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Regulation and Functions of Clusterin: A Protector Against Stress

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Radiation therapy is a common treatment for many types of tumors. Therefore, it is vital to understand the cellular responses to radiotherapy in malignant cells, as well as the surrounding normal tissues in order to optimize antitumor efficacy. Clusterin (CLU) is a secreted glycoprotein that has been implicated in many normal biological processes as well as many pathological diseases, including cancer. Our laboratory identified the secreted form of clusterin (sCLU) as a protein/transcript that could be induced by doses of ionizing radiation (IR) as low as 0.02 Gy, suggesting a role for sCLU in the cellular response to IR. While the exact functions of CLU are complex, it has been suggested that sCLU, the fully processed and glycosylated form of the CLU protein, plays a role in cytoprotection after cellular stress. sCLU appears to provide cytoprotection against cellular injury and inflammatory responses potentially by acting as a molecular chaperone, clearing cellular debris or binding to inflammatory and growth suppressive cytokines, such as TGF-ß1. A better understanding of this protein and its various roles in cellular responses to stress will allow us to generate better treatments and therapies for many different pathological processes. The functions of sCLU and its role(s) in disease processes will be discussed.

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Introduction

Clusterin

Expression from the clusterin (CLU) gene results in the generation of a full-length CLU mRNA that encodes a secreted glycoprotein (secreted clusterin, sCLU). sCLU was originally identified in 1983 as a protein involved in cellular aggregation in ram rete testis fluid.[1,2] In 1987, Leger et al. demonstrated that CLU transcription was induced in the ventral rat prostate after castration and named it testosterone-repressed prostate message 2 (*TRPM-2*).[3] Since then, many groups working in diverse fields have identified/cloned CLU, resulting in a diverse nomenclature for this protein[1,3-11]; in 1998, at the first CLU workshop, an international team adopted clusterin as the official name of this gene. Our laboratory isolated full-length CLU mRNA as an X-ray-inducible protein/transcript (xip8), in which northern blot and nuclear run-on studies demonstrated enhanced transcript synthesis and steady state mRNA accumulation within IR-exposed human malignant melanoma cells.[11-13] Interestingly, we then identified a truncated version of the CLU mRNA as a Ku70 binding partner (KUB 1) using a yeast two hybrid screen with Ku70 as bait. Since Ku70 is predominantly localized to the nucleus, we proposed that this truncated form of the mRNA encoded a nuclear form of the CLU protein (nCLU). We then found that a 49 kDa nCLU protein resided in the cytoplasm of unirradiated MCF-7 cells and that after IR, this form translocated to the nucleus as a 55 kDa protein.[14] The post-translational modifications that result in this change in molecular weight are currently being investigated.

The human *CLU* mRNA contains two AUG start sites separated by 32 amino acids—therefore one message appears to encode two separate proteins, whose processing is dictated by the presence (sCLU) or absence (nCLU) of this leader peptide sequence (Figure 1).[15,16] When full-length CLU mRNA is read at its first AUG sequence, a leader peptide targets the protein to the endoplasmic reticulum (ER). This 60 kDa unmodified peptide (pre-sCLU) is cleaved at an α/ß cleavage site to yield two 40 kDa subunits that heterodimerize through the formation of five disulfide bonds to form the mature 80 kDa secretory form of the protein that is further processed and glycosylated in the Golgi apparatus.[5,15] sCLU separates as an 80 kDa protein or ~40 kDa proteins under SDS-PAGE non-reducing or reducing conditions, respectively.[14] Treatment of log-phase MCF-7

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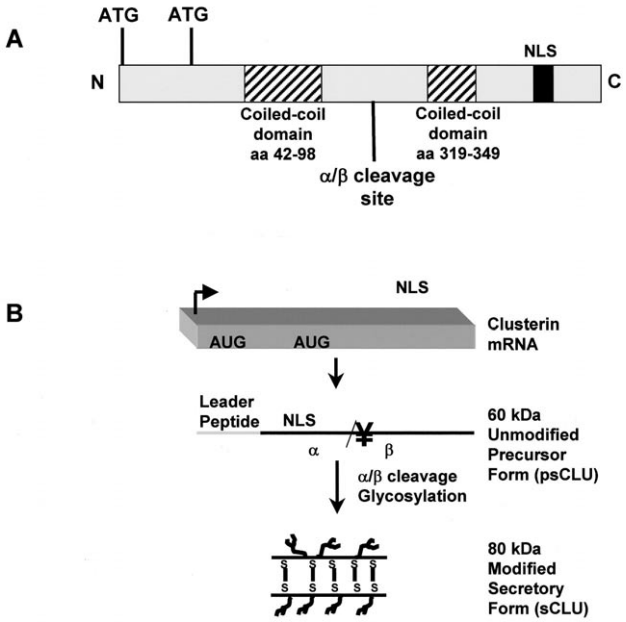

Figure 1. Schematic of CLU gene structure and protein processing. (A) Hashed boxes correspond to the two coiled—coil domains at amino acids 42-98 and 319-349, respectively. The black box corresponds to the functional nuclear localization site. (B) The human CLU mRNA contains two AUG start sites separated by 32 amino acids. When the CLU mRNA is translated from its first AUG sequence, a leader peptide targets the protein to the endoplasmic reticulum (ER) as it is being translated. This 60 kDa unmodified peptide (psCLU) is cleaved at an a/b cleavage site to yield two 40 kDa subunits that heterodimerize through the formation of 5 disulfide bonds to form the 80 kDa secretory form of the protein that is highly glycosylated in the Golgi apparatus. Translation from the second AUG start site results in the production of the nuclear form of CLU (nCLU).

cells with ≥ 2 cGy (2 rads) of ionizing radiation (IR) results in dramatic increases in *sCLU* message and protein. We initially noted the massive accumulation of the 60 kDa peptide that is presumably present in the ER and Golgi of IR-exposed cells within 24-72 h post-IR exposure.[14,17] Furthermore, the low levels of IR (≥ 2 cGy) required to induce *sCLU* and the dramatic accumulation of sCLU protein following taxol, PMA or thapsigargin (a sarcoplasmic reticulum Ca^{2+} -ATPase (SERCA) pump inhibitor that caused dramatic alterations in intracellular calcium homeostasis) exposures, strongly suggested that DNA damage was not required for the activation of this gene in MCF-7 breast cancer cells.[18]

More recently we noted that, due to alternative splicing of the *CLU* mRNA in which the leader peptide found in sCLU is eliminated by Exon I/II junctional splicing, *CLU* could be translated from the second, in-frame AUG start site in its mRNA, resulting in the formation of a 49 kDa pre-nuclear (pnCLU) protein. This precursor form of the nuclear clusterin protein is observed in the cytoplasm of control non-irradiated MCF-7 human breast cancer cells.[19] The pnCLU protein contains two putative nuclear localization signals (NLS); the first one located after the second AUG start site and the second located at the C-terminus. The C-terminal NLS appears to be the functional sequence.[19] We propose that these NLSs are kept concealed either through homodimerization or protein folding, through two coiled-coil domains, until after cellular stress.[19] After treatment of log-phase MCF-7 cells with ≥1.0 Gy of IR, the levels of pre-nCLU protein, as well as a ~55 kDa nuclear form of the protein (nCLU), dramatically increased 48 to 72 h[14,20] post-treatment. Analyses of nCLU (isolated from the nuclei of IR-exposed MCF-7 cells) revealed that the protein was not cleaved at its α/ß site, since its migration was not altered under reducing or non-reducing SDS-PAGE conditions. Furthermore, accumulation of nCLU protein appears to be sufficient for signaling cell death, even in the absence of IR exposure, and the protein appears to associate with the Ku70/Ku80 DNA double strand break repair machinery.[14] More recent data from our lab indicate that nCLU is also regulated by nuclear export sequences and signaling, wherein CRM-1 excludes nCLU from the nucleus (*Leskov and Boothman et al., submitted*). Our lab is currently working on the mechanism by which nCLU causes cell death.

Clusterin and disease

CLU has been implicated in many physiological processes such as lipid metabolism,[21,22] complement regulation,[10,23,24] cell differentiation,[25,26] reproduction,[1,15,27] tissue remodeling/regeneration,[28-33] cell adhesion,[34-36] and cell death.[14,37-42] CLU has also been implicated in many diverse disease processes including atherosclerosis,[43-45] Alzheimer's disease,[46-48] glomerulonephritis,[32,49,50] preeclampsia,[51] lupus erythematosus,[52] retinitis pigmentosa,[53,54] and scrapie.[37] Therefore, sCLU may be a good therapeutic target for a wide range of diseases. Many of these diseases involve dysregulation of the immune system. Indeed, sCLU has been shown to be an inhibitor of complement-mediated cytolysis through inhibition of C9 polymerization and insertion of the membrane attack complex into the plasma membrane of target cells.[24,55,56] Thus, down-regulation of sCLU would result in over activation of the complement system and an increase in inflammation. In agreement with this idea, Hogasen et al. demonstrated that decreased CLU mRNA levels in rheumatoid arthritis patients correlated with an increase in the terminal complement pathway.[57] Over-expression of CLU mRNA may also be harmful. Han et al. demonstrated that CLU-deficient mice had 50% less brain injury following neonatal hypoxic-ischemia insult, and that CLU localized to dying cells in wild-type CLU-expressing mice.[58] Gwon et al. demonstrated similar results in the rat retina following ischemia.[59] This disparity in CLU function is most likely due to a balance that is required between the two different CLU isoforms, the cytoprotective functions of sCLU *versus* the cytotoxic functions of nCLU. It is not yet understood how the cell regulates this balance (Figure 2).

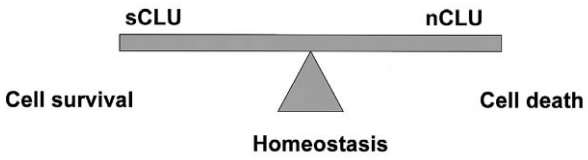

Figure 2. Schematic diagram of the homeostatic balance between sCLU and nCLU and therefore cell survival and cell death.

Elevated levels of secretory CLU (sCLU) protein and mRNA have been observed in several different types of human neoplasias and malignancies including prostate cancer,[60-62] pancreatic cancer,[63] hepatocellular carcinoma,[64] hemangiomas,[65] malignant lymphomas,[66-68] ovarian cancer,[69,70] breast cancer,[71] melanoma, colorectal carcinoma[72] and renal clear cell carcinomas.[73,74] Additionally, forced over-expression of sCLU in transformed cell lines resulted in an increased resistance to doxorubicin, cisplatin and taxol[60,75,76] and abrogation of *CLU* mRNA expression following antisense expression lead to modest chemo- and IR-sensitization in various cell lines.[75,777-80] More recently, it was shown that treatment of Her2 positive BT474 cells with antisense to sCLU in combination with Trastuzumab, a Her2 monoclonal antibody, resulted in a significant increase in apoptotic cells compared to cells treated with Trastuzumab alone.[81] These data support a cytoprotective role for sCLU and suggest that over-expression of endogenous sCLU provides a survival advantage for the tumors in which it is expressed. Indeed, Redondo et al. demonstrated a correlation between increased sCLU expression and a metastatic phenotype in breast cancer.[71]

Functions of clusterin

Clearly, delineating the functions of CLU will rely on the various functions of the two protein forms of the CLU gene. The defining property of CLU gene expression seems to be that it is induced under conditions that result in cellular stress. This fits with its proposed role as a molecular chaperone involved in the clearance of cell debris after cellular stress.[82-85] Indeed, it has been shown that exposure of fibroblasts to apoptotic vesicles and cellular debris can induce sCLU expression.[86] Bailey et al. demonstrated that heat shock-induced apoptosis in the testes of CLU-deficient mice occurred more rapidly and that clearance of cellular debris was slightly impaired compared to sCLU-expressing wild-type mice.[87]

Additionally, Humphreys et al. demonstrated that sCLU has properties similar to small heat shock proteins.[82] sCLU could protect the proteins, glutathione S-transferase and catalase, from heat-induced precipitation caused by stress-induced improper protein folding, through the formation of high molecular weight complexes.[82] Furthermore, Poon et al. demonstrated that the ability of sCLU to protect cells from precipitation was maximal at a slightly acidic pH.[88] This suggested a role for sCLU at sites of injury and inflammation, where the pH is slightly acidic to prevent against infection. It is intriguing to speculate on what role sCLU is performing as a secreted chaperone, since sCLU induction does not occur until several days after stress. It may be that sCLU is secreted from cells that survive cytotoxic stress, and is needed to clear cellular debris from dying cells in order to "turn over" factors that could be potentially cytotoxic or inflammatory.

The development of a *CLU* knock-out mouse allowed further insight into the function of this protein.[89] The *CLU* knock-out mice develop a more severe myocarditis, compared to wild-type mice, with substantial scarring after challenge with murine myosin,[89] and Han et al. demonstrated that CLU deficient mice had 50% less cell death after hypoxic/ischemic insult.[58] Additionally, Rosenberg et al. showed that CLU deficient mice had increased immune complex deposition in the kidneys, indicative of progressive glomerulopathy.[90] These data suggested a protective role for CLU during tissue injury and inflammatory responses. Additionally, *CLU* (also termed apolipoprotein J) and apolipoprotein E (*ApoE*) double knock-out mice showed a decrease in deposition of fibrillar ß-amyloid in the brain,[91] supporting a role for CLU in Alzheimer's disease and diseases of aging.

Regulation of clusterin expression

As previously mentioned, CLU was identified as an induced protein in the ventral rat prostate after castration and was thus termed testosterone repressed prostate message-2 (TRPM-2).[3] The hormonal regulation of CLU in the prostate has been well documented, but its potential hormonal regulation in other tissues is not well understood. In addition to prostate cancer, CLU has been shown to be over-expressed in breast, ovarian and endometrial cancers.[70,71,92] This suggests that CLU may also be regulated by estrogens. In 1998, Wunsche et al. demonstrated that CLU expression in RUCA-1 rat endometrial could be induced by treatment with estradiol. This same paper also demonstrated that CLU expression *in vivo* was also dependent on the presence of estradiol in endometrial carcinomas.[93] A more recent report has shown that CLU expression in RUCA-1 cells is dependent on the presence of estradiol and that this expression can be completely blocked by the presence of the pure anti-estrogen Faslodex (ICI 182,780). In contrast, the selective estrogen receptor modifier tamoxifen, which is commonly used for the treatment of breast cancer, was shown to also induce CLU in the endometrium.[91] They suggested that this induction of CLU by tamoxifen in the endometrium might be causally related to the high frequency of endometrial tumors seen in women treated with tamoxifen.

The signaling pathways that result in *CLU* induction after stress have not been elucidated. In 1992, Herrault et al. found that v-Src could induce transcription of the avian *CLU* gene.[94] The involvement of Src in human *CLU* induction has not been investigated. B-Myb has also been shown to bind and activate the *CLU* promoter,[95] but the physiologic relevance of this has yet to be determined. Finally, TGF-ß1 also appears to play a role in *CLU* gene and protein expression[96,97] potentially through c-Fos binding to an Ap-1 site located in the *CLU* promoter.[98] Additionally, extracellular CLU can bind to the TGF-ß1 receptor II, suggesting a possible feedback mechanism where CLU affects downstream TGF-ß1 signaling pathways. Recent data from our lab indicate that CLU promoter and transcription in response to TGF-ß1 is regulated by the activation of Smads 3 and 4 binding to three Smad Binding Elements within the CLU promoter. Activation of both AP-1 and IGF-1R signaling also appear to be involved.

Our recent studies revealed that sCLU plays a role in cell survival after exposure to IR. To further understand cellular responses to IR, we investigated the signaling pathways required for the IR-induction of sCLU. We report that IGF-1R activation after IR was required for

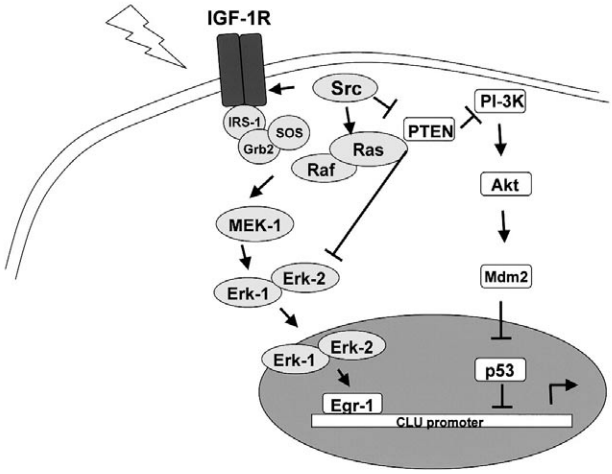

Figure 3. Model depicting potential cross-talk between the IGF-1R dependent signaling pathway leading to sCLU induction after IR and a potential pathway signaling to the p53 transc riptional repression of sCLU.

sCLU induction. We demonstrated that IR-induced sCLU was dependent on a novel reactivation of the Src/Mek/Erk signaling cascade 24-72 h after IR exposure, and that this signal culminated in the activation of the Egr-1 transcription factor (model, Figure 3). Interestingly, EGFR signaling was not apparently involved.

EGFR is over-expressed or constitutively activated in many types of tumors including colorectal, breast, pancreatic and ovarian cancers,[99] and is known to be a mediator of radio-resistance in several tumor types including glioblastoma multiforme and breast cancer cells through the activation of Erk-1/2.[100,101] As a result, many therapies have been developed that specifically target EGFR including monoclonal antibody therapies and small molecule inhibitors that specifically target the kinase domain.[102] Interestingly, the selective EGFR inhibitor, AG1478, did not block *CLU* promoter induction or regulate sCLU protein levels after 5 Gy, nor did it affect EGF-stimulated sCLU protein expression, possibly due to the involvement of other EGFR family members.

IGF-1R is another membrane receptor shown to be up-regulated after IR. IGF-1R activation results in mitogenic growth and cell survival,[103] and Gooch et al. demonstrated that treatment of cells with IGF-1 could prevent doxorubicin and taxol induced apoptosis.[104] Using AG1024, a selective inhibitor of IGF-1R, we were able to block sCLU induction after IR, demonstrating the requirement for IGF-1R activation for IR-induced sCLU expression. In a recent report, it was shown that AG1024 treatment of MCF-7 cells enhanced cell death after IR exposure.[105] Our data strongly suggest that activation of IGF-1R may mediate cell survival effects through the downstream induction of sCLU.

MCF-7 cells produce and secrete IGF-1 under serum-free conditions.[106] Since IGF-1R is often over-expressed in breast cancer,[107] the MCF-7 model appears to be a god one for investigating CLU gene expression. It was shown previously that peripheral lymph node stromal cells produce and secrete EGF and IGF-1, which can increase the growth of breast cancer cells.[108] It is possible that EGF and IGF secretion by lymph nodes can induce the tumorigenesis of neighboring breast tissue, especially cells that have up-regulated expression of EGFR or IGF-1R, through a paracrine mechanism. It is interesting to note that both EGF and IGF-1 were able to induce sCLU expression. Additionally, serum starvation increased the basal activity of the *CLU* promoter compared to cells grown in whole serum, suggesting a possible autocrine feedback loop induced by IR, where irradiated cells not only up-regulate IGF-1R, but also presumably increase production of the ligand, IGF-1. Consistent with a previous report,[103] we demonstrated induction of IGF-1R after IR, as well as a 20% increase in secretion of IGF-1, 24-72 h post-IR. Importantly, the induction of IGF-1R and its ligand provide a plausible explanation for the late induction of CLU after IR, as well as the increase in basal promoter activity after serum-starvation.

The Src-Raf-Mek-Erk-1/2 pathway is required for IR-induced sCLU activation culminating in the activation of the Egr-1 transcription factor. We demonstrated a novel re-activation of the MAPK cascade after IR that correlates with the temporal activation of sCLU. The physiological relevance of this biphasic activation of MAPK is unknown. EGFR may be required for the initial induction of MAPK after IR, and inhibitors to EGFR are known to potentiate the cytotoxicity of radiation therapy.[109] In addition, Lu et al. demonstrated that increased IGF-1R production in MCF-7 cells caused increased resistance to Herceptin (a monoclonal antibody to the Her2/neu receptor) induced cell death,[110] suggesting a role for IGF-1R signaling in the development of resistance to this type of therapy. Our data suggest that tumor cells that survive the initial phase of treatment may develop resistance to EGFR inhibitors, as a result of the late induction of MAPK signaling through IGF-R1 up-regulation, and potentially the up-regulation of sCLU. This suggests that the current antibody and small molecule therapies used to treat EGFR positive tumors may be optimized by the addition of inhibitors to the IGF-1R pathway.

NF-κB has also been shown to regulate sCLU expression. Saura et al. demonstrate that NF-κB is required for LPS stimulation of sCLU expression in glial cells[111] and Li et al. using microarray technology, demonstrate that TNF-stimulated *CLU* expression is dependent on the NF-κB/IKK complex.[112] Of interest, Santilli et al. demonstrated that sCLU can disrupt NF-κB signaling by stabilizing the inhibitors of NF-κB (IκBs).[113] This suggests a possible negative feedback loop, wherein NF-κB stimulates the expression of sCLU after LPS or TNF-α exposure that then acts to stabilize IκBs and silence NF-κB signaling.

Our laboratory has recently demonstrated that sCLU is transcriptionally repressed by the p53 transcription factor.[17] The p53 tumor suppressor gene is mutated in over half of all human tumors,[114] which commonly leads to a stable protein with loss of function. Wild-type p53 protein is stabilized after cellular stress and acts as a transcription factor for various downstream genes, including Bax, p21 and GADD45, resulting in either cell cycle arrest or apoptosis.[115-117] p53 can also act as a repressor of transcription, although the exact mechanism of repression varies. Forced expression of the papillomavirus E6 protein in MCF-7 human breast cancer cells, as well as HCT116 isogenically

matched colon cancer cell lines that differ only in their p53 status, were used to demonstrate a role for p53 in the transcriptional repression of sCLU in unirradiated cells. These data suggest that p53 responses after high doses of IR ($\geq$ 1.0 Gy) down-regulate the cytoprotective functions of this protein to allow for cell cycle checkpoint responses and for cell death in severely damaged cells.

The signaling pathway that relieves p53 repression, allowing for sCLU induction after IR, has not yet been elucidated. Recently, in a paper by Lu et al. it was shown that Src family kinases could inhibit the function of PTEN.[118] PTEN has been shown to be an inhibitor of IGF-1R activated MAPK[119] as well as the Akt signaling pathway.118 One function of Akt is to stabilize Hdm-2, allowing for degradation of p53. Additionally, Tanno et al. have shown that Akt activation can up-regulate IGF-1R.[120] The cross-talk between the IGF-1R, PTEN and Akt pathways may provide an intriguing connection between the signaling cascade resulting in sCLU induction after IR and the repressive effects of p53 (model, Figure 3). Preliminary data from our laboratory support a role for PTEN and Akt in the regulation of sCLU. Over-expression of a constitutively active Akt or expression of a catalytically dead PTEN in 1403 cells resulted in significantly higher basal levels of *CLU* promoter activity as compared to cells transfected with vector alone (Thakur et al., in preparation). This pathway appears to be in operation in MCF-7 cells that constitutively express AKT. In fact, we have recently shown that over-expression of Hdm-2 can repress CLU expression basally and after IR treatment. We also demonstrated that CLU up-regulation in MCF-7 cells involved the Egr-1 transcription factor, and that p53 may regulate CLU gene expression by interaction with Egr-1 (Thakur et al., in p reparation). Thus, the interactions between induction and repression signaling in the overall control of clusterin appear to be complex.

Recent data indicate that sCLU provides cytoprotection against doxorubicin, taxol and cisplatin in the treatment of cancer cells.[74,76,78,95] sCLU expression has been found to be elevated in many types of tumors, including prostate, colorectal and breast cancer.[61,63,71] In a recent paper by Chen et al. it was shown that *CLU* message and protein were elevated in intestinal tumors derived from mice containing the *Apc* min (multiple intestinal neoplasia) mutation.[72] IGF-1R and IGF-1 production are also elevated in many tumor types. Gleave et al. in a recent review, suggest using CLU and insulin-like growth factor binding proteins (IGFBPs) as targets for antisense therapy against prostate cancer.[121] Although they did not mention a possible connection between IGF-1R signaling and the induction of sCLU. Our data strongly suggests such a connection, which may explain the connection between elevated sCLU levels and the development of prostate cancer.

It is intriguing to speculate about a possible role for sCLU in bystander effects, after radiation or chemotherapeutic therapies. An increased production and secretion of sCLU by tumor cells into the lymph or vasculature system may provide a survival effect for neighboring or metastatic cancer cells. Additionally, secretion of IGF-1 by normal or tumor cells may provide an additional means for sCLU up-regulation.

Exploiting CLU induction

The exact regions of the *CLU* promoter that are required for IR induction have not yet been determined. Determining the exact element required for IR induction will allow us to potentially utilize the *CLU* promoter for combination gene targeting and radiation therapies. The *Egr-1* promoter linked to the herpes simplex virus thymidine kinase gene is currently being utilized in this manner to sensitize tumor cells for radio-therapy.[122-124] The IR-inducible element in the *CLU* promoter may be more useful since it is induced by much lower doses of IR and because it is repressed by p53. Since p53 is mutated in over 50% of all human tumors, using the IR-inducible 4250 *CLU* promoter with the p53 binding site will allow us to target this construct to be active in the tumor cells missing p53, while sparing the normal cells that still contain wild-type p53.

Conclusions

In summary, CLU gene expression is implicated in many normal biological processes and many pathological disease processes. The function of sCLU is complex, but it seems to play a role in cellular stress responses. sCLU appears to provide cytoprotection against cellular injury and inflammatory responses while the nuclear form of the protein appears to be cytotoxic. The regulation of this protein after stress is not understood. A better understanding of this protein and its various roles in cellular responses to stress will allow us to generate better treatments and therapies for many of these different pathological processes.

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