Review

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Clusterin in Cancer: Dual role as a Tumor Suppressor Gene and an Oncogene

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Clusterin (CLU), a heterodimeric and sulfated glycoprotein has been associated with various physiological functions. This molecular chaperone protein is ubiquitously expressed in diverse tissues and conserved across species. Differences in subcellular localization and possible existence of different CLU isoforms may contribute to its functional diversity. Increased or decreased expression of CLU has been observed in several cancers versus normal tissues and hence its role in tumorigenesis is controversial. Evidences from several studies imply that CLU may have a dual role as a tumor suppressor gene or an oncogene depending on the signal and cellular context. CLU possibly exerts its oncogenic role by inhibiting apoptosis, activating autophagy and modulating several signaling pathways like IGF-1/IGFR, EGFR, NF-kB, PI3K/AKT, TGFβ and select miRNAs. CLU may exert its tumor suppressive effects by regulating cell cycle and inducing apoptosis. In cancer, loss of heterozygosity (LOH), copy number loss at CLU locus, epigenetic modifications and expression of select miRNAs may lead to the downregulation of CLU. Custirsen (OGX-011), a second generation antisense oligonucleotide that inhibits CLU expression and increases sensitivity of cancer cells to chemotherapeutic drugs, is currently in phase III clinical trials. CLU is an attractive target in several cancers, however for effective targeting, it is essential to know whether it acts as an oncogene or a tumor suppressor gene in a specific tissue/cellular context. The current review attempts to discuss the two contrasting roles of CLU in cancer and associated regulatory mechanisms. This review also sheds light on the complex CLU splice variants, the varied functional attributes supporting the dual roles in cancer and limitations of the CLU research that warrant attention.

INTRODUCTION

Clusterin (CLU), a ubiquitously present sulfated chaperone glycoprotein was first isolated from ram rete testis fluid where it was shown to elicit clustering of Sertoli cells and also of erythrocytes *in vitro* from several species leading to its nomenclature 'Clusterin' (Fritz *et al.,* 1983). Despite 33 years of immense efforts by researchers to understand the diverse functions of this multifaceted

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protein CLU, it still remains an enigma. Since its discovery, several CLU homologues with different names and diverse physiological functions have been isolated from different species and tissues for example testosterone repressed prostate message protein 2 (TRPM2), sulfated glycoprotein 2 (SGP2), apolipoprotein J (ApoJ) and several others (Bettuzzi *et al*., 1989; de Silva *et al*., 1990; Léger *et al*., 1987). However "Clusterin (CLU)" is the acceptable name for all the above identified proteins.

In humans, the CLU gene (Fig. 1) encodes a mRNA of approximately 2 kb which directs the synthesis of a 449-amino acid primary polypeptide chain. CLU has been reported to be present in the body fluids of all vertebrates and is also one of the most abundant proteins (100-300ug/ml) found in human serum. Numerous biological functions have been associated with CLU including lipid transportation, membrane recycling, tissue differentiation and remodeling, cell–cell or cell-substratum interaction, cell proliferation, and cell death (Rosenberg *et al.,* 1995; Shannan *et al.,* 2006; Trougakos *et al.,* 2002; Wilson *et al.,* 2000). Altered expression of this important molecular chaperone CLU has been associated with aging, atherosclerosis, different neurological disorders including Alzheimers disease, cardiovascular and metabolic disorders and cancers of different origins. Diverse tissue specific distribution of CLU suggests that its expression is tightly regulated by different signaling pathways in normal and diseased conditions (Trougakos *et al.,* 2013).

In the light of new discoveries and information in the Clusterin field and the ongoing studies on the role of Clusterin in oral cancers in our laboratory, this review attempts to simplify and describe the CLU variants and the dual cell/tissue specific context dependent role of CLU as an oncogene or tumor suppressor gene in cancer and the constant challenges posed by this fascinating protein in understanding its complex role in cancer.

CLU Spliced Variants

The complexity and the low clarity on the existence of different CLU isoforms and its functions have challenged researchers for the past several years. Briefly, there are two major variants of CLU namely the predominant secretory form (sCLU) and intracellular forms which include the nuclear CLU (nCLU) and other non-secreted variants. These

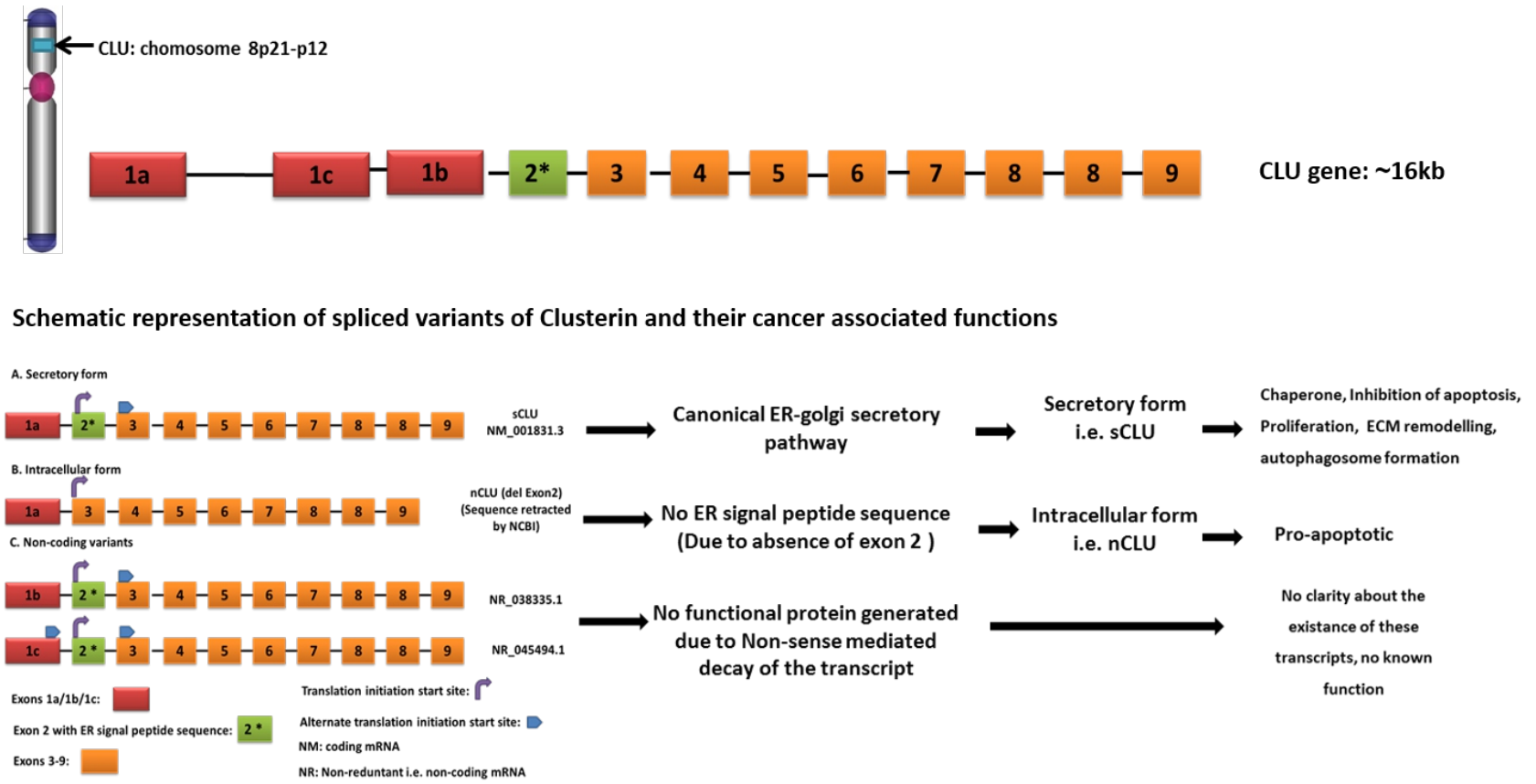

Figure 1: Schematic representation of spliced variants of Clusterin and their cancer related functions

CLU has following variants generated by alternate splicing event and differential use of exon 1:

A. Secretory form: Full-length variant generated by use of exon 1a

B. Nuclear form: N-terminally truncated variant generated by splicing of exon 1a to exon 3

C. Non-coding forms: These isoforms are predicted to use exon 1b and 1c, which do not code for functional protein due to nonsense mediated decay of these generated transcripts.

two isoforms have antagonistic functions i.e sCLU has prosurvival or antiapoptotic functions whereas nCLU has pro-death or pro apoptotic functions (Fig. 1) and are described below.

Secretory (extracellular) form i.e. sCLU (NM_001831.3)

This is the most predominant and commonly expressed anti-apoptotic isoform, synthesized as a full length secretory CLU via use of exon 1a and translation start site present upstream to signal peptide sequence on exon 2 (Prochnow *et al.,* 2013; Rizzi *et al.,* 2010). This signal peptide sequence of 22 amino acids encoded by exon 2 of CLU gene, directs the CLU protein to the ER where it undergoes N-linked glycosylation. Then this high mannose ER-precursor of 60kDa called pre secretory CLU (psCLU) enters the Golgi apparatus for further post translational modifications which include the addition of complex sugar moieties. The mature 80kDa CLU protein is further cleaved by a furin-like proprotein convertase which recognises the amino acid recognition motif RIVR to produce two polypeptide chains namely a N-terminal α-chain and C-terminal β-chain which are interlinked by five disulphide bonds thus yielding a heterodimeric mature secretory form (comprising of two sub units of 40 to 45kda each) (Jones *et al.,* 2002). Several groups have extensively studied the chaperone activity of sCLU.

The sCLU, a stress induced, ATP-independent extracellular chaperone protein is upregulated in several carcinomas like hepatocellular, lung, breast, bladder and in lymphoma, melanoma and downregulated in neuroblastoma, testicular seminoma and esophageal carcinomas (Chayka *et al.,* 2009; Koltai*,* 2014; Zhang *et al.,* 2003). It is not clear whether sCLU overexpression is a "cause" or "consequence" in the progression of a disease. Besides inducing proliferative and pro survival pathways as a signaling molecule, the cytoprotective role of sCLU is thought to be an outcome of the synergism of the chaperonic, scavenging and clearance activity of misfolded proteins and cellular debris. Different functional attributes of sCLU contributing to its pro-survival role in tumorigenesis are discussed further in detail, in this review.

Intracellular forms

In addition to the extracellular secretory form, several intracellular CLU forms

have been observed post stress and in damaged cells as described below.

nCLU (variant 1 del exon 2)

This putative nuclear pro-death form was initially demonstrated in MCF-7 breast cancer cell line and later on its occurrence was also demonstrated in prostate and colorectal carcinomas (Andersen *et al.,* 2007; Leskov *et al*., 2003; Rizzi *et al.,* 2010). This nCLU obtained by alternative splicing, generates N-terminally truncated isoform wherein exon 1 is spliced to exon 3 and thus lacks exon 2 bearing the ER signal peptide sequence, due to which the translation will initiate at the start site present on exon 3. Although the presence of three putative nuclear localization sequences (NLS) has been shown in nCLU, their presence was not found to be essential for its nuclear translocation (O'Sullivan *et al*., 2003). Interestingly, recent studies from our lab in oral cancer cell lines have demonstrated the localization of Clusterin in the nucleolus (unpublished data), which is a novel observation. Hence, whether nCLU is a different splice variant or is the sCLU which gets translocated to nucleus/nucleolus is not clear and warrants investigation. The nCLU has been shown to interact with Ku-70 of Ku-70/Ku-80 complex, thus impairing DNA repair and inducing apoptosis (Leskov *et al*., 2003). However, the sequence of nCLU is currently not available in NCBI database questioning the existence and the mechanism of nCLU transcript generation.

Stress induced intracellular non secreted CLU isoforms

Prochnow *et al*. (2013) demonstrated the generation of different CLU forms post stress and discussed the possible mechanisms for their generation: First they proposed that the post-translationally modified pre-mature CLU residing in endoplasmic reticulum is possibly re-translocated back to the cytoplasm. Secondly the authors proposed that the CLU transcript might use an alternative translation initiation site either present in exon 2, downstream to signal peptide sequence generating a truncated form of CLU or in exon 1, leading to a N-terminally elongated variant with a defect in the ER signal peptide sequence functionality, resulting in CLU accumulation in different intracellular organelles. Further these "non-secreted Clusterin isoforms" which are translated in negligible amounts

(about 0.34% of total CLU present in a cell) under stress conditions, possibly do not affect caspase 3/7 mediated apoptosis or NF-κB activity, thereby questioning their physiological relevance (Prochnow *et al.,* 2013). The only exception would be the hypoglycosylated form of CLU which interacts with GRP78, an ER stress associated protein which stabilizes the mitochondrial membrane, suggesting a possible role for CLU in unfolded protein response (UPR) and inhibition of apoptosis (Li *et al.,* 2013).

Non-coding/Non-redundant CLU isoforms

As shown in Fig. 1, these isoforms have been cited as Variant 2 (NR_038335.1) and variant 3 (NR_045494.1) in the NCBI database. These two variants are predicted to use exon 1b and 1c respectively and have been termed as "non-redundant or non-coding" isoforms as they do not code for a functional protein due to presence of an upstream ORF predicted to interfere with translation of the longest ORF due to which such a transcript generally undergoes nonsense mediated mRNA decay (NCBI database). Although variant 2 (NR_038335.1) is classified under non-coding isoforms, its presence was shown in the brain cells of Alzheimer's patients, suggesting a possible context dependent role for it which is yet to be explored (Ling *et al.,* 2012).

Thus, despite extensive efforts in the field of CLU research for the last several years, there is little clarity on the mechanism and regulation of different CLU transcript generation. As suggested by Essabbani *et al.* (2013), there might exist an "on demand alternative splicing" phenomenon generating the different isoforms in a context dependent manner.

Till date majority of the CLU research is focused on the prominent extracellular sCLU form and its chaperonic activities. One of the contributing factors for the low clarity on the existing CLU isoforms is the range of bands from 20-80kda obtained on a western blot following the use of different commercially available CLU antibodies. These bands are often found marked together as CLU in the antibody providing company data sheets. The development of CLU isoform specific antibodies may help to resolve the issue. However with the advent of new mass spectrometry based technologies it would now be possible to identify the different

forms of CLU seen on a gel and their post-translational modifications like glycosylation.

Structure of Clusterin

Despite the ubiquitous occurrence of extra and intracellular CLU forms and the ever increasing list of CLU interacting proteins, till date no crystallographic data is available for CLU. Several studies indicate that it has been very difficult to crystallize CLU protein due to its heavy glycosylation (almost 30% of the protein glycosylated) which is responsible for the "sticky" nature of this protein (Jones *et al.,* 2002). Also CLU exhibits a tendency to aggregate and form di, tetra and higher oligomers based on the pH, further adding to the difficulty in its crystallization. Hence majority of the available information on the secondary structure of CLU has been predicted through computational analysis, without any experimental support. sCLU exhibits a highly conserved primary structure across different species with highest homology displayed in the disulphide bonds and FC cleavage site (Bailey *et al.,* 2001).

Attempts have been made to characterize sCLU-client protein complexes using different techniques like size exclusion chromatography, dynamic light scattering, bis-ANS fluorescence spectroscopy, circular dichroism etc. These studies have shown the presence of 60% α-helices and also that CLU is likely to shield exposed hydrophobic regions of the client protein, resulting in the maintenance of secondary structure and stability of the same (Wyatt *et al.,* 2009). Further CLU structure has been predicted to be constituted of random coils and molten globule like regions as observed in proteins with ill-defined tertiary structure or in intrinsically disordered proteins like the heat shock protein family, essential for its chaperone functions. The amphipathic α-helical structure and intrinsically disordered molten globule structure attributes to its role as a "biological detergent", or scavenging/clearing agent which takes care of unfolded or undesired circulating macromolecules (Bailey *et al.,* 2001).

The sequence analysis of nCLU identified a conserved BH3 motif in its C-terminal coiled coil region (CC2) which interacts with Bcl2 family members as demonstrated by NMR analysis (Lee *et al.,* 2011). This is the only report till date which attempted to elucidate the interaction between nCLU

and Bcl2 family members using structural modeling and confirmed the proapoptotic function of nCLU by demonstrating its interaction with anti-apoptotic family members. Interestingly, the region of BH3 motif in CC2 region is common to both sCLU and nCLU, but it is the nCLU that interacts with Bcl2 family members and not the sCLU. Hence, it will be worth studying the interaction between sCLU and other BH3 motif containing family of proteins *in silico* which will help in understanding the basic CLU structure.

Functional aspects of Clusterin

Chaperonic functions of sCLU

sCLU was discovered as a molecular chaperone with extracellular activities like heat shock proteins and its expression is induced post stress via the CLE in its promoter. Through its chaperonic activity sCLU has been shown to play an important role in protein homeostasis in the cell to overcome stress conditions. sCLU prevents the aggregation of denatured proteins by binding to it in an ATP independent manner and forming high molecular weight soluble complexes (Rohne *et al.*, 2014). *In vitro* studies have demonstrated that sCLU facilitates uptake of these complexes in neighboring tissue cells for removal by lysosomes. sCLU interacts with scavenger receptors and contributes to removal of toxins in liver and kidneys. Interestingly studies demonstrate that the disulphide bonds of CLU are important for its maturation and correct folding but not for its chaperonic function. Similarly its glycosylation was demonstrated to be important for its correct polar secretion in cells but not for its chaperonic activity (Rohne *et al*, 2016).

Role for CLU in Phagocytosis

Interestingly another novel function of CLU as an opsonin in a process of efferocytosis i.e. phagocytosis of dying cell has been shown, suggesting a protective role for CLU in modulating immune response. CLU has been shown to bind on the blebs on late apoptotic cells and to histones accumulated in the cytoplasm of dying cells, which marks the cell for phagocytosis (Cunin *et al*., 2016). Another novel role of CLU in the clearance of excess of misfolded proteins has been reported in idiopathic pulmonary fibrosis (IPF), a lung disorder where excess of extracellular matrix gets accumulated. In this IPF condition, CLU has been shown to be downregulated,

which acts as a quality control regulator by binding to such misfolded proteins and promoting the phagocytosis process. In CLU-/- mice, impaired collagen/ECM clearance by macrophage driven phagocytosis has been demonstrated (Bernard *et al.,* 2015).

Role for CLU in Senescence

Recently the role of CLU in senescence was demonstrated. CLU has been shown to be transcriptionally up-regulated during both replicative senescence (RS) and stress induced premature senescence (SIPS). This upregulation of CLU occurs through the ATM/IGF-1/IGF-1R/MAPK/ERK-1/2/EGR-1 signaling pathway, which also overlaps with DNA damage response (DDR) pathway. Earlier it was deciphered that as sCLU is an anti-apoptotic protein, it may cause population doubling thereby preventing cell death. However knockdown of sCLU in middle aged and senescent cells did not exhibit apoptosis, suggesting that the anti-apoptotic function of sCLU may not be operative during senescence (Luo *et al.,* 2014).

CLU knockout studies

CLU knockout studies revealed that CLU knockout mice were fertile and had no obvious phenotype (Rosenberg *et al.,* 1995). Also mice development was not affected by the absence of CLU. However, these mice showed increased sensitivity to autoimmune myocarditis, suggesting a role for CLU in protecting the heart tissue from post inflammatory destruction. CLU-/- mice exhibited severe inflammation and changes in cellular pathology in experimentally induced murine autoimmune myocarditis as compared to CLU-expressing control mice (McLaughlin *et al.,* 2000). In contrast in another study, in the absence of CLU, mice were found to be partially protected after hypoxic injury, suggesting that CLU appears to have a negative role in neuronal survival (Han *et al.,* 2001).

$CLU^{-/-}$ mice showed impaired morphogenic and functional features of regenerating pancreas. These mice exhibited loss of regenerating capacity of the beta cells resulting in a hyperglycemic condition, implying a role for Clusterin in promoting regeneration following pancreas injury and in *in vitro* beta-cell regeneration (Lee *et al.,* 2011). Studies demonstrated that damage to testicular cells is increased after heat shock in $CLU^{-/-}$ mice and additionally the clearance of damaged cells is also impaired (Bailey *et al.,* 2002). Further, in

ageing $CLU^{-/-}$ mice, progressive glomerulopathy characterized by accumulation of insoluble protein deposits in kidneys was observed indicating that CLU may inhibit age-dependent accumulation of protein deposits in the glomeruli (Rosenberg *et al.,* 2002).

Role of CLU in tumorigenesis

Over the past 15 years a significant amount of data has been generated on CLU expression in different tumor tissues, however the discrepancy of its role in cancer still prevails. Overexpression of CLU in some cancers indicates its role as an oncogene, while its repression or downregulation in other cancers conversely indicates that it may have a tumor suppressive function. This review is an attempt to conciliate and address the available information on Clusterin's apparently contradictory and possibly context dependent and tissue specific role in cancer.

Evidence for Clusterin as a tumor suppressor gene

The first *in vivo* evidence for the possible role of CLU as a tumor suppressor came from the work by Thomas-Tikhonenko *et al*., 2004 which demonstrates that CLU-null mice are prone to development of skin cancers. Further studies by Davoli *et al.* (2009) demonstrated that siRNA mediated knockdown of sCLU leads to cell cycle progression with increase in proliferation markers. Additional support for the tumor suppressor function of CLU was provided by the TRansgenic Adenocarcinoma of Mouse Prostate (TRAMP) mice which exhibited aggressive tumor development when crossed to CLU-/- mice due to inactivation of one or both *CLU* alleles in TRAMP mice. Interestingly the TRAMP/CluKo mice exhibited enhanced tumor spreading and homing, early metastases in ectopic sites and decreased survival. Further 30% of these mice died by 28 weeks versus none of the TRAMP only group. These studies thus suggest CLU to be a negative modulator of prostate cancer and a putative haploinsufficient tumor suppressor gene.

Studies by Chayka *et al.* (2009) demonstrated that CLU acts as a negative modulator of growth in neuroblastoma. The authors showed that MYCN amplification via the activation of miR17-92 cluster brings about sCLU suppression. Intriguingly the penetrance of neuroblastomas arising in MYCN-transgenic mice was significantly

increased after deletion of the CLU gene, suggesting it to be a tumor suppressor protein. Further confirmation for this came from the studies showing that sCLU siRNA-transduced neuroblastoma cells exhibited increased metastases when xenografted in mice with concomitant activation of NF-ƙB signaling and epithelial to mesenchymal transition (EMT).

Andersen *et al.* (2007) reported the downregulation of CLU isoforms in colorectal carcinoma (CRC). Using genome-wide analysis they showed LOH and concomitant copy number loss at the CLU locus 8p21 in 67% CRC cases. Further analysis revealed that TCF1-mediated Wnt-signaling along with loss of copy number at CLU locus is responsible for the observed CLU downregulation (Schepeler *et al.,* 2007). CLU expression was also reported to be significantly lower in testicular seminoma as compared to normal testis. Testicular seminomas are one of the most sensitive tumors being responsive to radiotherapy and chemotherapy. This further supports the role of sCLU as a cytoprotective protein, protecting cells from death due to anti-tumor therapy (Liu *et al.,* 2013). Studies carried out by Chen *et al*. (2014) to identify host immune response protein candidates in the sera of oral squamous cell carcinoma patients, revealed that CLU is one of the downregulated genes. Preliminary data from our lab have demonstrated downregulation of sCLU in oral tumor tissues as compared to normal oral mucosa. Studies are ongoing to elucidate the mechanism of CLU downregulation and its role in oral cancers.

Clusterin-positive patients with pancreatic cancer exhibited significantly longer survival as compared to Clusterin-negative patients indicating that down-regulation of CLU may be involved in the progression of pancreatic cancer (Xie *et al*., 2002). However this observation is not consistent with current reports where Clusterin has been shown to confer chemoresistance in pancreatic cancers suggesting a role as an oncogene (Kong *et al.*, 2012; Tang *et al.,* 2012). Such contradictory reports add to the complexity of the subject and the dilemma whether CLU is a tumor suppressor or an oncogene.

The following functions/regulation of sCLU might attribute to its tumor suppressive functions/role.

Epigenetic regulation of CLU expression

Several evidences suggest that regulation of CLU expression at genomic level is effected through either epigenetic mechanism or large- scale deletion of the gene. Rat fibroblasts transformed with Ha-Ras exhibited downregulation of Clusterin mediated by deacetylation of CLU promoter followed by methylation via the MEK/ERK signaling pathway (Lund *et al.,* 2006). Earlier reports have demonstrated that CpG island methylation or histone deacetylation in the proximity of the *CLU* gene leads to the downregulation of Clusterin in neuronal cells, tumor endothelial cells and prostate cancer (Hellebrekers *et al*., 2007; Nuutinen *et al*., 2005; Rauhala *et al*., 2008). Another report in hepatocellular carcinoma demonstrated regulation of CLU through acetylation/ deacetylation of histone H3 within the CLU promoter (Liao *et al.,* 2009). In 2014, Park *et al.* (2014) studied the transcriptional regulation of nCLU in response to hypoxia, where binding of HIF1-α to the three putative hypoxia responsive elements (HREs) was shown, to induce nCLU expression followed by apoptosis in prostate cancer cell line PC3, but not in LNCaP cells. Further analysis revealed that *CLU* promoter was not methylated in PC3 cells; but was methylated in LNCaP cells suggesting that nCLU expression is regulated by direct binding of HIF-1α to HRE sites and is epigenetically controlled by methylation of its promoter region. Similar studies in breast carcinoma demonstrated absence of CLU expression in normal breast tissue due to methylation of CLU promoter, while in breast carcinoma tissues CLU promoter was found to be demethylated resulting in its overexpression (Serrano *et al.,* 2009). Recently, Amente *et al*. (2015) demonstrated that MYCN mediated downregulation of CLU was a result of the interaction of MYCN with lysine specific demethylase-1 (LSD1), which has been shown to be essential for repression of CLU gene expression.

Regulation of CLU by microRNAs

miRNAs are small (~ 22 nucleotides), non-coding single stranded RNA molecules involved in post-transcriptional gene regulation, by binding to the 3'-UTR region of targeted mRNA. These miRNAs act generally in a context dependent manner either as an oncogene or tumor suppressive miRNA (Erhard *et al*., 2014).

In neuroblastoma, Chayka *et al.* (2009) demonstrated that, CLU is negatively regulated by the protooncogene MYCN through the activation of the miR 17-92 cluster. This was further supported by a report which showed that the expression of two microRNAs in that cluster, miR-17-5p and miR-92, is upregulated by MYCN expression in SH-EP neuroblastoma cells. Further analysis using miRanda, a web based algorithm revealed that CLU mRNA was a target for miR-17, miR-18a and miR-19a which is known to be induced by c-MYC in a human B-cell line. However further validation using luciferase assay and miR mimics could not demonstrate direct binding of these miRs to the 3'UTR region of CLU, suggesting that it might possibly target some upstream CLU activator, thereby downregulating CLU expression (Sala *et al*., 2009).

Different miRNA microarray studies have revealed the overexpression of miR-21 in head and neck squamous cell carcinoma (HNSCC) (Shiiba *et al*., 2010) and further studies have indicated CLU to be potential target of miR-21. CLU was found to be downregulated following the expression of miRNA-21 in normal and HNSCC cell lines and tissues, thereby modulating cell growth properties (Mydlarz *et al.,* 2014). These reports suggest that miRNAs may have a key role in regulating CLU levels, defining the tumor suppressive function of CLU in a context dependent manner.

Modulation of NF-kB pathway by CLU

In 2003, Santilli *et al.* (2003) demonstrated that transfection of CLU in both normal and tumourigenic cells (LAN5 neuroblastoma cell line) caused stabilisation of NF-kB inhibitors, resulting in inhibition of NF-κB activity. Further, Devauchelle *et al*. (2006) demonstrated that CLU interacted with phosphorylated IkBα to prevent E3 ubiquitin ligase binding leading to IkBα stabilization, thereby preventing NF-kB translocation to the nucleus, thus implying CLU to be a negative modulator of NF- kB activity.

Evidence for Clusterin as an oncogene

Tumor cell survival and progression has been shown to be associated with increased levels of intracellular and secretory forms of CLU. The ability of CLU to function as an oncogene is mainly attributed by its ability to promote cell growth and inhibit apoptosis. Within the cell, sCLU blocks

apoptosis by binding to ku70-Bax complex, as a cytosolic retention factor and preventing its translocation to the mitochondria (Trougakos *et al.,* 2009). This interaction obstructs Bax oligomerization, which does not allow the release of cytochrome *c* from mitochondria and caspase activation. Further, sCLU was shown to inhibit the oncogenic c-Myc-induced apoptosis by interacting with conformation-altered Bax (Zhang *et al.,* 2005). Recently the role of CLU in prosurvival autophagy has been demonstrated where CLU was shown to interact with LC-3 via LIR-binding sequence within autophagosome membrane, which causes LC-3 lipidation and facilitates LC-3 and Atg-3 complex stabilization leading to autophagy initiation. In CLU-/- mice and prostate cancer cells with CLU knockdown, autophagy was shown to be attenuated, suggesting a role for CLU in pro-survival autophagy (Zhang *et al.,* 2014).

Sensibar *et al.* (1995) demonstrated the role of SGP-2/ sCLU in the prevention of cell death induced by TNF-α in LNCaP prostate cancer cell line. The high expression of CLU in renal cancer cells was significantly associated with pathological stage and grade of the tumor, and with poor overall and recurrence-free survival rate of patients (Miyake *et al*. 2002a). There are several indirect evidences in the literature which suggests that sCLU is an oncoprotein. Studies have shown that CLU silencing affected the chemosensitivity of human pancreatic cells to gemcitabine by either modulating NF-ƙB activity or inhibiting clusterin-dependent pERK1/2 activation (Kong *et al*., 2012; Tang *et al*., 2012). Further, over-expression of CLU in transitional cell carcinoma of the bladder was shown to prolong cell survival, resulting in enhanced metastatic potential *in vivo*, indicating its possible use as a marker for prognosis and tumor recurrence (Miyake *et al.,* 2002b).

Another evidence for the role of CLU in oncogenesis came from the studies by Chou *et al*. (2009) in lung adenocarcinoma, where its role in epithelial to mesenchymal transition was demonstrated and CLU was shown to be a positive indicator of the degree of invasiveness in lung adenocarcinoma cell lines. CLU silencing resulted in mesenchymal to epithelial transition (MET) as evidenced by the spindle-to-cuboidal morphological change, increased E-cadherin expression, and decreased fibronectin expression. The levels of slug protein, a zinc finger

containing transcription factor that represses E-cadherin, were reduced in the CLU silenced cell lines. Also the ERK levels correlated with that of slug and CLU. These studies indicate a role for Clusterin in EMT and ERK/Slug signaling. Overexpression of CLU and its role in invasiveness has been reported in laryngeal squamous cell carcinoma wherein siRNA knockdown of CLU was found to inhibit cell proliferation and induce apoptosis *in vitro* (Wang *et al.,* 2014). Studies demonstrate that B-MYB binds to and positively regulates the CLU promoter through a MYB-consensus element. In fibroblasts transfected with a dominant-negative B-MYB construct, which suppressed the thermal induction of CLU, thermal injury was prominently observed. B-MYB induced CLU has also been shown to confer doxorubicin resistance in human LAN5 neuroblastoma cells (Cervellera *et al.,* 2000; Santilli *et al.,* 2005).

Role of CLU in the recruitment of monocyte/macrophage infiltration at the tumor site and its role in invasion were studied by Shim *et al*. (2011). In monocytes and macrophages, CLU was shown to regulate MMP-9 expression via ERK1/2 and PI3K/AKT/NF-κB pathways, which contribute to the tissue reorganization by serving as a modulator for extracellular matrix degradation. Further CLU facilitated IκB degradation by SCF complex (E3 ubiquitin ligase complex) and nuclear translocation of NF-κB p65 (Zoubeidi *et al*., 2010) which is critical for MMP-9 expression. Thus CLU provides connecting link between two cellular processes i.e. inflammation and cancer by increasing NF-κB and MMP-9 levels. Recently, Li *et al.* (2016) have shown that CLU is induced by N, N'-dinitrosopiperazine (DNP), a known carcinogen responsible for the development of nasopharyngeal carcinoma (NPC). It was shown that post-DNP treatment, CLU, VEGF and MMP-9 levels increases and interestingly increase in VEGF and MMP-9 was via increased CLU expression. CLU was shown to interact with VEGF and MMP-9, which was responsible for invasiveness and metastasis.

These pro-survival functions of sCLU might attribute to its oncogenic function, role in other diseased conditions, and also to the increased resistance of cancer cells to different chemotherapeutic agents, like doxorubicin, cisplatin and taxol (Djeu *et al.,* 2009). This is evident from the observation that depletion of sCLU by

antisense or small interfering RNA caused hypersensitization of cancer cells to paclitaxel or IR (Criswell *et al.,* 2005; So *et al.,* 2005).

CLU induction via regulatory pathways

The complex mechanism of transcriptional regulation of CLU gene and the existence of more than one regulatory promoter region may be responsible for the varied expression pattern of CLU proteins. Studies by Wong *et al.* (1994) revealed that the proximal promoter region of CLU (P1) showed presence of different cis-regulatory elements including AP-1, AP-2, and SP-1 motifs. Additionally, a long domain of 14bp conserved among different species called as Clusterin element (CLE), was found to be related to heat-shock response element (HSE), which differed by just a single base. Further, another putative promoter region located in intron 1 of CLU (P2) was predicted to have a TATA box, cAMP responsive element (CRE) and CAAT box sequences. These predicted regulatory elements present in the promoter region of CLU may possibly have a role in the regulation of CLU in a context dependent manner, which needs to be validated experimentally.

The different regulatory pathways involved in CLU induction are described below and illustrated in Fig. 2.

NF- kB pathway

Zoubeidi *et al*., 2010 showed that, CLU facilitated degradation of inhibitors of NF- ƙB i.e. IkB and Copper metabolism gene MURR1 domain-containing protein (COMMD1) in response to different cellular stress by SCF E3 ubiquitin ligase complex, thereby enhancing NFκB activity in prostate cancer cell line (Fig. 2A). Thus, NF-κB induces further sCLU expression turning on a positive feedback loop.

TGF-β signaling

The TGF-β signaling pathway also plays a key role in sCLU induction via activation of transcription factors like AP-1 and EGR-1 which are well documented to activate sCLU transcription. TGF- β signaling has also been shown to induce de-repression of sCLU transcription mediated by c-FOS (Jin and Howe, 1999). sCLU has been shown to bind to both TGF- β type-I and II receptors by yeast two-hybrid screening and transmit signaling via the conventional pathway. TGF- β treatment

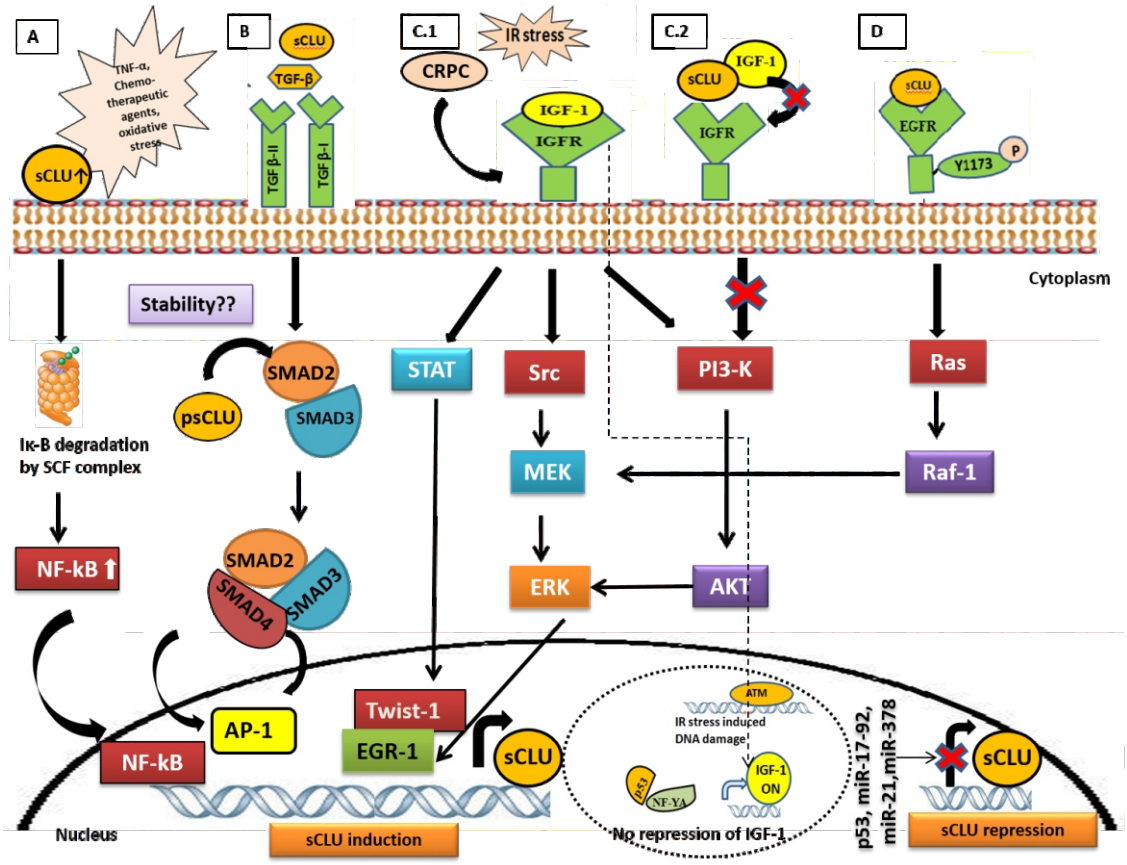

Figure 2: Schematic representation showing different regulatory pathways involved in sCLU induction
sCLU has been shown to bind to different receptors on the cell membrane, activating different cellular pathways. A) Under stress conditions like increase in TNF-α, chemotherapy etc. sCLU levels increases which causes degradation of NF-kB inhibitors, activating this pathway. B) sCLU can also bind to both TGF-beta receptors and can activate the pathway mediated by SMAD2/3 and SMAD4 complex. psCLU binds to SMAD2/3 intracellularly, maintaining their stability probably by preventing their proteasomal degradation. C.1) In different stress conditions like IR exposure, DNA damage induced ATM is activated which causes de-repression of IGF-1 mediated by p53-NF-YA complex. This activates pro-survival pathway i.e. IGF-1/IGF-1R which in turn activates MEK/ERK pathway leading to activation of EGR-1, a well-known transcription factor known to activate sCLU transcription. C.2) IGF-1 binding to IGF-1R can also activate PI3K/AKT pathway, which is blocked by binding of sCLU to IGF-1 extracellularly. D) sCLU binds to EGFR and activates Ras dependent Raf-1/MEK/ERK pathway.

causes translocation of CLU from the cytoplasm to nucleus in the HepG2 and CCL64 epithelial cell lines (Reddy *et al.,* 1996). psCLU has been shown to modulate the stability of SMAD2/3 by binding to it intracellularly. Thus the overexpression of CLU enhanced TGF- β induced transcriptional activity resulted in increased amounts of Smad2/3 proteins (Fig. 2B). This increased stability of Smad2/3 is not due to direct binding of CLU to Smad2/3; but because CLU possibly prevents the proteasome mediated degradation of Smad2/3 (Lee *et al.,* 2008). Recently a role for CLU as a mediator of the TGF-β induced epithelial

to mesenchymal transition (EMT) was demonstrated. Studies revealed that Twist-1 mediated TGF-β-induced CLU expression by binding to E-box elements in the distal promoter region of CLU gene (Shiota *et al.,* 2012).

IGF-1/IGF-1R signaling

It is well documented that CLU is induced post treatment with low non-toxic doses of IR (0.02-0.5 Gy), suggesting a role for CLU in radiation adaptive responses, characterized by increased radioresistance. Survival of damaged cells after IR leads to genomic instability (Klokov *et al.,* 2004). IGF-1/IGF-1R signaling pathway is one of the most common pro-survival pathway constitutively upregulated in several types of cancer. Studies to investigate whether sCLU induction occurs via this pathway revealed that IR stress induced DNA damage causes activation of Ataxia telangiectasia-mutated kinase (ATM), which causes de-repression of IGF-1 transcription mediated by p53-NF-YA complex. As a result of this IGF-1 levels increase leading to the activation of IGF-1/IGF-1R pathway which further activates downstream targets like Src MEK/ERK or PI3K/AKT (Ammar and Closset, 2008; Zhang *et al.,* 2014) which inturn activates EGR-1 transcription factor and further induction of sCLU transcription (Figs. 2C.1 and 2C.2) (Goetz *et al.,* 2011). This provides a connecting link between p53 mediated suppression of sCLU post IR induction and IGF-1/IGF-1R signaling (Criswell *et al.,* 2005).

Interestingly under stress conditions like serum deprivation, sCLU has been shown to bind to and sequester IGF-1 extracellularly, to prevent IGF-1 binding to IGF-1R, thus negatively modulating the PI3K-AKT pathway (Jo *et al.,* 2008). In hepatocellular carcinoma, high expression of CLU has been shown to be associated with poor survival and high tumor recurrence, wherein CLU overexpression has been shown to activate PI3K/AKT pathway by interacting with EIF3I, leading to the further activation of MMP13 and to metastasis. Interestingly knockdown of CLU was shown to affect the CLU-EIF3I/AKT/MMP13 axis, suppressing metastasis (Lee *et al.,* 2016). CLU is overexpressed in castration resistant prostate cancer (CRPC) where the pro-survival pathway like IGF-1/IGF-1R pathway is well studied wherein sCLU

has been shown to be induced via the STAT-Twist-1 signaling in this pathway (Takeuchi *et al.,* 2014).

EGFR pathway

Studies by Shim *et al.* (2009) suggest a role for CLU in astrogliosis or reactive astrocytosis in which an abnormal increase in the number of astrocytes occurs due to loss of nearby neurons caused by accidental injury, ischemia, autoimmune disorder or neurodegenrative disorders, mediated via the EGFR pathway. Their studies revealed that sCLU binds to epidermal growth factor receptor (EGFR), transmitting mitogenic signal downstream via the Ras dependent Raf/MEK/ERK pathway in rat astrocytes (Fig. 2D). It is not known whether the activated ERK further activates EGR-1(early growth response-1), a well-documented transcription factor for sCLU transcription, leading to a positive feedback loop inducing cell growth and proliferation.

Regulation of CLU by miRNA

In non-small cell lung carcinoma (NSCLC), CLU has been shown to be upregulated and confer resistance to chemotherapeutic agents like cisplatin. Recently, miR-378 has been shown to target CLU, which chemosensitizes NSCLC cells highlighting its therapeutic importance (Chen *et al.,* 2016).

From the above information, it is still unclear whether the opposing functions of CLU reported in the literature are due to the use of different antibodies by different groups, the lack of antibodies specifically recognizing different forms of CLU, the type of cell lines, patients, etc studied or whether it indicates that CLU can act as a tumor suppressor or oncogene, depending on the type of cancer and its phase of progression. It is possible that the prominent role of CLU in the different normal tissues may be a determining factor of its role as a tumor suppressor gene or oncogene in the malignant tissues.

Targeting CLU for treatment of advanced cancers

In majority of the cancers, the conventional treatment modalities include surgery, chemotherapy, radiotherapy and alternatively in case of prostate and breast cancers, hormone ablation therapy. Overall, about one third of the cancer patients show recurrence and resistance to different anti-cancer therapeutics. One of the important

contributing factors for this development of resistance would be overexpression of certain pro-survival factors including stress induced cytoprotective chaperonic sCLU, which is upregulated in several cancers as mentioned earlier in this review. It has been speculated that sCLU might confer resistance to the different therapies by modulating several cellular processes like apoptosis, cell cycle checkpoints, inflammation etc. Hence, targeting sCLU may help to improve the efficacy of current therapeutic strategies by sensitizing the cancer cells to the different therapeutic agents.

Custirsen (OGX-011), is a second generation anti-sense oligonucleotide (ASO) designed by OncoGeneX Technologies Inc. in collaboration with Isis Pharmaceuticals and is directed against the translation start site located in exon 2 of sCLU. ASO comprise of chemically modified stretch of DNA that targets specific mRNA, and further inhibits its translation by forming DNA/RNA duplex. However, a major disadvantage of using ASO is its instability and rapid intracellular degeneration. Custirsen is a phosphorothioate antisense oligo-nucleotide, which also has the 2'-MOE modification on the 4 bases on either end of the 21-mer phosphorothioate backbone. This ASO to CLU exhibited a significantly higher affinity for the target and better potency in terms of its increased half-life (7 days) and longer duration of its action as compared to first generation ASOs (Zellweger *et al.,* 2001). In a phase I clinical trial aimed to study the pharmacokinetics and pharmacodynamics of OGX-011 and its efficacy in treatment of patients with localized prostate cancer revealed that OGX-011 can be safely administered to humans at a dose of 640 mg (Chi *et al.,* 2008). Further studies have shown that OGX-011 improved the efficacy of radiatiotherapy, chemotherapy and hormone ablation therapy by inhibiting sCLU expression and enhancing apoptosis (Koltai *et al.,* 2014). Studies by Trembley *et al*. (2013), (Patent no.: WO 2013123588 A1) showed that co-targeting CLU and EGFR using their respective inhibitors i.e. h16B5 and Erlotinib is a promising strategy in non-small cell lung carcinoma (NSCLC) and prostate cancer patients

Concluding remarks

CLU, a stress-induced multifunctional glycoprotein is vital for maintaining cellular homeostasis, predominantly via

its role as a chaperone. Based on the available information in the literature, there is little clarity on the CLU isoforms and their functions in cancer and research is warranted in this area to decipher the same. The potentially conflicting evidence of overexpression and repression of CLU in different cancer tissues suggests a dual role for CLU as a tumor suppressor or an oncogene. The mechanism of CLU regulation is signal and cellular context dependent, deciphering which is a challenge. Although the existence of a nuclear CLU is controversial, the possible occurrence of hypoglycosylated and glycosylated forms with opposing functions and differential localization is speculated and may support its tumor suppressive and oncogene roles. Development of an antibody that distinguishes these two forms of CLU and deciphering its crystal structure may help in clarifying the dual role of CLU.

The complex role of CLU in cancer is far from being resolved. However with the advent of new technologies, it may be possible to gain some clarity in the role of CLU variants in cancer. Using high end mass spectrometry techniques, it may be possible to identify the different CLU variants detected post stress, in different types of tumors and cell lines. However the identification of these variants can be further strengthened by the development of variant specific antibodies for their antibody-based detection in the cells and tumors. Also, clarity on the functions of CLU variants in a specific cancer tissue can be obtained by performing knockdown/knockout studies of specific CLU variant and followed by rescue experiments. Using latest molecular imaging techniques, the route and destination of the labeled CLU proteins can be tracked in cancer versus normal cells to understand their cellular function. Identification of the sCLU interactome in normal versus tumor tissues will provide clues to its binding partners and possible functions in these tissues. High CLU expression has been associated with tumor progression, therapy resistance and poor prognosis and studies indicate that CLU can serve as a biomarker/predictor of response post drug treatment. However, caution needs to be exercised in the use of CLU ASO- Custirsen to target CLU in cancer and it would be important to ascertain whether CLU is a positive or negative modulator of carcinogenesis in the specific cancer tissue.

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