmed experimentally using the luciferase sensor assay or gain-of-function microRNA mimic screens (Dews *et al.*, submitted for publication). This suggests that CLU may not be a direct molecular target for miR-17-92 and that instead this cluster targets an upstream activator of CLU expression.

As mentioned above, in some cell lines CLU can be induced by the TGF-β signaling pathway (Jin and Howe, 1997, 1999). This idea had been also promulgated by David Boothman and his colleagues (Bey *et al.*, 2006). Thus, it was tempting to propose that perhaps downregulation of CLU by miR-17-92 is in fact lack of activation by TGF-β. Indeed, very recent work from the Thomas-Tikhonenko laboratory demonstrated that Myc-overexpressing cells contain defects in several key components of the TGF-β signaling pathway, including TGF-β receptor II and activating Smads (Bierie and Moses, 2006; Massague, 2008). As predicted previously (Volinia *et al.*, 2006), miR-17-5p and miR-20 reduce levels of the type II TGF-β receptor (TGFBR2) and miR-18 was found to target Smad4 and in some cell lines—Smad2. Overall, weakened TGF-β signaling in Myc and/or miR-17-92 overexpressing cells resulted in very poor induction of CLU by TGF-β.

B. MYB

MYB, similarly to MYC, is a family of transcription factors which includes the tissue-specific c-MYB and A-MYB and the ubiquitous B-MYB, a positive regulator of cell proliferation and survival (Lipsick *et al.*, 2001; Oh and Reddy, 1999; Sala and Watson, 1999). Interestingly, B-MYB is overexpressed or amplified in various types of human cancer suggesting that it too is a protooncogene (Nakajima *et al.*, 2008; Raschella *et al.*, 1999; Sala and Watson, 1999). In the Sala laboratory, it has been shown that B-MYB binds to and positively regulates the CLU promoter through a MYB-consensus sequence. It has also been shown that CLU mediates, at least in part, the antiapoptotic effects of B-MYB. B-MYB-induced CLU can confer resistance to doxorubicin killing of human LAN5 neuroblastoma cells. Furthermore, thermal injury is more pronounced in fibroblasts transfected with a construct expressing dominant-negative B-MYB, which also blunts thermal induction of CLU (Cervellera *et al.*, 2000; Santilli *et al.*, 2005). These results are in agreement with evidence correlating decreased expression of secreted CLU and B-MYB with apoptosis induced by all-*trans*-retinoic acid in smooth muscle cells (Orlandi *et al.*, 2005).

C. NF-κB

NF-κB is a multifunctional transcription factor that has central importance in immunity and cancer. NF-κB is activated in response to external stimuli—such as engagement of the TNF-α receptor by its ligand, and by the IKK kinases alpha, beta and gamma (the latter known as NEMO) which phosphorylate the inhibitors of κB (IκBs), liberating the transcriptionally active NF-κB molecule (Gilmore, 2006; Perkins, 2007). The first evidence that NF-κB regulates CLU expression was provided by Kenneth Marcu and coworkers. In their study, the authors carried out a systematic analysis to isolate all NF-κB target genes in mouse embryo fibroblasts. They used a molecular inhibitor of NF-κB in the presence or absence of TNF-α, a classical NF-κB inducer. Among the plethora of genes activated by NF-κB, CLU was one of the most highly regulated (Li *et al.*, 2002). Interestingly, knockout of either one of the three IKKs resulted in lack of activation of CLU, suggesting that its activation is dependent on the whole NF-κB signalsome.

These results were later confirmed by another group that showed that CLU can be induced in glial and astrocyte cells by the bacterial lipopolysaccharide LPS (Saura *et al.*, 2003). LPS is a known activator of NF-κB, and

the use of aspirin or MG132 as indirect means to inhibit NF-κB resulted in the inhibition of CLU expression. Intriguingly, it was later shown that CLU regulates NF-κB activity in a negative manner by stabilizing IκBs (Devauchelle *et al.*, 2006; Santilli *et al.*, 2003; Savkovic *et al.*, 2007; Takase *et al.*, 2008a,b). This leads to the hypothesis that CLU participates in a negative loop in which transcriptional activation of CLU is evoked to dampen NF-κB activity. This would be especially important when there is a need to control the secretion of potentially harmful cytokines regulated by NF-κB. This hypothesis is corroborated by the study in which it has been shown that abnormally low CLU levels cause excessive NF-κB activation and pathological cytokine secretion in rheumatoid arthritis (Devauchelle *et al.*, 2006).

D. Egr1

The group lead by David Boothman was the first to show that secreted CLU is induced by ionizing irradiation (Yang *et al.*, 2000). The same group later showed that irradiation leads to the activation of a signaling pathway that emanates from two growth factors receptors: EGFR and IGFR. It was demonstrated that IGFR, but not EGFR, mediates the induction of secreted CLU in response to irradiation (Criswell *et al.*, 2005). Notably, the Src/Map kinase cascade that is triggered downstream of IGFR ultimately signals to the transcription factor Egr1, which, in turn, binds to the CLU promoter and induces upregulation of CLU mRNA. In this context, secreted CLU is induced as a protective response to damaging stress since knockdown of CLU by RNAi accelerates cell death.

E. Stat1

Stats are a group of transcription factors implicated in transducing survival or apoptotic signaling downstream of a class of receptor-associated molecules called JAKs. In an Affymetrix screen to search for genes involved in conferring resistance to the chemotherapeutic drug docetaxel, Djeu and coworkers identified CLU and Stat-1 as docetaxel-inducible genes that inhibit drug-induced apoptosis. Interestingly, Stat-1 seems to lie upstream of CLU since its depletion by siRNA induces a 50% reduction of CLU expression in prostate cancer cells (Patterson *et al.*, 2006). It is not clear whether Stat-1 can directly regulate CLU gene expression, but the presence of putative Stat-binding sites in the CLU promoter suggests that this could be the case.

F. GLI and TCF

Recent studies have placed CLU downstream of the two protooncogenic transcription factors activated by the signaling molecules Hedgehog and Wnt, GLI-2 and TCF-1, respectively. Hedgehog and Wnt play important roles in normal development and cancer (Jiang and Hui, 2008; Nusse, 1992; Polakis, 2000). Signaling emanating from these developmental factors is relayed to nuclear transcription factors belonging to the GLI and TCF families. Abnormal activation of GLI is often detected in medulloblastomas with disruption of the Sonic Hedgehog antagonist *Patched*, which results in constitutive activation of Hedgehog signaling (Villavicencio *et al.*, 2000). TCF family members are usually activated in epithelial tumors in which Wnt signaling is increased by stabilization of β-catenin, an essential partner in transcriptional regulation (Ilyas, 2005; Rask *et al.*, 2003).

The group led by Marin Gleave has found that knockdown of GLI-2 in prostate cancer cells results in suppression of proliferation and increased apoptosis and concurrent inactivation or activation of several genes. Interestingly, CLU protein expression increased after treatment with GLI-2 antisense oligonucleotides, although CLU mRNA expression did not change (Narita *et al.*, 2008). In another study, it was found that TCF-1 mediates activation of a short mRNA isoform of CLU (Schepeler *et al.*, 2007). In both studies, no attempts were made to understand the functional role of CLU in these signaling pathways and whether CLU is a positive or negative modulator of Wnt and Hedgehog pathways remains to be determined.

IV. ONCOGENIC SIGNALING AND EPIGENETIC REGULATION OF CLU EXPRESSION

As mentioned in previous paragraphs, c-Myc, N-Myc, and Ras cause silencing of CLU expression, probably facilitating tumorigenesis. The mechanisms of repression by Myc family members appear to be complex and are still a matter of active investigation. Recent studies suggest that RAS-mediated silencing is epigenetic. Analysis of gene expression in rat fibroblasts transformed with activated Ha-RAS revealed that several genes, including CLU, are silenced. Interestingly, RAS first induces deacetylation of the CLU promoter followed by methylation of a CpG island located in proximity of the transcription start site via MEK/ERK signaling (Lund *et al.*, 2006). Curiously, as mentioned in the previous section, the Boothman group has shown that an IGFR-dependent MEK–ERK–EGR signaling pathway mediates activation of CLU by ionizing radiation (Criswell *et al.*, 2005). The apparent contradiction could be explained if one hypothesized that the

MEK–ERK pathway could feed into different downstream effectors in a stimulus-dependent manner (i.e., *irradiation*-induced MEK–ERK activates gene transcription via transcription factors whereas *ras*-induced MEK–ERK inactivates gene expression by inducing histone deacetylases).

Other research groups have observed epigenetic silencing of CLU in transformed cells and cancer. For example, Nuutinen *et al.* have shown that CLU is silenced by gene methylation and deaceylation in human neuroblastoma or neuronal cell lines (Nuutinen *et al.*, 2005). In murine and human prostate cancer cell lines CLU expression is silenced by gene methylation and/or histone deacetylation (Rauhala *et al.*, 2008). In line with these results, the Bettuzzi group had previously shown that CLU expression is downregulated during progression of human and murine prostate cancer and that CLU promotes slowdown of prostate cell proliferation (Bettuzzi *et al.*, 2000, 2002; Caporali *et al.*, 2004; Scaltriti *et al.*, 2004b).

Moreover, CLU is one of the genes most highly induced by histone deacetylase inhibitors and inhibitors of DNA methylation in tumor endothelial cells. Most notably, suppression of CLU expression by shRNA drives increased proliferation, migration, and sprouting of tumor endothelial cells (Hellebrekers *et al.*, 2007). Overall, these results invoke a scenario in which oncogenic stimuli provoke chromatin rearrangements that result in suppression of genes, like CLU, that are implicated in restraining tumor proliferation and angiogenesis. Indeed, most recent work from Thomas-Tikhonenko laboratory provides direct evidence that CLU overexpression severely limit neovascularization of murine and human colon carcinomas (Dews *et al.*, submitted for publication) potentially affecting tumor metabolism (see chapter "The shifting balance between CLU forms during tumor progression," of vol. 104).

V. CONCLUDING REMARKS

These are exciting times for researchers studying CLU and cancer. CLU is emerging as an important player in human cancer, although its role is more complex than anticipated. Regarded initially as a mere extracellular chaperone or a scavenging protein, CLU has been proven to be an important mediator of cell signaling as well. Its documented ability to interfere with NF-κB, PI3 kinase, or MAP kinase signaling could perhaps explain its role as a tumor modifier. Cancer cells often hijack cellular signaling to their advantage and become "addicted" to a specific molecular pathway. By interfering in a negative or a positive manner with such pathways, CLU could either promote or restrict neoplastic disease. In the light of recent evidence gathered using mouse models of human cancer where CLU has been genetically

ablated, we hypothesize that CLU is mainly required to restrict the early stages of mammalian tumorigenesis and metastatic spread while assisting established tumors in becoming chemo- and radioresistant.

While the mechanism by which CLU acts as a tumor suppressor gene is not entirely clear, there is some evidence to suggest that suppression of the NF-κB signaling could be involved. It is tempting to speculate that very aggressive clones of cancer cells arising after chemotherapeutic drug treatments or natural selection could reactivate the expression of CLU. This hypothesis was recently corroborated in experimental models in which initial upregulation of CLU was found to induce clonogenic toxicity, thus killing the majority of prostate cancer cells, while the rare surviving clones were expressing CLU solely in the cytoplasm. This could lead to the development of antiapoptotic properties and the ability to survive the mitotic catastrophe, if only at the cost of acquiring an altered phenotype with impaired mitosis, endoreduplication, and genetic instability (Scaltriti *et al.*, 2004a,c). Therefore, high expression of secreted or cytoplasmic CLU could be advantageous because it confers increased resistance to killing by anticancer drugs or enhances tumor cell survival in specific niches. The opposite roles played by CLU in early versus late stages of tumorigenesis could also explain why epigenetic inactivation of CLU, but not gene rearrangements or mutations, is commonly detected in mammalian cancers.

ACKNOWLEDGMENTS

Research in the authors' laboratories has been supported by: Sporting Aiding Medical Research for Kids (SPARKS) and Neuroblastoma Society (A.S.); FIL 2008 and FIL 2009, University of Parma, Italy, AICR (UK) Grant No. 06-711, and Istituto Nazionale Biostrutture e Biosistemi (INBB), Roma, Italy (S.B.); US National Institutes of Health grant CA 122334 (ATT).

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