C. Final Progress Report:

We completed all of the Aims of our prior grant, DE-FG-022178-16, that ended in March, 2006. The data that resulted led to publications on the regulatory processes and functions of sCLU in cells after low dose exposures of IR (see below). Our accomplishments include:

1. In Aim 1, we discovered a low dose IR-inducible, delayed IGF-1/IGF-1R/Src/Raf/MAPK/Egr-1 signaling pathway that activated the CLU promoter. In Aim 2, we discovered that sCLU was a pro-survival factor in MCF-7 cells, wherein expression knockdown using siRNA specific for sCLU enhanced lethality after clinically relevant doses of IR.
2. In Aim 1, we generated MCF-7 cells stably containing the hCLU promoter-firefly luciferase reporter, and then developed technology to visualize CLU promoter activity in cells in culture. We then developed an IR-inducible transgenic mouse containing the human CLU promoter controlling the firefly luciferase reporter for *in vivo* bioluminescent, noninvasive imaging of CLU promoter activity before and after IR. Using these mice, we showed CLU promoter activity induced after IR (by ≥10 cGy) in specific organs (bone marrow, colon, thymus, skin, spleen) **with temporal *and* dose-response induction characteristics identical to IR-induced endogenous sCLU protein levels.** (below, Refs. **b,c**). The human and mouse CLU promoters are highly conserved, particularly in functional domains.

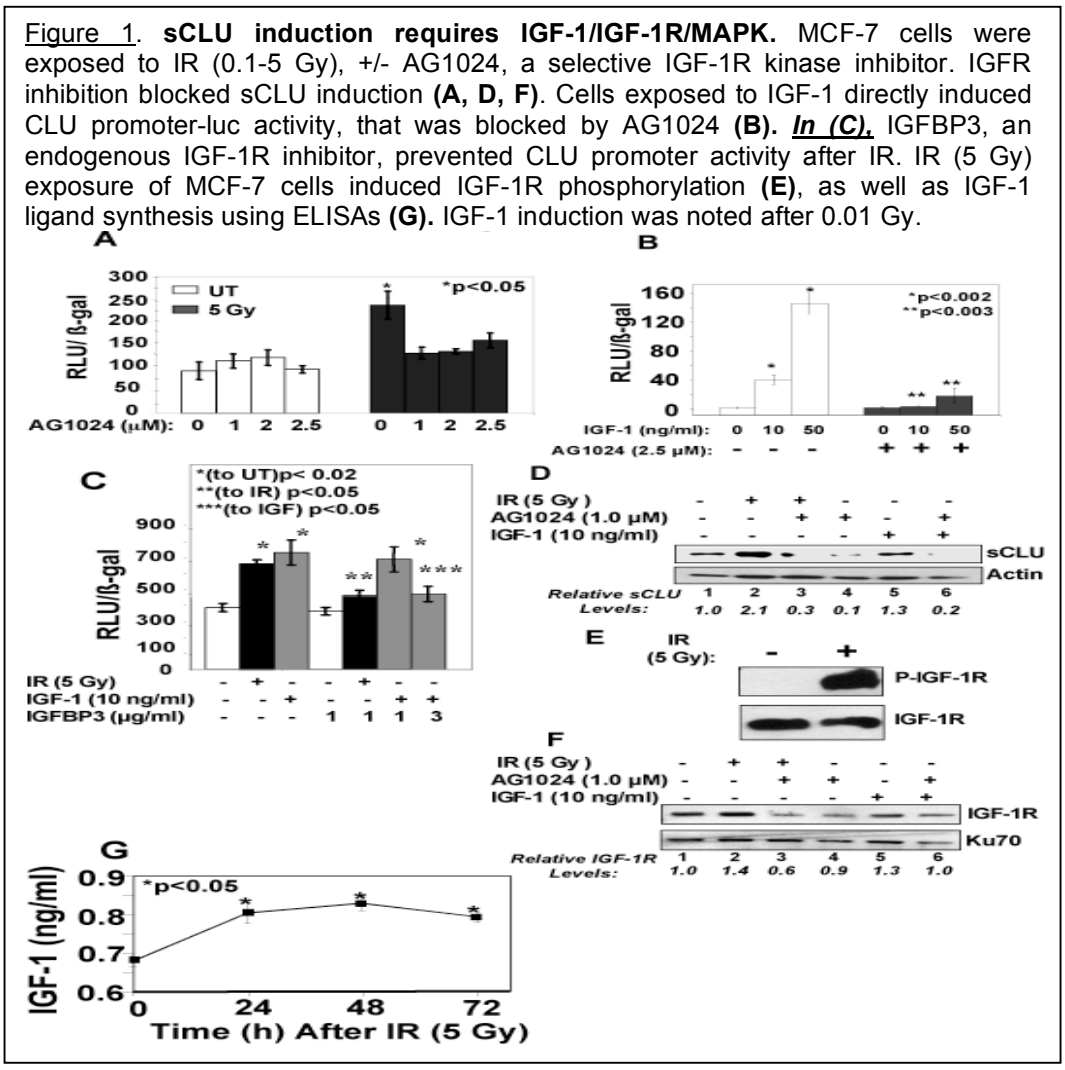

Figure 1. **sCLU induction requires IGF-1/IGF-1R/MAPK.** MCF-7 cells were exposed to IR (0.1-5 Gy), +/- AG1024, a selective IGF-1R kinase inhibitor. IGFR inhibition blocked sCLU induction **(A, D, F)**. Cells exposed to IGF-1 directly induced CLU promoter-luc activity, that was blocked by AG1024 **(B).** ***In (C),*** IGFBP3, an endogenous IGF-1R inhibitor, prevented CLU promoter activity after IR. IR (5 Gy) exposure of MCF-7 cells induced IGF-1R phosphorylation **(E)**, as well as IGF-1 ligand synthesis using ELISAs **(G).** IGF-1 induction was noted after 0.01 Gy.

3. In Aim 1, we discovered that p53 suppressed sCLU expression (Refs **d**). We subcloned a longer region (-4250 to +1) of the human

CLU promoter and fused this in-frame with firefly luciferase to elucidate transcription factors that regulate CLU promoter activity and to elucidate the mechanism(s) by which p53 suppresses CLU promoter activity (Refs. **a**). This construct enables transient transfection of cells for analyses in any cell of choice. We also developed a lentivirus system for sCLU expression in CLU- cells, and for expression of small hairpin RNA specific to CLU (shRNA-sCLU) for stable sCLU-specific knockdown; nuclear clusterin levels are not affected (Ref. **a**).

4. In Aim 2, we developed LNCaP cells stably over-expressing sCLU and demonstrated that these cells were resistant to IR compared to CLU-deficient vector alone LNCaP cells. A similar LNCaP system was previously reported by Gleave et al., (*169, 280*). These cells will be used in our new Aims 2 and 3 to investigate the role(s) of sCLU in bystander and adaptive responses after low doses of IR.
5. In Aim 3, we discovered that TGF-ß1 treatment of human cancer cells induced sCLU in a signal transduction process that depended on Smad 4 and Smad 3 activation. We defined Smad Binding Elements (SBEs) in the CLU promoter and characterized Smads 3 and 4 binding. (Ref. **e**). We are now in a position to test the hypothesis that sCLU suppresses TGF-ß1 signaling in Aim 2.
6. In Aim 3, we showed that unlike IR, TGF-ß1 exposure of cells induced sCLU in cells with functional p53, and we provided evidence that induction by TGF-ß1 was the result of activated Smad-dependent, increased Hdm-2 expression, which down-regulates p53 allowing induced CLU promoter activity (Ref. **f**). We now provide direct, preliminary evidence that sCLU can suppress TGF-ß1 induction of CLU promoter activity (below). These data support a role for sCLU as a negative feedback factor to suppress TGF-ß1 responses. Potential bystander effects of sCLU will be examined in Aim 2.
7. In Aims 1 and 2, we defined the mechanism by which the nuclear clusterin (nCLU) pro-death protein was synthesized and activated (Ref. **g**). We recently discovered that nCLU regulation is controlled by nuclear export sequences (NESs), as well as CRM1-mediated processes (Refs. **h,i**).

Articles Published: Peer-reviewed (9 papers published, in press, or submitted):

a. Criswell T, Beman M, Araki S, Leskov K, Cataldo E, Mayo LD, Boothman DA. Delayed activation of IGF-1RSrc/MAPK signaling after IR regulates clusterin expression, a pro-survival protein. J Biol Chem. 2005; *280(14):*14212-21.

b. Klokov D, Criswell T, Sampath L, Leskov KS, Frinkley K, Araki S, Beman M, Wilson DL, , Boothman DA. Clusterin: a protein with multiple functions as a potential ionizing radiation exposure marker. Int Congress Series. 2004; *27:*2784-93.

c. Sampath, L, Klokov, D, Wilson D, and Boothman, DA. Bioluminescent imaging of CLU promoter activity *in vivo* and *in vitro*. Molecular Imaging, Submitted, 2004.

d. Criswell, T, Klokov, KS, and Boothman, DA. Transcriptional repression of clusterin by the p53 tumor suppressor protein. Cancer Biology and Therapy, ***2(4***): 25-31, 2003.

e. Araki S, Leskov K, Criswell T, and Boothman DA. TGF-ß1 induction of the human clusterin promoter through Smad binding elements. Mol. Cell, Submitted, 2006.

f. Araki, S., Leskov, K, Mayo, L, and Boothman, DA. Regulation of human double minute 2 (Hmd2) by TGF-ß1 and its role in inhibiting p53 to allow expression of the pro-survival sCLU protein. Mol. Cell, Submitted, 2006.

g. Leskov, KS, Klokov, DY, Li, J, Kinsella T J, and Boothman, DA. Synthesis and functional analyses of nuclear clusterin: a cell death protein. JBC ***278***: 11590-11600, 2003.

h. Leskov, K., Araki, S., and Boothman, DA. CRM 1-mediated nuclear export of nuclear clusterin (nCLU), a pro-death protein activated by ionizing radiation. JBC, *Submitted*, 2006.

i. Araki S, Israel S, Leskov KS, Criswell TL, Beman M, Klokov DY, Sampath L, Reinicke KE, Cataldo E, Mayo LD, Boothman DA. Clusterin proteins: stress-inducible polypeptides with proposed functions in multiple organ dysfunction. Br J Radiol. 2005; *27:*106-13.

j. Ai H, Pink JJ, Shuai X, Boothman DA, Gao J. Interactions between self-assembled polyelectrolyte shells and tumor cells. J Biomed Mat Res. 2005; *73(3):* 303-12.

k. Sutton, D., Kim, S., Shua, X., Leskov, K., Marques, JT, Williams, BRG, **Boothman, DA,** and Gao, J. Efficient suppression of secretory clusterin levels by polymer-siRNA nanocomplexes enhances ionizing radiation lethality in human MCF-7 breast cancer cells *in vitro*. 2006; Intern. J Nanomedicine. 1 (2): 155-162.

l. Shannan, B, Seifert, M, Leskov, K, Willis, J, **Boothman, DA,** Tilgen, W, and Reichrath, J. Challenge and promise: roles for clusterin in pathogenesis, progression and therapy of cancer. 2006; *Cell Death and Differentiation 13:* 12-19.

j.

Related Peer- and non-peer-reviewed articles published as a result of this grant:

1. Leskov, K, Antonio, S., Criswell, T., Yang, C-R., Kinsella, TJ, and Boothman, DA. Radiation-inducible clusterin (CLU): A molecular switch between life and death. Radiation Research ***156***: 441-442, 2001.

2. Leskov, K., Criswell, T.A., Antonio, S. Li, J., Yang, C-R., Kinsella, T.J., and Boothman, DA. When X-ray-inducible proteins meet DNA double strand break repair. Seminars in Radiation Oncology ***11***: 352-372, 2001.

3. Criswell, T., Leskov, K., Miyamoto, S., Luo, G-B., and Boothman, DA. IR-inducible transcription factors in mammalian cells at clinically relevant doses. Oncogene, ***22(37***): 5813-5827, 2003.

4. Klokov, D, Criswell, T, Leskov, KS, Araki, S, Mayo, L, and **Boothman, DA.** IR-inducible clusterin gene expression: a protein with potential roles in ionizing radiation-induced adaptive responses, genomic instability, and bystander effects. 2004; *Mutation Research,* **568(1):** 97-110.

5. Sun, W, Sawada, M, Hayes, P, Leskov, K, Boothman, DA, and Matsuyama, S. Ku70 suppresses the apoptotic translocation of Bax to mitochondria. Nature Cell Biology 5(4): 320-329, 2003.