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14. ABSTRACT
Prostate Cancer is the leading cancer diagnosed and the second leading cause of cancer death of men in the United States. The incidence of prostate cancer is closely associated with aging. Recent evidences suggested that the causes of cancer development are not limited to mutations within cancer cells, but also involve in alterations of cancer microenvironment. Senescent cells are irreversibly growth arrested, but remain metabolically active. Senescent cells, especially senescent fibroblasts in the stroma may provide a beneficial environment for tumor growth through secretion of certain factors. In our studies, we found that expression of insulin-like growth factor 1 (IGF-1) and secretory clusterin (sCLU) are increased in senescent fibroblasts, and increasing of sCLU expression in senescent fibroblasts is mediated through IGF-1 signaling pathway and regulated by ATM. Both IGF-1 and sCLU are known tumor promoting factors, and are closely associated with progression of various cancers. Increased IGF-1 signaling is reported to be a poor prognostic factor of breast cancer and overexpression of sCLU protects cells from various stressors induced apoptosis. Thus, we hypothesize that accumulation of senescent fibroblasts would lead to alterations in the microenvironment that could promote cancer growth and progression through secretion of IGF-1 and sCLU. To test this hypothesis, we established an in vivo co-culture mouse model by co-injection of cancer cells with young or senescent normal fibroblasts into mice prostate.

15. SUBJECT TERMS
clusterin, IGF-1, senescence, prostate cancer
<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Introduction</td>
<td>4</td>
</tr>
<tr>
<td>Body</td>
<td>4</td>
</tr>
<tr>
<td>Key Research Accomplishments</td>
<td>10</td>
</tr>
<tr>
<td>Reportable Outcomes</td>
<td>10</td>
</tr>
<tr>
<td>Conclusion</td>
<td>11</td>
</tr>
<tr>
<td>References</td>
<td>12</td>
</tr>
<tr>
<td>Appendices</td>
<td>12</td>
</tr>
</tbody>
</table>
Introduction
Prostate Cancer is the leading cancer diagnosed and the second leading cause of cancer death of men in the United States. The incidence of prostate cancer is closely associated with aging. Senescent fibroblasts could accumulate in prostate due to aging or stress-induced premature senescence induced by chemo-/radio-therapy. In our preliminary data, we have demonstrated that senescent fibroblasts have increased insulin-like growth factor-1 (IGF-1) and secretory clusterin (sCLU) expression. In this grant, we want to test our hypothesis: accumulation of senescent cells would lead to alterations in the microenvironment that could promote cancer growth, and allow progression of cancers to metastatic disease. Our work will focus on two aspects, 1. To elucidate the signaling pathway that regulates IGF-1 and sCLU expression during replicative- and stress-induced premature senescence in normal human fibroblasts; 2. To determine the functional roles of IGF-1 and sCLU expressed in replicative- or stress-induced premature senescent human fibroblasts on prostate cancer growth and metastasis.

Body
1. sCLU is induced during both replicative- and stress-induced premature senescence.
As shown in Fig.1A, normal human lung fibroblasts, IMR-90, are sensitive to oxygen level in the medium. Under high oxygen culturing condition (5% CO₂ in air (about 20% O₂)), population doubling of IMR-90 cells decreased significantly faster than it was cultured under low oxygen condition (5% CO₂, 2% O₂). IMR-90 cells cultured under high oxygen were actually undergoing oxidative stress-induced premature senescence. Unlike IMR-90, normal human fibroblasts, BJ are not sensitive to oxygen level in the medium. Population doubling had no changes between BJ cells cultured in high oxygen condition and low oxygen condition (Fig. 1B). BJ cells undergo replicative senescence. When cultured under high oxygen condition, sCLU expression was increased in both senescent IMR-90 and BJ cells that underwent stress-induced premature senescence (SIPS) and replicative senescence (RS) respectively (Fig. 1). These results indicate sCLU is induced during both RS and SIPS.

Besides RS and SIPS, cells can also undergo senescence through activation of oncogenes like, Ras, c-Myc, BRAF et. al. To investigate the regulation of sCLU expression during oncogene induced senescence, young IMR-90 cells was transduced with lentivirus that contains Ras12V (constitutive active) expression construct. Interestingly, IMR-90 cells with Ras12V expression was growth arrested and showed decreased sCLU expression.
(Fig. 2). The Activation of Ras was also evidenced by activation of Erk. This result is consistent with what reported in the literature (1). These results suggested that sCLU induction is only happened during RS and SIPS, but not during oncogene induced senescence.

2. sCLU is a sensitive measure of oxidative stress.
As shown in figure 1A, IMR-90 cells are sensitive to oxidative stress and undergo SIPS when cultured in 20% O₂. IMR-90 cells at early passage, passage 13 (P13), had relatively low sCLU level when cultured in 20% O₂. When it went on senescence at P21, sCLU level was further increased as expected (Fig. 3A). However, the same passage of IMR-90 cells cultured in 2% O₂ expressed much lower sCLU level. To further study the oxygen effect on sCLU expression in IMR-90 cells, we first cultured IMR-90 cells in 2% O₂, then switched them to 20% O₂ and our results showed that sCLU was induced 24 hours after switched to 20% O₂, and picked after 72 hours (Fig. 3B). We also got the similar results using HCT116 (human colon cancer cell line) and H1299 (human lung cancer cell line) cells. These results suggested that relatively high oxygen culture condition (20% O₂) indeed induces oxidative stress, and sCLU is a very sensitive to oxidative stress and could be served as a marker of oxidative stress.

3. ATM is required for sCLU induction during senescence.
To investigate the role of ATM in CLU induction during senescence, we aged fibroblasts, AT2052 from Ataxia-Telangiectasia (AT) patient, which are ATM deficient. As shown in Fig. 4A, AT fibroblast, AT2052 had low basal expression of CLU in comparison with IMR-90 (ATM wild type). And senescent AT2052 cells did not have increased CLU expression, while senescent IMR-90 did (Fig. 4A). Same results were found in the medium from young/senescent AT2052 and GM03487 cells.

Figure 3. sCLU is a sensitive measure of oxidative stress. A. IMR-90 cells undergo SIPS in high oxygen culture condition and express increased level of sCLU in comparison with cells cultured in low oxygen condition. B. sCLU is induced IMR-90 cells after switch from 2% O₂ to 20% O₂ culturing condition.

Figure 4. A. sCLU was not induced during senescence in ATM deficient AT2052 cells. B. IR induced sCLU in young and middle aged IMR-90 cells, but no further increasing of sCLU expression in senescent IMR-90 cells. C. IGF-1 induced sCLU expression and Erk activation in ATM deficient AT2052 cells. D. IGF-1 induced sCLU in both ATM⁺ and ATM⁻ cells. E. IGF-1 induced sCLU in ATM deficient GM03487 cells.
and IMR90 cells (Fig. 4A). These results indicated that ATM is a mediator of senescence induced sCLU expression.

ATM is a key mediator in both RS and SIPS, and it is also an important regulator of cellular DNA damage response (2). Our previous results also showed that DNA damage induces sCLU expression through IGF-1/Src/MAPK/Egr-1 signaling pathway, and this pathway is also involves in sCLU induction during senescence (3). Thus the same signaling pathway could be used in both DNA damage induced sCLU and senescence induced sCLU. To test this, we exposed young, middle aged, senescent IMR-90 to ionizing radiation (IR). As shown in Fig. 2B, sCLU expression was induced by IR treatment in young IMR-90; middle aged IMR-90 has higher basal sCLU level, and IR treatment further increased sCLU expression. However, sCLU was high in senescent IMR-90, but no further induction after IR treatment. In addition, Erk activity was also responded in a similar way in young, middle aged, and senescent IMR-90 after IR treatment (Fig. 4B). These results indicated that senescence-induced sCLU shares the same signaling pathway as DNA damage induced sCLU. Since the signaling pathway is already activated in senescent cells, there is not further increasing of sCLU level after IR treatment.

To further investigate the role of ATM in sCLU induction during senescence, we first tested if IGF-1/Src/MAPK/Egr-1 signaling pathway is intact in AT cells. AT fibroblasts, AT2052 and GM03487 (normal human fibroblasts from AT patient), immortalized ATM deficient fibroblast (ATM\(^{-/-}\)) and matched ATM proficient fibroblast (ATM\(^{+}\), the ATM\(^{-/-}\) fibroblast with exogenous introduced ATM), were treated with IGF-1 for indicated time, and the induction of expression sCLU was observed as expected in AT2052, GM03487, ATM\(^{-/-}\) and ATM\(^{+}\) cells (Fig. 4C-4E). And IR treatment only induces sCLU expression in ATM\(^{+}\) cells, but not in GM03487 and ATM\(^{-/-}\) cells (Data not shown). These results suggested that the IGF-1/Src/MAPK/Egr-1 signaling pathway is intact in AT cells, thus ATM regulation is upstream of IGF-1 in senescence-induced sCLU. To further prove the role of ATM in sCLU induction during senescence, we treated senescent IMR-90 with AAI, an inhibitor of both ATM and ATR. As expected, AAI inhibited CLU expression in senescent IMR-90 cells, and significantly decreased sCLU level in the medium (Fig. 5A). Furthermore, AAI treatment also decreased IGF-1 level in senescent IMR-90 cells (Fig. 5B).
5B). These results supported our model that ATM is not only a key regulator of RS/SIPS after DNA damage or telomere shortening, but also act upstream of IGF-1 in senescence induced sCLU (Fig. 6). Our results indicated that ATM mediates sCLU induction during senescence through regulation of IGF-1 level, hence modulated IGF-1R/MAPK/Egr-1 signaling pathway (Fig. 6).

4. sCLU protects cells from senescence.
We have established that cells will increase sCLU expression during senescence through ATM/IGF-1/MAPK pathway. sCLU is known to function as an extracellular chaperone and protects cells from apoptosis by binding to Bax and inhibiting its pro-apoptotic effect. Whether sCLU plays a role in cellular senescence is still not clear. To understand why sCLU is upregulated during senescence and what is its function in cellular senescence, we generated an inducible sCLU knockdown system by using a lentivirus vector, pTRIPZ-shCLU, that expresses a sCLU specific shRNA (shCLU) and knockdown sCLU only in the presence of doxycycline. While in absence of doxycycline, shCLU will not be expressed and sCLU level is not affected. This sCLU shRNA sequence targets specifically to sCLU, and does not affect nuclear clusterin (nCLU) (Fig. 7), a cytosolic form of clusterin that induces cell death when induced and translocated to the nucleus (4, 5). IMR-90 cells at early passage were first transduced with inducible shCLU vector (pTRIPZ-shCLU) containing lentivirus, then selected with puromycin to generated pool populations. The pTRIPZ-shCLU containing IMR-90 cells from the same selected pool clones were then cultured with or without doxycycline, and the population doubling and percentage of SA-β-gal positive cells were monitored alone time. In this way, the “age” difference of matched pair of selected pool population was minimized, and the start points of IMR-90 cells with or without sCLU knockdown were the same.

To investigate the effect of sCLU knockdown on senescence, we first confirmed the effect of sCLU shRNA using Western blot analysis (Fig. 7A8A). The induced sCLU shRNA effectively decreased sCLU expression in pTRIPZ-shCLU containing IMR-90 cells cultured with doxycycline, while sCLU level in cells cultured without doxycycline were not affected (Fig. 8A). We then monitored the population doubling and percentage of SA-β-gal positive cells in pTRIPZ-shCLU containing IMR-90 cells
continually cultured with or without doxycycline. Interestingly, the cells with sCLU knockdown showed decreased population doubling (Fig. 8A), suggesting that loss of sCLU expression sensitized IMR-90 cells to senescence. This is further confirmed by the result that the percentage of SA-β-gal positive cells in IMR-90 cells with sCLU knockdown increased about two fold in comparison with the cells without sCLU knockdown (Fig. 8B). To verify that these results were not affected by addition of doxycycline or lentivirus transduction, we generated pool clones using the same vector but contain a non-silencing shRNA (pTRIPZ-non silencing). As shown in Fig. 8C&D, the non-silencing shRNA had no effect on sCLU expression, and the population doubling and the percentage of SA-β-gal positive cells were no significant difference between the non-silencing shRNA containing IMR-90 cells cultured in presence or absence of doxycycline. These results suggested that the doxycycline and lentivirus had no obvious effect on cellular senescence, and confirmed that decreased sCLU expression is the cause of facilitated senescence in IMR-90 cells with induced sCLU knockdown. These results suggested that in addition to protect cells from apoptosis, sCLU also plays a role in protect cells from senescence (Fig. 6).

5. Senescent fibroblasts promote tumor cells growth.
To investigate the growth promotion effect of senescent fibroblasts, we labeled A549 cancer cells with RFP and selected out single clones. The RFP intensity reading of labeled A549 cells is well correlated with cell number (Data not Shown). Then we cultured same amount of RFP labeled A549 cells with young or senescent IMR-90 fibroblasts in 0.2% FBS medium and monitored cancer cell growth by reading RFP intensity. As shown in Fig. 9, A549 cells mixed with senescent fibroblasts grew much faster than cells mixed with young fibroblasts, while no significant growth in A549 cells alone. This result suggested that senescent fibroblasts indeed promote cancer cell growth. Further experiment will be performed using labeled prostate cancer cells, PC-3 and DU145 to test the effect of senescent fibroblasts on prostate cancer cell growth, and IGF-1 blocking antibody or IMR-90 with IGF-1 knockdown will be used to test the effect of fibroblasts secreted IGF-1 on growth promotion.

6. Prostate orthotopic cancer mouse model.
We have established a subcutaneous xenograft mouse model to test the effects of human fibroblasts on cancer cell growth. Our results shown that cancer cells co-injected with senescent fibroblasts grew faster than cancer cells co-injected with young fibroblasts. Two cancer cell lines, A549 and MDA-MB-231 were tested and senescent fibroblasts promoted both A549 and MDA-MB-231 cancer cells growth when co-injected into mice subcutaneously (Fig. 10).

To test the effect of senescent fibroblasts on prostate cancer cells, we first labeled prostate cancer cell lines, PC-3 and DU145 with luciferase reporter construct and
verified the expression of luciferase by measuring its activity using a luminometer. We then determined the minimal amount of PC-3 or DU145 needed to establish orthotopic prostate tumor in mice. For both PC-3 and DU145, 1 x 10^4 cells was chose to establish orthotopic tumor when co-injected with young/senescent fibroblasts, and this amount of cells is about the threshold that an orthotopic tumor can be established when injected alone.

We co-injected 1 x 10^4 PC-3 cells with 10 times more young/senescent fibroblasts into mouse prostate and monitored the tumor growth by measuring the BLI. As shown in Fig. 11A, the tumor formed by PC-3 cells co-injected with both young and senescent fibroblasts grew faster than the tumor formed by PC-3 cells alone. However, there was no significant difference between tumors formed by PC-3 cells with young or senescent co-injection. This indicated the co-injection of fibroblasts does benefit tumor growth, but senescent fibroblasts did not show additional tumor promoting effect. PC-3 cells form orthotopic prostate tumor much aggressive, and PC-3 cells lack tumor suppressor PTEN, which may interfere the signaling of IGF-1 and sCLU. Both A549 and MDA-MB-231 are PTEN positive and senescent fibroblasts showed significant tumor promoting effect in these cancer cells in vivo.

Thus, to further investigate whether senescent fibroblasts promote prostate tumor growth, we utilized another prostate cancer cell line, DU145, which, unlike PC-3, forms orthotopic tumor slower and contains tumor suppressor PTEN. As we determined previously, the threshold of DU145 cells to establish an orthotopic tumor is about the same as PC-3 cells, the growth of tumor formed by DU145 was much slower. We co-injected 1 x 10^4 DU145 cells with 1 x 10^5 young/senescent fibroblasts into mouse prostate. As shown in Fig. 11B, senescent fibroblasts showed some tumor promoting effect on DU145 cells, but young fibroblasts showed much more significant tumor promoting effect. These results again suggested
that fibroblasts do promote orthotopic tumor growth, while the effect of senescent fibroblast was not clear. Given that the cancer cells at orthotopic site had sufficient nutrition supplies from circulation, the growth promoting factors secrete by senescent fibroblasts was dispensable. But at subcutaneous site, cancer cells were in a harsh environment with limited nutrition supplies, the growth promoting factors provided by senescent fibroblasts gave the cancer cells essential advantage to survive and grow.

7. Senescent fibroblasts secreted sCLU might not play a role in its tumor promoting effect.
Since senescent fibroblast failed to show growth promoting effect in orthotopic tumors, we utilized A549 subcutaneous mouse model to test the effect of sCLU on tumor growth. To test this, we first generated a sCLU stable knockdown IMR-90 pool clones by transducing young IMR-90 cells with retrovirus that contains sCLU shRNA (shCLU) expression construct and selected with paramycin for one week. A control pool clone was also generated using the same vector that contains a non-silencing shRNA (scr). After both scr and shRNA IMR-90 cells were senescent, we co-injected them with A549 cells subcutaneously and monitor the tumor growth by measuring the tumor with caliper. As shown in Fig. 12A, the growth of A549 tumor was not affect by sCLU level in co-injected IMR-90 senescent cells. Since A549 itself expresses significant amount of sCLU, this might compensate the lost sCLU from senescent cells. To minimize this effect, we generated A549 sCLU knockdown clone and repressed more 90% percent of sCLU in these A549 cells (A549-shCLU). Then, A549-shCLU cells were co-injected into mice with scr or shCLU containing senescent IMR-90 cells. Surprisingly, remove of sCLU in both A549 and senescent IMR-90 cells did not decrease tumor growth (Fig. 12B). All together, these results indicated that sCLU from senescent fibroblasts might not be the factor that promotes tumor growth.

Key Research Accomplishments

- sCLU is induced during both replicative senescence (RS) and stress-induced premature senescence (SIPS), but not in oncogene induced senescence.
- sCLU is a very sensitive marker for oxidative stress. It can be induced by modulate oxidative stress.
- ATM is required for RS/SIPS induced sCLU expression, and ATM acts upstream of IGF-1/Scr/MAPK/Egr-1 signaling pathway through regulation of IGF-1 expression.
- sCLU protects cells from senescence. Cells with sCLU knockdown undergo senescence faster.
Reportable Outcomes

Publications:

Abstracts and presentations:
1. Luo X., Suzuki M., Goetz E., Zou Y. and Boothman D.A. 7th Annual Postdoctoral Research Symposium, University of Texas Southwestern Medical Center at Dallas, Dallas, TX, February, 2010.

Conclusion

Our current results have demonstrated that sCLU is induced during both replicative senescence and stress-induced premature senescence. This indicates that IGF-1/sCLU induction could happen in senescent cells generated from aging or therapeutic stresses. Our results also demonstrated that ATM acts upstream of IGF-1 to mediate sCLU induction during senescence, and ATM inhibitor, AAI, represses both IGF-1 and sCLU expression in senescent fibroblasts. This inhibitor would useful in our in vivo experiments to test the effect of senescent fibroblast secreted IGF-1 and sCLU. Our results also showed that sCLU protects cells from senescence, and cells with sCLU
knockdown undergo senescence faster, suggesting cells increase sCLU to counteract “aging”.

References


Appendices
ATM-dependent IGF-1 induction regulates secretory clusterin expression after DNA damage and in genetic instability

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Secretory clusterin (sCLU) is a stress-induced, pro-survival glycoprotein elevated in early-stage cancers, in particular in APC/Min-defective colon cancers. sCLU is upregulated after exposure to various cytotoxic agents, including ionizing radiation (IR), leading to a survival advantage. We found that stimulation of insulin-like growth factor-1 (IGF-1) and IGF-1R protein kinase signaling was required for sCLU induction after IR exposure. Here, we show that activation of Ataxia telangiectasia-mutated kinase (ATM) by endogenous or exogenous forms of DNA damage was required to relieve basal repression of IGF-1 transcription by the p53/NF-YA complex, leading to sCLU expression. Although p53 levels were stabilized and elevated after DNA damage, dissociation of NF-YA, and thereby p53, from the IGF-1 promoter resulted in IGF-1 induction, indicating that NF-YA was rate limiting. Cells with elevated endogenous DNA damage (deficient in H2AX, MDC1, NBS1, mTR or hMLH1) or cells exposed to DNA-damaging agents had elevated IGF-1 expression, resulting in activation of IGF-1R signaling and sCLU induction. In contrast, ATM-deficient cells were unable to induce sCLU after DNA damage. Our results integrate DNA damage resulting from genetic instability, IR, or chemotherapeutic agents, to ATM activation and abrogation of p53/NF-YA-mediated IGF-1 transcriptional repression, that induces IGF-1–sCLU expression. Elucidation of this pathway should uncover new mechanisms for cancer progression and reveal new targets for drug development to overcome resistance to therapy.

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Keywords: secretory clusterin; ATM; IGF-1; p53; ionizing radiation; genetic instability

Introduction

Prior to neoplastic transformation, initiated normal cells undergo a re-programming of inherent gene expression, permitting subsequent cancer promotion and progression. These changes typically result in upregulation of proteins that provide pro-survival signals and down-regulation of cell-death initiation events. One pro-survival pathway commonly and constitutively upregulated in human tumors is the insulin-like growth factor-1 (IGF-1)/IGF-1R tyrosine kinase signaling cascade (Ryan and Goss, 2008). We found that exposure of cells to IGF-1 induced secretory clusterin (sCLU) expression, and that IGF-1R signaling was essential for sCLU upregulation after IR exposure. In addition to IR, sCLU was induced after exposure to chemotherapeutic agents and ultraviolet (UV) radiation (Boothman et al., 1993; Miyake et al., 2000); however, the role of IGF-1/IGF-1R signaling in sCLU induction after these agents was not investigated. Although we noted that wild-type p53 repressed basal sCLU expression and partially inhibited sCLU upregulation after IR exposure (Criswell et al., 2003), a link between IGF-1R signaling and p53 suppression was not elucidated. The initiating signals from DNA damage to upregulation of IGF-1/IGF-1R signaling also remain undefined.

sCLU is a heavily glycosylated, 80 kDa secreted protein that functions in both an extra- and intracellular manner to promote cell survival. sCLU is synthesized as a 60-kDa precursor protein (psCLU) that is targeted to the ER. Within the ER, psCLU is cleaved approximately into half, linked together by five disulfide bonds, and is heavily glycosylated to form mature sCLU (80 kDa). sCLU production appears as two major protein forms by western blotting. The band at ~60 kDa corresponds to psCLU and represents the protein before maturation. The ~40-kDa smear corre-
sponds to one half of the mature sCLU at varying states of glycosylation (Jones and Jomary, 2002). Expression changes in psCLU and sCLU are always concordant, and both forms consistently match CLU promoter activity (Criswell et al., 2003), allowing any one of these forms to be monitored for changes in sCLU expression.

Outside the cell, sCLU acts as a molecular chaperone, binding to stress-induced unfolded proteins, lipids and amyloid, among other molecules, and works to clear cell debris after tissue trauma (de Silva et al., 1990; Boggs et al., 1996; Wilson and Easterbrook-Smith, 2000). Within the cell, sCLU can bind Bax and prevent its translocation to the mitochondria, thereby blocking apoptosis (Zhang et al., 2005). Due to its pro-survival functions, sCLU overexpression is partially responsible for increased resistance of cancer cells to various stresses, including doxorubicin, cisplatin and taxol (Miyake et al., 2000). 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). Aside from exogenous stress, sCLU is constitutively elevated in many early-stage cancers, during replicative senescence, and in Alzheimer’s disease (Chen et al., 2003). These observations strongly suggest that elevated sCLU levels in cancer, as well as induction of sCLU after cytotoxic agent exposures, may result in the consequent resistance of cancer cells to therapy, including cancer stem cells. Therefore, antisense constructs specific for sCLU (for example, OGX-011) were developed and are now in phase II/III clinical trials in combination with standard chemotherapies for prostate cancer (Chi et al., 2010). Thus, understanding sCLU regulation after DNA damage and in genetically unstable cells should lead to a better understanding of resistance to therapy and open avenues for improving cancer therapy.

Here, we show that p53 and NF-YA are necessary to suppress basal IGF-1 transcription by binding together at a single NF-Y consensus site within the IGF-1 promoter. Although p53 levels were stabilized and elevated after IR exposure, loss of p53/NF-YA binding from the IGF-1 promoter after IR exposure allows for IGF-1 induction to, in turn, activate IGF-1R signaling and sCLU induction. Interestingly, we noted that genetically unstable cells have heightened basal IGF-1 and sCLU levels and concomitant elevated Ataxia telangiectasia-mutated kinase (ATM) signaling, not unlike cells undergoing a DNA damage response. We found that ATM was the DNA damage sensor kinase responsible for IGF-1–sCLU upregulation after endogenous or exogenous DNA damage. Collectively, our findings show that the ATM–IGF-1–sCLU expression axis is an important pro-survival pathway activated in response to DNA damage, which has important implications in carcinogenesis, tumor progression and resistance in cancer therapies.

Results

p53 negatively regulates basal IGF-1 expression

p53 has an important and essential role in responding to cell stress by either activating or repressing gene expression, controlling checkpoint responses, and inducing apoptosis. Data from our laboratory suggested that p53 suppressed basal sCLU expression; however, sCLU was moderately induced after IR exposure, even though p53 was stabilized (Criswell et al., 2003). The mechanism of p53-mediated repression of basal sCLU expression was not explored. Since prior data suggested that IGF-1 signaling may regulate sCLU expression (Criswell et al., 2005) and p53 was regulated, through Mdm-2 expression, by IGF-1 (Mayo and Donner, 2001), we examined the ability of p53 to regulate IGF-1 transcription. To test this, the IGF-1 promoter fused to luciferase reporter (IGF-1–LUC) (Mittanck et al., 1997) was transfected into a series of genetically matched cell lines altered in functional p53 (Figure 1a). p53-null (PC3 and HCT116 p53−/−) and p53 knockdown (RKO7 shp53, HCT116:3-6 shp53, HBEC 3kt shp53) cells had higher basal IGF-1 promoter activity compared with matched WT p53-expressing cells (white bars; PC3 (stably expressing WT p53), RKO7 SCR, HCT116 SCR, HCT116:3-6 SCR and HBEC 3kt). Immortalized human bronchial epithelial (HBEC 3kt, WT p53) cells expressing mutant R273H p53 (mp53) also had elevated basal IGF-1 promoter activity. Similarly, IGF-1 mRNA and protein expression was higher in HCT116:3-6 shp53 compared with SCR cells (Figures 1b and c). These data suggested that p53 transcriptionally repressed basal IGF-1 in a dose-dependent manner, as complete loss of functional p53 allowed for greater IGF-1 expression than in cells where p53 was knocked down.

Since IGF-1R signaling was activated by IR exposure (Criswell et al., 2005), we examined whether IGF-1 was elevated after IR treatment, and if its expression was dependent on p53 status. In RKO7 SCR and shp53 cells, IGF-1 protein and promoter activity were induced in both cell lines after IR exposure. However, IGF-1 was induced to a significantly greater extent when p53 was knocked down (Figures 1d and e). These data strongly suggested that p53 transcriptionally repressed basal IGF-1 expression, while only partially blocking IR-induced IGF-1. These data also suggested that the p53 repression mechanism of IGF-1 (and sCLU) was fundamentally different than other p53 targets, such as Chk2, since Chk2 is further suppressed after IR exposure due to stabilization of p53 (Matsui et al., 2004).

p53 and NF-Y-mediated transcriptional regulation of IGF-1

Since IGF-1 was induced in WT p53 cells after IR exposure even though p53 was stabilized, we investigated the mechanism of p53 suppression, and whether this changed after IR. Interrogation of the human IGF-1 promoter for potential regulatory elements repressed by p53 led to the identification of a single NF-Y consensus site found at −438 bp in the IGF-1 promoter (Figure 2a). NF-Y sites are bound by NF-Y proteins, which are trimeric transcription factor complexes comprised of NF-YA, -YB and -YC proteins. NF-Y can interact with p53 to transcriptionally repress specific genes, including Cdc2 and Chk2 (Yun et al., 1999; Matsui et al., 2004).
To determine whether the NF-Y consensus site in the IGF-1 promoter was required for transcriptional repression by p53, we mutated the site (Figures 2a and b). Dramatically increased promoter activity in HCT116 cells was observed with the NF-Y mutant IGF-1 promoter (NF-Y MUT), similar to the activity of the WT IGF-1 promoter in HCT116 p53−/− cells (Figure 2b). Thus, p53 suppressed basal IGF-1 promoter activity through an NF-Y binding site in HCT116 cells.

To examine whether NF-Y alone could repress the IGF-1 promoter in the absence of p53, NF-YA was overexpressed in HCT116 cells. NF-YA overexpression
in HCT116 cells resulted in modest repression of the IGF-1 promoter; however, NF-YA was not able to suppress in the absence of p53 (HCT116 p53−/−, Figure 2c), consistent with studies showing that NF-YA mediates the interaction between p53 and DNA (Imbriano et al., 2005). Finally, chromatin immunoprecipitation analyses were performed to examine the binding of p53 and NF-YA, -YB or -YC to the IGF-1 promoter. After IR exposure, p53 protein levels were stabilized (Figure 2d); however, p53 and NF-YA binding to the IGF-1 promoter decreased. NF-YB and NF-YC binding increased 72 h after IR exposure (Figure 2e). Intriguingly, NF-YB binding to the IGF-1 promoter decreased 6 h after IR exposure; however, as IGF-1 and sCLU are most robustly induced 48–72 h after IR exposure, decreased binding at 6 h may not affect IGF-1 expression. As a positive control, increased p53 binding to the p21 promoter was noted after IR exposure (Figure 2e). These data suggest that IGF-1 induction after IR exposure in WT p53 cells was due to loss of p53 and NF-YA occupancy on the IGF-1 promoter, and thereby loss of repression. These data strongly suggested that NF-YA is rate limiting and p53 alone is not capable of repressing IGF-1.

p53 suppression of the IGF-1–sCLU pathway
To show that IGF-1 signaling was upstream of sCLU expression, cells were treated with AG1024, an IGF-1R tyrosine kinase inhibitor, before IR exposure. AG1024 blocked IR-induced CLU promoter activity in RKO7 shp53 cells, while having no effect on IGF-1 promoter induction (Figure 3a). The overall lower induction of IGF-1 and CLU promoter activity observed in the WT p53 RKO7 cells was probably due to the low serum conditions required for AG1024 efficacy. To determine if both IGF-1 and CLU expression were similarly regulated by p53, HCT116 and HCT116 p53−/− cells were transiently transfected with WT flag-tagged p53 cDNA (WT p53) and IGF-1– or CLU–LUC reporters before IR exposure. Basal IGF-1 and CLU promoter activities were significantly higher in HCT116 p53−/− cells compared with WT p53 cells (Figure 3b), consistent with previous results. Overexpression of non-lethal p53 levels partially blocked IR-induced IGF-1 and CLU promoter activities in both cell lines (Figure 3b). CLU and IGF-1 promoter activities and sCLU protein induction after IR exposure were also partially blocked by p53 in RKO7 cells (Supplementary Figures S1A–C). Since both IGF-1 and CLU were similarly regulated by p53, an inter-dependent relationship between the expression patterns of these two genes was revealed, showing that upstream IGF-1 expression directly controlled sCLU synthesis.

Induction of sCLU by DNA damage
Since IGF-1R signaling was required for sCLU induction after IR exposure, we examined whether this pathway was activated after other forms of DNA damage. In RKO7 cells, induction of both the intracellular, ~60-kDa precursor sCLU (psCLU) and the ~80-kDa mature sCLU (appearing as an ~40-kDa smear) were noted after exposure to IR, H2O2 or topoisomerase I or IIz poisons, topotecan (TPT) or VP16, respectively (Figures 4a and c). Induction of sCLU was inhibited by AG1024 pre-treatment (Figure 4a). IGF-1 expression and phosphorylation of ERK were observed after DNA damage (Figure 4b and Supplementary Figures S2A–C), consistent with IGF-1R–mitogen-activated protein kinase (MAPK) activation after IR exposure (Criswell et al., 2005). These data suggested a common DNA damage-induced pathway of IGF-1 induction that activated IGF-1R–MAPK signaling, leading to sCLU expression.

To determine whether DNA double-strand breaks (DSBs) were necessary for sCLU induction, cells were exposed to aphidicolin (Aph), a DNA polymerase
α inhibitor, before treatment with various DNA-damaging agents. Topotecan, a topoisomerase-I poison, causes DNA–protein crosslinks that are converted to DSBs during DNA replication, which are prevented by Aph (D’Arpa et al., 1990). Aph abrogated TPT-induced sCLU protein expression and CLU promoter activity in RKO7 cells (Figures 4c and d). In contrast, Aph did not affect sCLU induction for the agents that directly caused DSBs. These data suggested that DSBs were minimally sufficient to induce sCLU.

The IGF-1–sCLU expression axis is upregulated in genetically unstable cells

Noting that DSBs were minimally required for IGF-1–sCLU expression (Figures 4c and d) and that genetically unstable cells commonly exhibit constitutive DSBs, we examined whether genetic instability, in general, led to an increase in sCLU expression. Various genetically unstable cells were examined for IGF-1 and sCLU expression compared to stable, genetically matched cells (Figures 5a and b). Cells deficient in H2AX (H2AX−/−) have defective localization of BRCA1 and 53BP1, deficiencies in homologous recombination and persistent DSBs (Celeste et al., 2002). Elevated basal expression of sCLU was noted in H2AX−/− MEFs compared to genetically matched and stable, WT MEFs. Similarly, sCLU expression was constitutively elevated in MDC1−/− MEFs, which have defects in ATM recruitment (but not activation), leading to increased DSBs and genetic instability (Lou et al., 2006). sCLU expression was also elevated in fifth generation MEFs defective for the RNA component of mouse telomerase, mTR (Figure 5a); mTR−/− MEFs are genetically unstable due to a failure to extend telomeres, revealing DSBs that lead to chromosomal rearrangements and end-to-end fusions (Hao and Greider, 2004). Genetically unstable NBS1 hypomorphic MEFs (NBS1−/−) (Williams et al., 2002) and mismatch repair-deficient (MMR, hMLH1-deficient) HCT116 cells (Wagner et al., 2008) also exhibited elevated basal sCLU expression compared with their matched, genetically stable WT counterparts (Figure 5b).

We then examined whether IGF-1 signaling was elevated in cells with endogenous genetic instability. Elevated IGF-1 and CLU promoter activity was noted in H2AX−/− MEFs, compared with WT control cells (Figure 5c). Higher levels of intracellular IGF-1 in H2AX−/− MEFs were noted (Supplementary Figure S2D), with constitutively elevated basal phosphorylation of IGF-1R, AKT1 and ERK (Figure 5d and Supplementary Figure S2E). Exposure of both MEFs to exogenous IGF-1 induced phosphorylation of IGF-1R and its downstream target, AKT1 (Supplementary Figure S2E), confirming that IGF-1 signaling was intact in WT MEFs. Finally, AG1024 exposure reduced basal sCLU expression in H2AX−/−, MDC1−/− and mTR−/− MEFs (Figure 5e, and Supplementary Figures S2F and G), suggesting that IGF-1/IGF-1R signaling was constitutively active in unstable cells, leading to sCLU expression. These data strongly suggested that the same IGF-1/IGF-1R/MAPK/ERK signaling pathway that was activated after exogenous DNA damage was responsible for stimulating basal sCLU expression in genetically unstable cells.

sCLU induction following DNA damage is mediated by ATM

Since IGF-1–sCLU expression was elevated in response to DNA damage, we wanted to delineate the DNA damage sensor responsible for upregulating this expression axis. Since ATM and ATR signaling are activated in response to the DNA-damaging agents used, we suspected that these two PIKKs might be involved in IGF-1–sCLU expression. Genetically unstable MEFs were examined for upregulation of ATM signaling by

**Figure 4** IGF-1–sCLU is induced after exogenous DNA damage. (a) Serum-starved MCF-7 cells were pre-treated with 4 μM AG1024 or DMSO and then treated with IR (5 Gy), H2O2 (50 μM), VP16 (10 μM) or TPT (2.2 μM) for 5 h or DMSO (UT). Whole-cell extracts were collected 72 h later for immunoblotting. (b) MCF-7 cells were treated as in (a), media collected at 48 h, and IGF-1 levels assessed by ELISA. *Treated vs untreated. (c) MCF-7 cells were pre-treated with Aph (100 ng/ml or DMSO for 2 h before the agents mentioned in (a), and extracts harvested at 48 h for western blotting. (d) MCF-7 cells were transiently transfected with CLU-LUC and RSV-β-gal and treated as in (e). For (b, d), data were graphed as means ± s.d. *P≤0.05. RL, relative levels.
analyzing phosphorylation of H2AX (γ-H2AX) and auto-activation of ATM (phosphorylation of S1981). Neither H2AX−/− nor MDC1−/− MEFs showed γ-H2AX staining due to their known abrogation of this response (Lou et al., 2006), but had elevated basal pATM S1981 levels (Figures 5f and g). WT MEFs were responsive to IR exposure, showing elevated pATM S1981 and γ-H2AX staining (Figures 5f and g). Additionally, both pATM S1981 and γ-H2AX were elevated in RKO7 cells exposed to TPT, which were abrogated by APh pretreatment (Supplementary Figure S3A), ATR signaling, as determined by Chk1 phosphorylation on serines 317 and 345, was elevated in H2AX−, MDC1−, and mTR− deficient cells (Supplementary Figure S3B). These data suggested that both ATM and ATR signaling were elevated under the same conditions in which IGF-1–sCLU expression was upregulated.

The requirement for ATM or ATR activation in IGF-1/sCLU expression was examined by pre-treating cells with AAI, a dual ATM and ATR kinase inhibitor (see Bentle et al., 2006). AAI blocked pATM S1981 and γ-H2AX staining in irradiated MEF and human cells (Supplementary Figures S4A and B), and lowered basal sCLU protein expression in genetically unstable cells (Figure 6a). AAI also prevented sCLU induction in MCF-7 cells following IR or TPT exposure (Figure 6b). Thus, either ATM or ATR were possible DNA damage sensor kinases directing IGF-1–sCLU induction.

To delineate between ATM and ATR, sCLU induction was examined in genetically defined, SV-40 immortalized AT fibroblasts (ATM−/−/−) compared with genetically matched, ATM-reconstituted AT fibroblasts (ATM+/+/+). Although ATM−/− cells are genetically unstable (Kojis et al., 1991) and sensitive to IR (Ziv et al., 1997), sCLU basal expression was equal to ATM−corrected (ATM+/+) fibroblasts (Figures 6c and e, and Supplementary Figure S5A), presumably due to the presence of IGF-1 in the culture medium. Importantly,
ATM<sup>−/−</sup> fibroblasts did not induce sCLU after IR exposure, unlike ATM<sup>+/−</sup> fibroblasts (Figure 6c). Similarly, sCLU expression was not induced in GM2052 primary AT fibroblasts after IR exposure (Figure 6d). As an important control, both ATM<sup>−/−</sup> and ATM<sup>+/−</sup> fibroblasts were able to induce sCLU and showed phosphorylation of IGF-1R and AKT1 by exogenous IGF-1 (Supplementary Figures S5A and B), indicating that both cell lines were proficient in IGF-1–sCLU expression, but not in signaling from DNA damage to sCLU induction.

The possible role of ATR signaling in IGF-1–sCLU expression was examined by exposing ATM<sup>−/−</sup> and ATM<sup>+/−</sup> fibroblasts to UV (1 or 3 J/m²), which predominately activates ATR. Similar to IR, sCLU induction was observed only in ATM<sup>+/−</sup> cells after UV treatment (Figure 6e), probably due to ATR-dependent activation of ATM after UV exposure (Stiff et al., 2006). As a control, p53-mediated DNA damage-inducible expression of the human homolog of Mdm2 was noted in both ATM<sup>−/−</sup> and ATM<sup>+/−</sup> cells after UV exposure (Figure 6e) (Perry et al., 1993). Also, Chk1<sup>S137</sup> phosphorylation in UV-irradiated ATM<sup>−/−</sup> and ATM<sup>+/−</sup> cells was noted (Figure 6e and Supplementary Figure SSC), confirming ATR activation (Zhao and Piwnica-Worms, 2001). These data strongly suggested that ATM, and not ATR, was necessary for sCLU induction after IR exposure.

Since p53 and NF-YA binding to the IGF-1 promoter were lost after IR exposure in HCT116 cells, p53/NF-YA binding was examined in ATM<sup>−/−</sup> vs ATM<sup>+/−</sup> cells before and after IR. As in HCT116 cells (Figure 2e), both p53 and NF-YA were lost from the IGF-1 promoter after IR exposure in ATM<sup>−/−</sup>, although no change was detected in ATM<sup>+/−</sup> cells (Figure 6f). These data strongly suggested that ATM signaling was important for loss of NF-YA and p53 from the IGF-1 promoter after IR exposure, alterations required for IGF-1–sCLU upregulation in response to DNA damage.

Since ATM and ATM-activated proteins (for example, activated Chk2) phosphorylate p53 at serines 15 and 20 after IR exposure, we examined whether these post-translational modifications of p53 altered its ability to repress IGF-1 or sCLU. HCT116 p53<sup>−/−</sup> cells were transiently transfected with WT p53, or p53 constructs containing serine 15 or 20 mutated to non-phosphorylated alanine mutants (S15A, S20A), or the phospho-mimic aspartate (S15D) or glutamate (S20E). All serine 15 and 20 mutants repressed IGF-1 and CLU promoter activities similar to WT p53 (Supplementary Figures S5D and E), even though these are obvious direct and indirect targets of ATM kinase activity after DNA damage. As a positive control, the p21 promoter (el-Deiry et al., 1993) was induced by WT and to a lesser extent by S15A, S15E and S20A p53 mutants (Supplementary Figure S5F) (Dumaz and Meek, 1999). Mutation of S20 to glutamate enhanced p53-mediated activation of the p21 promoter over WT. Additionally, mutation of p53 at S20 blocked the ability of the DO1 p53 antibody to detect p53 (Supplementary Figure S5F, right). Thus, our data strongly suggested that activation of ATM signaling, and not ATR, was sufficient to induce IGF-1–sCLU expression, a common damage-inducible pro-survival
expression axis initiated by genotoxic stress or genetic instability.

Discussion

Genetic instability is a hallmark of cancer initiation, triggering changes that induce a switch from normal to uncontrolled growth. Engagement of survival pathways within these aberrant cells is important for cancer promotion and progression. Our results integrate a novel signaling pathway, initiating from ATM, that activates the pro-survival IGF-1–sCLU axis due to endogenous or exogenous DNA damage (Figure 6g). All cytotoxic agents that induced DSBs and activated ATM kinase led to IGF-1 induction and subsequent activation of IGF-1R, leading to sCLU expression. In normal cells, transient upregulation of IGF-1–sCLU expression after DNA damage is most likely beneficial, blocking apoptosis, providing a mitogenic stimulus and mobilizing glucose utilization to maintain cellular homeostasis until DNA repair is concluded. When this pathway is uncontrolled, however, like in genetically unstable cells or by loss of p53 function, IGF-1–sCLU upregulation may promote resistance of tumors to IR or chemotherapies and allow initiated cells to progress.

Several studies have suggested links between ATM, p53 and IGF-1 signaling, but this is the first report to show that p53, in conjunction with NF-YA, directly suppresses the IGF-1 promoter (Figures 6f and g). Other studies suggested that p53 suppressed IGF-1; however, no mechanistic data were provided (Bocchetta et al., 2008; Sulkowski et al., 2009). Additionally, ATM upregulates IGF-1R expression, and loss of IGF-1R, in turn, blocks ATM activation after IR exposure (Macaulay et al., 2001; Shahrabani-Gargir et al., 2004). These observations appear to explain the biphasic activation of IGF-1R–MAPK signaling observed in MCF-7 cells after IR exposure (Criswell et al., 2005). Initial IGF-1 signaling by ATM activation could trigger a more robust positive feedback loop, wherein IR-induced IGF-1 signaling activates AKT1. AKT1 stimulation then leads to p53 degradation via activation of Mdm2 (Leri et al., 1999), resulting in robust but delayed synthesis of IGF-1 and sCLU. This may also explain the slower kinetics of IGF-1 induction in RKO7 SCR cells, compared with RKO7 shp53 cells, because the RKO7 shp53 cells already have lowered p53 (Figure 1e).

NF-Y A–C proteins bind p53 and suppress transcription of target genes involved in cell cycle regulation, such as Cdc2 and Chk2 (Yun et al., 1999; Matsui et al., 2004). These genes are suppressed after DNA damage by p53 stabilization (Matsui et al., 2004). In contrast, IGF-1–sCLU expression was induced after low- (≥2 cGy) and high-dose IR exposures, even though p53 was stabilized (Klokov et al., 2004). This was concomitant with the simultaneous loss of NF-YA and p53 binding to the IGF-1 promoter. NF-YB and NF-YC binding increased after IR exposure (Figure 2e), consistent with their abilities to bind TFIIID independently of NF-YA and promote transcription (Bellorini et al., 1997). Even though the mechanism of IGF-1 suppression by p53 and NF-YA is different from other known targets, it is not incongruous with the known functions of the NF-Y subunits.

Since we uncovered a novel mechanism of p53/NF-YA-mediated suppression of IGF-1 transcription, we propose several possibilities for this loss of suppression after IR exposure, including post-translational modification of NF-YA and/or p53. Cdk2 phosphorylates NF-YA, and mutation of these sites blocked its ability to bind DNA (Yun et al., 2003). We propose a model whereby Cdk2 promotes NF-YA phosphorylation in the basal state, enhancing p53/NF-YA binding to DNA and repressing IGF-1 promoter transactivation. After IR exposure, ATM-mediated p53 stabilization promotes p21 expression. p21, in turn, suppresses Cdk2, blocking NF-YA phosphorylation and releasing NF-YA from the IGF-1 promoter (Figures 2e and 6f). Additionally, acetylation, phosphorylation or other post-translational modifications of p53 may have an impact on its ability to bind NF-YA and repress IGF-1 transcription. Studies to elucidate the roles of NF-YA and p53 post-translational modifications are ongoing. Collectively, our data indicate that ATM signaling results in NF-YA modification to prevent or reverse its binding to the IGF-1 promoter, suggesting that NF-YA is rate-limiting and cells retain the ability to induce IGF-1 expression and downstream sCLU production after IR exposure, even though p53 is stabilized.

p53 null cells, or cells with p53 knockdown, induced IGF-1–sCLU to a much greater extent after IR exposure compared with WT p53 cells. These data suggest that a positive transcription factor, in addition to loss of p53/NF-YA from the IGF-1 promoter, is required for IGF-1 induction. There are several candidate factors, including NF-YB/YC itself, AIB1 or TGFβ1 signaling, that may regulate IGF-1 expression. NF-Y is a known IR-activated transcription factor (Peng et al., 2007), and NF-YB/YC complexes may act as positive transcription factors for IGF-1 induction after IR exposure, consistent with increased NF-YB/YC binding to the IGF-1 promoter (Figure 2e). On the other hand, levels of the steroid receptor coactivator, AIB1, are directly correlated with IGF-1 expression (Wang et al., 2000). AIB1 binds to the IGF-1 promoter with AP-1 (Yan et al., 2006), suggesting that changes in these complexes may regulate IGF-1 expression after IR exposure. Finally, TGFβ1 induces sCLU (Jin and Howe, 1997) and TGFβ1 signaling may be activated after IR exposure (Andarawewa et al., 2007), TGFβ1 signaling may be involved in IR-induced IGF-1 expression. Current studies are ongoing in our lab to elucidate the positive factors in induction of IGF-1 after DNA damaging agents.

In conclusion, we elucidated the complete signaling pathway from DNA damage to sCLU expression. By determining the signaling pathways involved in IGF-1–sCLU upregulation, important targets for anti-tumor therapy emerge, as well as the possibility of using IGF-1 and sCLU as markers for genetic instability and cancer progression.
Materials and methods

Cell treatments

Cells were plated overnight, treated with TPT, H$_2$O$_2$ or VP16 (Sigma, St Louis, MO, USA) for 5 h at the indicated doses, and medium replaced. Pre-treatment with Aph (Sigma) was done for 2 h before treatment. For IGF-1 (R&D Systems, Minneapolis, MN, USA) and AG1024 treatments (EMD Chemicals, Gibbstown, NJ, USA), cells were serum-starved (0.5% FBS) overnight, and exposed to IGF-1 or AG1024 at the indicated doses. A 15Cs Mark I-68 irradiator (JL Shepherd and Associates, San Fernando, CA, USA) (3.87 Gy/min) was used for IR exposures. Mock and DMSO-treated cells were used as controls (UT).

Plasmids and site-directed mutagenesis

The IGF-1 promoter fused to luciferase was from Dr P Rotwein (Mittancck et al., 1997). The CLU promoter fused to luciferase was previously described (Criswell et al., 2005). Flag-tagged CMV-p53 cDNA was created by subcloning p53 cDNA into the pcDNA3.1-N-term-Flag construct. NF-YA cDNA was provided by Dr R Mantovani (Mantovani et al., 1994). Site-directed mutagenesis of p53 cDNAs and the IGF-1 promoter was performed using PCR-based mutagenesis, detailed in the Supplementary Material.

Luciferase reporter assays

Cells were transiently transfected with various promoter luciferase constructs as indicated, and with RSV-β-gal as a transfection control, using Fugene 6 (Roche, Indianapolis, IN, USA). Treatments, where indicated, were performed 24 h after transfection. Luciferase activities were analyzed using Luciferase Assay Reagent (Promega, Madison, WI, USA). β-Galactosidase activity was determined using Galacto-Star reagent (Life Technologies, Carlsbad, CA, USA).

Enzyme-linked immunosorbent assays

IGF-1 was detected using capture and detection antibodies, MAB291 and BAF291 (R&D Systems). Samples were normalized by cell number.

Flow cytometry

Cells were fixed with 1% formaldehyde, permeabilized with 100% ethanol, stained with pATMS1981 (Rockland, Gilbertsville, PA, USA), γ-H2AX (Millipore, Billerica, MA, USA), and IGF-1 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) antibodies and FITC-tagged secondary antibodies (Life Technologies), and counterstained with propidium iodide. Data were graphed as percent cells staining positive ± s.d.

Chromatin immunoprecipitation

Cells were fixed in 1% formaldehyde, sonicated to shear chromatin, and incubated overnight with antibodies against p53, NF-YA, NF-YB and NF-YC (Santa Cruz). Protein A/G beads were added the next day, then washed extensively. Chromatin was eluted from beads, cross-links reversed and DNA purified. Primers are listed in Supplementary Material.

Western blotting

Western blotting was described previously (Criswell et al., 2005). GAPDH, β-actin or α-tubulin was probed as loading controls. Relative expression was calculated from X-ray films using Image J, comparing the relative density of experimental conditions with a loading control. Control values were set to 1.

Statistics

Statistics were calculated using paired Student’s t-tests (n≥ 3), and each experiment was performed at least three times.

Conflict of interest

The authors declare no conflict of interest.

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Low dose IR-induced IGF-1-sCLU expression: a p53-repressed, TGFβ1-induced expression cascade that interferes with TGFβ1 signaling to confer survival

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Abstract

To better understand tissue responses after low IR doses, we generated a reporter system using human clusterin promoter fused to firefly luciferase (hCLUp-Luc). Secretory clusterin (sCLU), an extra-cellular molecular chaperone, induced by low doses of cytotoxic agents, clears cell debris promoting survival. Low dose IR (>2 cGy) exposure induced hCLUp-Luc activity with peak levels at 96 h, consistent with endogenous sCLU levels. As doses increased (>1 Gy), sCLU induction amplitudes increased and time to peak response decreased. sCLU expression was stimulated by IGF-1, but suppressed by p53. Responses in transgenic hCLUp-Luc reporter mice after low IR doses showed that specific tissues (i.e., colon, spleen, mammary, thymus, bone marrow) of female mice induced hCLUp-Luc activity more than male mice after whole body ≥10 cGy. Tissue-specific, non-linear dose- and time-responses of hCLUp-Luc and endogenous sCLU levels were noted. Colon maintained homeostatic balance after 10 cGy. Bone marrow responded with delayed, but prolonged and elevated expression. Intraperitoneal administration of the α-TGFß1 (1D11) antibody, but not a control antibody (13C4), immediately following IR exposure abrogated CLU induction responses. Induction in vivo also correlated with Smad signaling via activated TGFß1 after IR. Mechanistically, media with elevated sCLU levels suppressed signaling, blocked apoptosis and increased survival of TGFß1-exposed tumor or normal cells. Thus, sCLU is a TGF-ß1-induced pro-survival, potential bystander factor expressed in certain exposed tissues that, in turn, abrogates TGFß1 signaling and may promote wound healing and likely contributes to a pro-tumor growth microenvironment.
Introduction

DNA damage- and senescence-induced secreted proteins (i.e., an ‘induced secretome’) have been identified as an important response to low doses of ionizing radiation (IR) (Arufe et al 2011, Freund et al 2011, Rodier et al 2011). Identifying low dose IR-responsive expression pathways that ultimately result in secreted proteins that could have biological impacts on exposed, as well as non-exposed, cells would strongly suggest that not all cells have to be irradiated for entire tissues to respond \textit{in vivo}. To date, few pathways activated \textit{in vivo} in response to low doses of IR have been elucidated.

Recently, we delineated a unique pathway of regulation of insulin-like growth factor 1 (IGF-1) expression in response to low doses of IR \textit{in vitro} (Criswell et al 2003, Criswell et al 2005, Goetz et al 2011). We showed that IGF-1 was suppressed by p53/NF-YA complexes in its basal state at one unique NF-Y regulatory site in the IGF-1 promoter, whose binding was lost after low dose IR exposures in an ATM-dependent manner in cells deficient in p53 function, resulting in secretory clusterin (sCLU) expression. Regulation of IGF-1 expression, and thus sCLU, was rate-limited by p21 in an ill-defined ATM-dependent modification of NF-YA, even though p53 stabilization and protein induction occurred (Goetz et al 2011). IGF-1-sCLU expression was linked to cytoprotection of irradiated cells \textit{in vitro} and tissues \textit{in vivo}, however, the underlying mechanisms of cytoprotection are unknown (Cardona-Gomez et al 2001).

sCLU is a known low dose IR-inducible secreted protein that is regulated by IGF-1 signaling and can confer an ‘adaptive response’ (Criswell et al 2003, Criswell et al 2005). Since numerous cancers constitutively over-express sCLU as a pro-survival and pro-growth factor, strategies using antisense oligomers (Chi et al, Zoubeidi et al) or siRNA nanoparticles (Sutton et al 2006) have been developed to enhance chemo- and radio-therapies against human prostate and lung cancers. sCLU can bind and sequester BAX in the endoplasmic reticulum (ER) of cells, thereby preventing drug- and radiation-induced apoptosis (Araki et al 2005, Shannan et al 2006, Zhang et al 2005). Thus, low dose IR-inducible IGF-1-sCLU expression from cells \textit{in vitro} or in tissues \textit{in vivo} could afford significant cytoprotective bystander functions.

Since we previously linked IGF-1 signaling to expression of pro-survival sCLU levels, we hypothesized that expression of the IGF-1-sCLU axis would: (i) allow development of a potent and ultrasensitive biodosimeter of live cells using the human clusterin (CLU) promoter linked to
firefly luciferase (hCLUp-Luc) expression for bioluminescence imaging (BLI); BLI allows for non-invasive temporal quantitative imaging of cells and tissue in real time and greatly increases detection sensitivities in vitro and in vivo; and (ii) result in expression of a secreted protein expression axis that would have significant pro-survival bystander effects in vivo.

Here, we demonstrate that cells in vitro and transgenic mice in vivo containing the hCLUp-Luc reporter can be used as an extremely sensitive and potentially important reporter system to repeatedly image responses of irradiated live cells and tissues. We present compelling evidence that sCLU expression in specific radiation-sensitive tissues in vivo is controlled by low dose IR activation of TGFβ1. The IGF-1-sCLU expression axis is long-lived and extremely responsive to low doses of IR, as well as to other cytotoxic agents (Goetz et al 2011), making it ideal for future use as a ‘biomarker’ for biological responses to low dose IR exposures. The exogenous reporter responses matched endogenous sCLU protein expression in dose-response and temporal kinetics, and tissues differed in dose- and time-responses to low (i.e., 2 cGy) versus higher IR doses. Importantly, induction of sCLU at low doses of IR, from 1-100 cGy, were linear for cells in vitro and tissue in vivo, however, expression of sCLU could not be extrapolated from high doses, and non-linear responses were clearly indicated at doses >1 Gy. Finally, sCLU induction is part of a TGFβ1-induced negative feedback regulatory bystander loop that is induced by TGFβ1, but in turn, can suppress TGFβ1 signaling and promote survival, creating a microenvironment that may ultimately promote tumor growth.

Materials and Methods

Chemicals and plasmids. AG1024 (IGF-1R tyrosine kinase inhibitor) was obtained from EMD Chemicals (Gibbstown, NJ). IGF-1 was obtained from R&D Systems (Minneapolis, MN). TGFβ1 and ultrapure luciferin were obtained from the Sigma/Aldrich Chemical Co. (St. Louis, MO), and used in luciferase assay reagent (LAR) assays (Promega, Madison, WI). The human 1403 bp CLU promoter fused to luciferase (hCLUp-Luc) was previously described (Criswell et al 2003, Criswell et al 2005, Klokov et al 2004). Flag-tagged CMV-p53 cDNA was created by subcloning p53 cDNA into the pcDNA3.1-N-term-Flag construct. Constitutive-active PTEN (PTEN CA) and kinase-deficient ATK1 (AKT KD) were obtained from Dr. Lindsey Mayo (Indiana University).
**Antibodies and immunoblotting.** Antibodies specific to mouse (M18) and human (B5) sCLU, phosphorylated Smad3 (p-Smad3) or total Smad3 (t-Smad3), γ-H2AX, p53 and α-tubulin were purchased from Santa Cruz. Antibodies for GAPDH and α-tubulin, were used for loading. Immunoblotting was performed as described (Criswell et al 2005) and relative protein levels were quantified from x-ray films using NIH image J software as described (Criswell et al 2005). α-TGF-β1 murine monoclonal antibody (1D11) that neutralizes the three TGF-β isoforms (Dasch et al 1989), and an isotype-matched IgG1 monoclonal antibody (13C4, raised against Shigella toxin) were provided by Genzyme Corporation (Framingham, MA).

**Cell lines, treatments and survival assays.** Human MCF-7 breast cancer cells and a stably transfected 1403 MCF-7 clone (MCF-7 cells containing stably integrated 1403 bp CLU promoter fused to firefly luciferase) were cultured in DMEM (BioWhittacker; Walkersville, MA) containing 10% fetal bovine serum (HyClone; Utah, USA). Human HCT116 cells were grown as described (Li et al 2008, Wagner et al 2008) in 10% FBS-DMEM. Human mammary epithelial cells (HMECs), life-extended using hTERT and CD4 over-expression, were kindly obtained from Dr. David Euhus (UT Southwestern). All cells and stable derivatives were maintained at 37 °C at 5% CO₂-95% air. For TGFβ1 or IGF-1 treatments, cells were serum-starved (0.5% FBS) overnight, and exposed to IGF-1 or TGFβ1 at the indicated doses in normal serum-DMEM. Cell irradiations were performed using a JL Shepherd ¹³⁷Cs Mark I-68 irradiator (3.87 Gy/min) with appropriate shielding. All cells were free from mycoplasma infection.

**Transfections and luciferase assays.** Cells were transiently transfected with hIGF-1p-Luc or hCLUp-Luc reporter constructs and RSV-β-gal as a transfection control using Fugene 6 (Roche) (Goetz et al 2011, Trougakos et al 2009a). Treatments, where indicated, were performed 24 h after transfection. Luciferase activities were analyzed using LAR (Promega, Madison, WI). β-Galactosidase activity was determined using Galacto-Star reagent (Life Technologies, Carlsbad, CA). All experiments were normalized for protein amounts using Bradford assays (Bio-Rad). For stable hCLUp-Luc MCF-7 cells, cells were co-transfected with the hCLUp-Luc construct together with 2-fold excess pcDNA-3 that contained a G418-
Cells were then selected with G418 and resistant clones isolated as described (Criswell et al 2005). Isolates were then treated with IR, other cytotoxic agents (Criswell et al 2005, Goetz et al 2011) or TGFβ1 and analyzed for hCLUp-Luc reporter activities, as well as endogenous sCLU expression. A clone with identical hCLUp-Luc activities monitored by luciferase activities (Criswell et al 2005, Goetz et al 2011) and endogenous sCLU protein expression by Western blotting, was selected and examined for low dose IR induction. HCT116 and HMEC cells were transiently knocked down for sCLU expression using an siRNA-sCLU directed to the coding sequence for the CLU mRNA leader peptide sequence (Goetz et al 2011). siRNA-nonsense/scrambled (shRNA-Scr) was used as a control. Knockdown of sCLU levels were confirmed by Western blotting using α-tubulin as a control.

Bioluminescence Imaging (BLI) in vitro. BLI imaging was described (Klokov et al 2004). Suspensions of log-phase MCF-7 hCLUp-Luc cells were mock- or IR-treated with various doses of IR. After IR, cells were seeded onto 10 cm or 96-well black-coated dishes (Corning Life Sciences, Acton, MA) to eliminate light scatter during imaging, and allowed to grow as needed. Initially, optimization of imaging of trypsinized and pelleted cells was performed to obtain the best signal-to-noise ratio for detecting hCLU promoter activity, since high density suspensions may inhibit luciferase light emission (Supplemental Figure 1). Cells were harvested by trypsinization 72 h after IR treatment, and cell suspensions of various concentrations were made in LAR (Promega). Cell suspensions (~10^7) were added to wells of a 96-well plate and imaged immediately. For repeated imaging of cells on different days, we also optimized imaging of luciferase activity in intact MCF-7 hCLUp-Luc cell monolayers. Experiments were performed at various times post-IR (Criswell et al 2005, Klokov et al 2004) by adding LAR to cells, mounting plates in a light-tight box using an ultrasensitive CCD camera chilled with liquid N₂ (Xenogen Vivovision IVIS Lumina Imaging system). Since integration times for CLU promoter activity after very low IR doses exceeded conventional integration times for CCD camera BLI (~33 msecs), all images contained random cosmic ray events, resulting in high valued pixel intensities. To eliminate cosmic ray contamination, we developed and implemented a correction algorithm using an adaptive median filtering program for pixel substitution (Supplemental Figure 2). The code was written using MatLab, version 6.5 software.
Photomicrographs were processed to generate overlay colored images using Matlab software and gray scale values (GSVs) were calculated and background subtracted using NIH Image J software.

**Human CLU promoter-luciferase (hCLUp-Luc) transgenic mice.** A 1403 bp promoter region of the human CLU gene was amplified using the Elongase enzyme (Gibco) by PCR and the following primers: 5'-GATCCATTCCCGATTCTCATCG-3' and 5'-GCGTTGTGGGCACTGGGAG-3'. The insert was then cloned into the Smal site of pA3luc (a generous gift from Dr. R. Pestell, Thomas Jefferson University) and sequenced. hCLUp-Luc was used to generate the stable 1403 MCF-7 cell line (Criswell et al 2005). To generate transgenic mice, the hCLUp-Luc fragment (~3.4 kb) was excised using BamHI restriction, purified, sequenced and submitted to the CWRU transgenic animal facility, where it was microinjected into fertilized mouse 129/FVBN oocytes and ~300 fertilized oocytes were implanted into FVBN female mice. Tail clippings from offspring under three weeks of age were analyzed using PCR for the presence of 1403 hCLUp-Luc construct using the following primers: 5'-GCCTCCCAAAGTGCTAGGATTACA-3' and 5'-GAGTATCCTGTGGCCTGTGATTA-3'. Male and female hCLUp-Luc mice were analyzed, but female mice were investigated in more depth due to their higher hCLUp-luc activity in specific organs after IR exposure.

**Bioluminescence imaging (BLI) in vivo and ex vivo.** Transgenic hCLUp-Luc reporter mice were mock-irradiated or treated with whole body γ-irradiation (0.02 - 5 Gy) using a Shepard Mark Irradiator equipped with a $^{137}$Cs source at dose rates: 3.88 Gy/min (for doses >0.5 Gy) or 0.42 Gy/min (for doses <0.5 Gy). Mice (three/group) were imaged using BLI (Klokov et al 2004). Briefly, male or female FVBN mice were anesthetized using isoflurane, hair was removed by mild shaving, and 2.5-3.0 mg D-luciferin was intraperitoneally (i.p.) administered. BLI images of mice were captured 10-30 mins later using a liquid $N_2$-cooled Xenogen Vivovision IVIS Lumina Imager for 30 sec to 2 mins. After whole body imaging, mice were sacrificed and tissues extracted and imaged ex vivo using BLI. For time-course and dose-response BLI ex vivo imaging studies, transgenic female (~18 gram) hCLUp-Luc reporter FVBN mice (3/group) were used, at least in duplicate, for each datum point. Data from several
different experiments performed at different times and doses were compiled. Results were highly consistent within and between experiments, with a minimal n=6.

**Western blot analyses of irradiated tissues.** Mice (3/group) were treated with whole body IR at doses of 0.02 to 1 Gy. Tissues (colon/small intestine, bone marrow, muscle, spleen, and lung) were extracted 24 or 72 h later and homogenized (Klokov et al 2004). Tissue homogenates were centrifuged (5,000 X g, 15 min, 4°C) and protein samples diluted to 2 µg/ml. Extracts were combined from each of 3 mice/group. Proteins were then analyzed by Western blotting and blots are representative of three experiments with similar results.

**Flow cytometry.** Cells were fixed with 1% formaldehyde, counterstained with propidium iodide (PI) and analyzed for cell cycle changes by FACS using a Coulter Epics XL flow cytometer (Beckman Coulter Electronics; Miami, FL). Data were analyzed using ModFit LT, version 2.0 software (Verify Software House; Topsham, ME) (Criswell et al 2003, Criswell et al 2005).

**Statistical analyses.** All experiments were independently performed at least three times in duplicate, unless otherwise indicated. Student’s t tests and ANOVA power analyses were performed with the aid of Dr. Jin Xie, Biostatistics Core, Simmons Comprehensive Cancer Center.

**Results**

**hCLUp-Luc, a low dose IR-sensitive reporter construct.** We hypothesized that the human clusterin promoter linked to a firefly luciferase reporter (hCLUp-Luc) would be a sensitive reporter system capable of responding to very low doses of IR. To generate such a ‘biodosimeter’, a 1403 bp hCLU promoter region was cloned upstream from the firefly luciferase reporter, creating hCLUp-Luc. hCLUp-Luc was stably integrated into MCF-7 breast cancer cells, and BLI used to quantify induction. BLI was compared to standard luciferase assays. Responses of cells with integrated hCLUp-Luc directly mimicked endogenous gene expression in dose-response and time-course studies as reported in MCF-7 cells (Criswell et al
Responses of stably integrated hCLUp-Luc MCF-7 cells were identical to various human and mouse cells transiently transfected with hCLUp-Luc to characterize time-course and dose-response expression using BLI or standard luminometer assessments.

We first optimized BLI of hCLUp-Luc expression from irradiated (0.02 – 5.0 Gy) stably integrated MCF-7 cells for increased signal/noise ratios, compensating for cosmic ray background using repeated measurements (Supplemental Figures 1A, 1B and 2A-2D). We then explored expression in dose-response and time-course studies (Figure 1). Log-phase MCF-7 cells were irradiated with various IR doses (0, 0.2, 0.5 1.0 and 5.0 Gy) and induction assessed 72 and 96 h (Figures 1A, 1B at 96 h) later using BLI. Significant hCLUp-Luc promoter activity was noted after 20 cGy, and expression dramatically increased with increasing IR doses. Linear expression with dose (r=0.987) was noted with exposures between 20 to 100 cGy, but hCLUp-Luc reporter activity and sCLU protein expression responses reached plateau levels at doses >1 Gy (Figures 1B and 1B insert); exogenous reporter and endogenous sCLU protein expression were consistently expressed with increasing doses of IR (Figure 1B); in insert, psCLU and sCLU represent related immature and mature sCLU protein forms, respectively, as described (Goetz et al 2011). In time-course studies, exposure of MCF-7 cells with 10 cGy caused >2-fold induction of sCLU at 96 h. As cells were exposed to higher doses of IR (up to 5 Gy), hCLUp-Luc promoter induction responses were significantly faster, with ~3-fold induction, 24 h after 5 Gy (Figure 1D). Thus, both the magnitude and temporal kinetics of CLU induction were directly proportional to the dose of IR to log-phase MCF-7 cells, consistent with expression of the endogenous CLU gene (Criswell et al 2003, Criswell et al 2005). Similar responses were noted in all mouse and human cells examined, even when transient transfections were used instead of stable clones (Criswell et al 2003, Criswell et al 2005). In general, expression of hCLUp-Luc and sCLU protein expression was linear with dose up to 1.0 Gy (r=0.987, Figure 1B), but plateaued with higher doses.

p53 represses hCLUp-Luc promoter activity. Wild-type p53 (wtp53) HCT116 cells transiently transfected with the hCLUp-Luc promoter reporter and imaged using BLI showed significantly less hCLUp-Luc activity compared to isogenic p53-/- HCT116 cells, where p53 was
soma
tically knocked out (Figure 2A) as previously found for endogenous sCLU (Criswell et al 2003, Criswell et al 2005). p53 function can also be affected by alterations in AKT or PTEN, whose activities enhance or prevent, respectively, the efficacy of Mdm2 to degrade p53 (Araki et al 2010). Alterations that compromised p53 function (e.g., PTEN CA or E6 over-expression) led to elevated hCLUp-Luc activities, whereas conditions that enhanced p53 suppressor activity (i.e., down-regulation of AKT with AKT KD expression or forced over-expression of p53) repressed hCLUp-Luc activities in stable hCLUp-Luc MCF-7 cells (Figure 2B). Similar responses were noted in HCT116 cells using luminometer assessments (Supplemental Figures 3, 4).

**Regulation by IGF-1.** We reported that CLU promoter transactivation and sCLU expression were regulated upstream by IGF-1R signaling in p53-compromised cells, such as in MCF-7 cells that express endogenously elevated Mdm-2 levels (Criswell et al 2005). We confirmed that IGF-1 and CLU promoter activities correlated well in temporal expression kinetics after IR (0.1 Gy) using BLI (Figure 2C). Although the response from the hIGF-1p-Luc reporter was slightly faster than hCLUp-Luc, both promoter reporters were dramatically increased in response to low doses of IR. Similar data were found by standard luminometer measurements using protein levels or RSV-ß-gal expression normalized (Criswell et al 2005). In separate studies, addition of AG1024 dramatically suppressed CLU promoter and sCLU protein expression, while IGF-1 promoter and ligand expression were not affected (Goetz et al 2011).

**A low dose IR-sensitive transgenic hCLUp-Luc promoter mouse.** We then generated a transgenic hCLUp-Luc reporter mouse in an FVB/N genetic background and examined the responses of age (10 week)- and weight (18 gram)-matched male and female mice after exposure to whole body low doses of IR (e.g., 0.1 Gy). Irradiation of female versus male mice induced BLI expression in specific internal organs of both genders at 24 h. Greater responses were, however, routinely noted in female *versus* male mice (Figure 3A). Increased expression of hCLUp-Luc activity in female mice may be related to the known testosterone-repression of this gene; the CLU gene is also referred to as testosterone-repressed prostate message-2 (TRPM-2) (Trougakos et al 2009a). *Ex vivo* imaging of internal organs of female mice demonstrated dramatic induction of hCLUp-Luc promoter activities in bone marrow, spleen,
colon, thymus (Figure 3B) and mammary glands (not shown). In contrast, imaging of muscle, heart, liver or pancreas did not demonstrate significant hCLUp-Luc activities by BLI at any time. BLI or ex vivo imaging of control non-irradiated mice or tissue showed little hCLUp-Luc expression. Importantly, even though all mouse tissues express functional wt53, sCLU was noted.

**Induction of sCLU in vivo is mediated by TGFβ1.** Since sCLU can also be induced by activated TGFβ1 (Jin and Howe 1999), and low dose IR exposure can stimulate TGFβ1 (Nguyen et al 2011), we examined whether sCLU responses in vivo by hCLUp-Luc reporter and endogenous sCLU induction were prevented by intraperitoneal administration of a α-TGFβ1 (1D11, 10 mg/Kg) versus control IgG antibody (13C4, 10 mg/kg) (Figure 3C); the 1D11 antibody neutralizes all three forms of TGFβ (Dasch et al 1989). Both endogenous sCLU, as well as hCLUp-Luc reporter, responses were suppressed in colon, bone marrow, spleen and mammary gland tissue by injected α-TGFβ1 antibody, whereas the control 13C4 isotype antibody had no affect (Figure 3C). Data in Figure 3C represent a composite of experiments in which endogenous sCLU induction by Western blotting and hCLUp-Luc activities were simultaneously monitored.

**IGF-1-sCLU induction in vivo is due to activated TGFβ1 signaling in response to low doses of IR.** Since p53 represses IGF-1-sCLU expression after IR, but not after TGFβ1 (Doi et al 2011), we suspected that induction of hCLUp-Luc in vivo in transgenic mice was due to activation of TGFβ1 signaling (whole body irradiation (0.1 Gy) of female FVBN mice) (Figures 4A-4F). The most responsive tissues within female hCLUp-Luc reporter mice were colon and bone marrow, where temporal induction was monitored after 0.1 vs. 1.0 Gy (Figures 4A, 4B). Bone marrow and colon tissue responses differed in their temporal induction and longevity of CLU expression after 0.1 Gy, where colon hCLUp-Luc reporter activities and sCLU protein expression peaked at 24 h, but waned by 48 h (Figures 4A, 4E). In contrast, bone marrow sCLU responses were significantly delayed in comparison, showing peak levels 72 h after 0.1 Gy, and remained elevated for at least 96 h post-treatment (Figures 4B, 4F). CLU induction in bone marrow was more rapid after a higher dose (1.0 Gy) of IR (Figures 4B and 4F), and hCLUp-Luc reporter activities and endogenous sCLU expression levels were linear (r=0.965).
with IR doses between 2-10 cGy at 72 h (Figures 4D, 4F). At doses >0.1 to 1.0 Gy, sCLU induction levels reached plateau levels and were non-linear with IR dose (Figure 4D), as previously reported for endogenous sCLU protein expression (24, 32). Overall, sCLU induction correlated with the sensitivities of internal organs (bone marrow, spleen, thymus, mammary glands and colon) to whole body IR exposure. As TGFβ1 signaling appeared to be involved in CLU gene and sCLU expression responses (Figure 3C), Western analyses of mouse colon (Figure 4E) and bone marrow (Figure 4F) confirmed activation of Smad signaling (elevated phospho-Smad3/total Smad3 levels) with delayed kinetics consistent with sCLU expression; note that in Figures 4E and 4F, psCLU is the ~60 kDa precursor form of mature sCLU protein (~40 kDa) that correlated well with CLU promoter activation and mRNA expression after low doses of IR (Criswell et al 2005). Thus, IGF-1-sCLU induction in vivo appears to be linear with very low doses (10-100 cGy), but non-linear with higher doses, of IR. Thus, induction of sCLU levels in vivo was the result of activated TGFβ1 signaling consistent with prior reports (Cipriano et al 2011, Kirshner et al 2006, Wang et al 2011). TGFβ1 activation and downstream signaling is likely a mechanism for relieving p53 repression of IGF-1 expression via Smad-activated transcriptional increases in Mdm2 (Araki et al 2010).

**sCLU suppresses TGFβ1 signaling.** In an attempt to investigate the physiological role of sCLU in TGFβ1-responsive human colon cancer cells, TßRII-deficient HCT116 cells versus cells restored for TßRII expression (HCT116 3-6) were used (Davis et al 1998). HCT116 3-6 cells were then knocked down for sCLU expression using siRNA-sCLU or mock-transfected using siRNA-Scr, and interrogated for changes in TGFβ1-induced signaling, growth arrest, and lethality (Figure 5). Specific loss (~70% knockdown) of sCLU levels in HCT116 3-6 cells transfected with siRNA-sCLU was noted compared to HCT116 3-6 cells transfected with siRNA-Scr oligomers (Figure 5A). Interesting, cells knocked down for sCLU were significantly more sensitive to TGFβ1 growth suppression, as well as long-term survival, compared to shRNA-Scr HCT116 3-6 cells (Figure 5B). Conversely, addition of exogenous sCLU to the medium significantly repressed TGFβ1 signaling in TßRII- HCT116 cells transfected with the TßRII receptor (compare lanes 8,9, Figure 5C). However, TGFβ1 signaling was restored by co-addition of a polyclonal antibody specific to human sCLU (B5 sCLU antibody, lane 10, Figure 5C). Indeed, sCLU addition prevented TGFβ1-induced G₁ cell cycle checkpoint arrest.
in TGF-β1-treated TßRII+ HCT116 3-6 cells, which was restored by addition of polyclonal B5 antibody specific for the mature form of human sCLU (Figure 5D).

Since HCT116 cells are deficient in Bax expression and lack apoptotic responses after various cytotoxic agents (He et al 2003), and sCLU is thought to protect cells from Bax-induced apoptosis (Trougakos et al 2009b, Zhang et al 2005), we also explored responses in life-extended HMECs that were, or were not, knocked down for sCLU expression as in Figure 5 (Figure 6). Indeed, sCLU knockdown (Figure 6A) augmented TGF-β1 lethality (Figure 6B), which correlated with dramatic increases in apoptosis as monitored by TUNEL+ stained cells (Figure 6C). Addition of medium from irradiated MCF-7 cells that contained elevated levels of sCLU (MsCLU) as in Figure 5, suppressed apoptosis (Figure 6C) and restored the survival of TGFβ1-exposed HMECs (Figure 6B). Addition of excess polyclonal B5 antibody directed against human sCLU (AbB5-sCLU), which cleared the protein from medium as monitored by Western analyses, prevented sCLU function to block TGFβ1 activity and restored both apoptosis (Figure 6C) and lethality (Figure 6B) caused by TGFβ1 exposure. Thus, as with HCT116 cells (Figure 5), sCLU mediates a pro-survival bystander effect in HMECs by preventing TGFβ1-induced signaling, and therefore, downstream growth arrest, apoptosis and lethality (Figure 6).

Discussion

Since both IGF-1 and sCLU are secreted proteins that play roles in survival (IGF-1 and sCLU) and clearance of cell debris from traumatized tissue (sCLU) (Nachtrab et al 1998), we hypothesize that this expression axis plays a major role in wound healing and recovery in vivo (Trougakos et al 2009a, Trougakos et al 2009b), as well as promoting a pro-growth microenvironment for tumor growth. Thus, monitoring IGF-1-sCLU levels may allow assessment of the biological effects of low dose IR exposures, and be used for assessment of normal versus abnormal recovery responses that affect potential health risks to individuals. Transient IGF-1-sCLU expression may be healthy in normal cells, but constitutive induction in vivo may indicate acquired damage-induced or mutation-mediated long-term genetic instability,
as recently shown for repair-compromised cells (Goetz et al 2011). Responses in colon versus bone marrow after exposure to 10 cGy were intriguing in this context.

We recently linked induction of sCLU *in vitro* after low doses of IR, as well as after low doses of other DNA damaging agents in human and rodent cells (Criswell et al 2003, Criswell et al 2005), to the specific induction of IGF-1 by ATM activation (Goetz et al 2011) or TGFß1. IGF-1-sCLU induction is long-lived and proportional to IR exposures between 10-100 cGy *in vitro* (Figure 1) and *in vivo* (Figure 4). Due to its extreme sensitivity to IR and other cytotoxic agents, we decided to use the hCLUp-Luc reporter to monitor induction kinetics of this low dose stress-response gene as a first step in the development of a potential sensitive biodosimeter that may one day be used to assess risk to human health. sCLU expression is complex, with known induction responses after a variety of cell stress events, all culminating in ATM-IGF-1 (Goetz et al 2011) or TGFß1-Smad-IGF-1 stimulation. Our recent data on endogenous IGF-1-sCLU expression strongly suggested a complex expression axis that was basally repressed by wtp53 (Goetz et al 2011). Data presented in Figure 2 also indicate that alterations in PI3K and AKT affect sCLU expression, consistent with a role of AKT affecting p53 function (Garkavtsev et al 2011) by altering Mdm2 levels (Araki et al 2010). sCLU induction *in vivo* most likely occurs through exposure of tissue *in vivo* to activate TGFß1, which can abrogate p53 repression through increased Mdm2 levels as we reported (Araki et al 2010).

IR induction of IGF-1-sCLU expression via activation of TGFß1 *in vivo* offers a new wrinkle to an already complex story of IR-ATM-IGF-1-IGF-1R regulation. Exposure of human and rodent cells *in vitro* up-regulated IGF-1R levels through ATM activation (Peretz et al 2001, Saatman et al 1997, Shahrabani-Gargir et al 2004). Furthermore, links between ATM and TGFß1 exposures were reported, whereby TGFß1 signaling augmented ATM activation (Cipriano et al 2011, Kirshner et al 2006, Wang et al 2011), and activation of TGFß1 by an as yet poorly described ROS pathway after low doses of IR seem extremely important in carcinogenesis (Nguyen et al 2011). Since p53 repressed IGF-1-sCLU expression (Criswell et al 2005, Goetz et al 2011) so efficiently *in vitro*, we were surprised to find the robust up-regulation of this expression axis in normal tissue of low dose IR-exposed mice, particularly in tissues that are typically hypersensitive to IR (Figures 3 and 4). Our data suggest that these induction responses are mediated by activated TGFß1, and its downstream activation of Smad3 signaling (Figures 3C, 4). Indeed, Smad signaling was only noted in tissues
expressing hCLUp-Luc reporter activity and sCLU protein (Figures 3 and 4), with tissue hCLUp-Luc induction kinetics that matched endogenous sCLU protein induction. This is consistent with prior findings of low dose IR activation of TGFß1 (Kirshner et al 2006). By activating TGFß1 \textit{in vivo}, which most likely further augments ATM activity, p53 function and phosphorylation of NF-YA are promoted through elevated p21 levels that ‘permits’ IGF-1 promoter induction (Doi et al 2011, Goetz et al 2011). p21 inhibits cdk-induced NF-YA phosphorylation required for p53/NF-YA binding to the IGF-1 promoter (Doi et al 2011, Goetz et al 2011). Thus, TGFß1-activated Smad signaling in tissues hypersensitive to IR induced IGF-1-sCLU expression, presumably required for tissue recovery. Induction in normal tissues is transient and not identical in various exposed tissue (i.e., bone marrow vs colon, Figure 4). Different tissues may have different risks after low IR doses. For example, analyses of mammary and lung tissues after various high LET exposures is currently underway in our lab. The danger for cells within specific tissue is that if damage persists, as a result of genetic instability through loss or faulty repair or permanent mutation or memory, IGF-1-sCLU expression could be ‘permanently’ turned on. In genetically unstable cells, such as neoplastic-initiated cells, IGF-1-sCLU expression can be dramatically elevated compared to matched stable cells due to loss of p53 function and ATM activation (Goetz et al 2011). Permanent IGF-1-sCLU elevation is an early marker of genetic instability in tissues of individuals exposed to low doses of IR or other toxins (Goetz et al 2011), which may allow development of an altered microenvironment that promotes tumor growth and progression, including EMT and metastasis.

\textbf{Conflict of Interest.} The authors declare no conflict of interest.

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**Figure Legends**

**Figure 1.** Development of the hCLUp-Luc reporter as a sensitive indicator of low dose IR exposures. Stable 1403 hCLUp-Luc MCF-7 cells were monitored for time-course and dose-response promoter reporter activities using BLI imaging, where numerous readings were taken from the same living cells. **In A, B,** Dose-response induction of hCLUp-Luc. Significant hCLUp-Luc activity was noted after 0.2 Gy (20 cGy) exposure, with linear X-fold increases with IR doses between 0.2-1.0 Gy. Inset, endogenous psCLU and sCLU protein expression in MCF-7 hCLp-Luc cells. Relative sCLU levels (RL_{sCLU}) were assessed using NIH J imaging of western blots as described (Goetz et al 2011). **In C,D,** Time-course hCLUp-Luc induction with IR dose. Note that longer times were required for induction as IR doses decrease. Real-time images (A,C) and corresponding quantitation of three experiments performed in triplicate (B,D, means ±SE) are shown. *p<0.05; **p<0.01. Time-course and dose-response increases in hCLUp-Luc activities were identical to endogenous increases in the precursor form of CLU (psCLU), as well as mature sCLU protein expression, in MCF-7 cells (Criswell et al 2005, Goetz et al 2011, Yang et al 1999, Yang et al 2000).

**Figure 2.** CLU expression is repressed by p53 and upstream factors that alter p53 function. **In A,** Wild-type p53 HCT116 (wtp53) and an isogenic HCT116 cell line in which p53 was somatically knocked out (HCT116 p53-/-) were transiently transfected with hCLUp-Luc. Cells were irradiated with various IR doses and 24 h later analyzed for hCLUp-Luc activity using BLI, 72 h post-IR. Relative hCLU-Luc expression in Gray Scale Values (GSVs X 10^5) were monitored and results (means ±SE) shown for three experiments repeated in triplicate each. Note slight increases in hCLU-Luc activity in HCT116 p53+/+ cells, but significantly greater levels in HCT116 p53-/- cells; there was no difference in overall IR sensitivity in HCT116 p53-/- versus HCT116 p53+/+ cells (Davis et al 1998). **In B,** wtp53 MCF-7 cells were co-transfected with hCLUp-Luc and either vector alone (VO), constitutive-active PTEN (PTEN CA), dominant-negative AKT1 (AKT kinase-deficient, AKT KD), wtp53, or E6 expression vectors. Cells were then irradiated with 0.1 Gy 24 h later and hCLUp-Luc activities monitored 48 h later using BLI. Note that any treatment that functionally inactivated p53 in a direct (i.e., E6) or indirect (PTENCA) manner, suppressed hCLUp-Luc activity. In contrast, vectors that express factors that enhance p53 function (i.e., wtp53 or AKTKD) suppressed basal and IR-
induced hCLUp-Luc activities. Reported are gray values for treated over control levels, where the level of expression of the mock-irradiated vector only control was set at 1.0. In C, MCF-7 cells were transfected with hIGF-1p-Luc or hCLUp-Luc promoter reporters, then mock-treated or exposed to 0.1 Gy, 24 h later and analyzed using BLI at various times (h) later; the power of BLI is that the same cells are repeatedly assessed at 8, 32, 48 and 72 h post-IR. Note simultaneous increases in CLU and IGF-1 promoter activities in MCF-7 cells after 0.1 Gy exposure. Results (means ±SE) presented are from three separate experiments, each repeated in triplicate. *p<0.05; **p<0.01.

Figure 3. hCLUp-Luc transgenic mice are responsive to low doses of IR in specific hypersensitive tissues. Age (10 weeks)- and weight (18 gram)-controlled hCLUp-Luc reporter transgenic FVB/N mice were mock-treated or exposed to 0.1 Gy IR and imaged using BLI 24 h later. In A, Images of male and female mice exposed to IR. Mice were exposed to 0.1, 1.0 and 5.0 Gy and at various times intraperitoneally (i.p.) injected with 10 mg/kg luciferin. At 24 h, internal organ hCLUp-Luc responses were noted with all doses used. Images after 0.1 Gy were shown for representation. In B, Female mice shown in A were sacrificed and internal organs removed and placed in PBS containing 2.5 mg/ml luciferin for ex vivo BLI. Note hCLUp-Luc expression was elevated in bone marrow, spleen, thymus, and colon at 24 h. Responses from mammary tissue were also noted, but not shown. Responses were not found in muscle, lung, heart or liver. Images are representative of experiments performed at least three times. In C, female mice (3/treatment group) were either mock-irradiated or exposed to whole body IR (1.0 Gy) as described above and then injected with or without the α-TGFβ1 (1D11) antibody or a control antibody (13C4), each at 10 mg/Kg, and hCLUp-Luc or endogenous sCLU levels monitored by imaging or Western analyses 96 h later. sCLU induction at 96 h was blocked by the α-TGFβ1 antibody that blocks all three forms of the TGFβ1 family (Dasch et al 1989), whereas the control IgG 13C4 antibody had little affect. Experiments were repeated twice with 3 animals/treatment group and tissues pooled as indicated. Endogenous sCLU levels and hCLUp-Luc reporter activities were monitored, and levels quantified. Data are a composite of three independent experiments of CLU/sCLU expression. **p<0.01.
Figure 4. **TGFβ1 signaling in vivo correlates with sCLU induction after low doses of IR.** In **A, B,** Ten week-old (18 gm) female FVB/N mice were exposed to IR (0.1 or 1.0 Gy) and colon and bone marrow tissues extracted at various times as indicated. In **C, D,** Dose-response (0.1 - 1.0 Gy) studies were also performed in female FVBN hCLUp-Luc transgenic mice and reporter expression monitored at specific times (24 or 72 h) as indicated. In **A-D,** Data (means +SE, n=6) are from experiments performed at least two independent times with three mice/group each. Tissues were pooled and then analyzed. In **E, F,** Steady state protein level changes in psCLU and sCLU, phosphorylated or total Smad3 (P-Smad3 or t-Smad3, respectively), γ-H2AX and α-tubulin were monitored by Western analyses. Note increased levels of P-Smad3/tSmad3, indicative of activated TGFβ1 signaling after 0.1 or 1.0 Gy at 24 or 72 h post-IR in both colon and bone marrow tissue samples. Activated Smad signaling (indicated by elevated P-Smad3/t-Smad3 levels) corresponded well with increased sCLU protein levels. Shown are representative Western blots for colon and bone marrow from experiments performed three times in triplicate with similar results.

Figure 5. **IR-induced sCLU levels confer resistance to TGFβ1 signaling.** In **A,** TβRII-responsive HCT116 3-6 cells were transfected with siRNA-Scr or siRNA-sCLU oligomers to mock transfect or knock down sCLU levels, respectively; siRNA-sCLU is specific for the leader peptide in sCLU mRNA and did not alter nCLU levels (Criswell et al 2005). sCLU levels were knocked down ~70% versus parental or siRNA-Scr transfected HCT116 3-6 cells. In **B,** sCLU knock down cells were significantly more susceptible to TGFβ1-induced lethality by colony forming ability assays. In **C,** addition of sCLU-containing conditioned medium from IR-treated MCF-7 cells to TβRII-transfected HCT116 cells repressed TGF-β1 signaling by TGFβ1-responsive SBE2X2-tkLuc reporter activity, which was blocked by B5, a human sCLU-specific antibody (lanes 8 vs lanes 9 and 10). HCT116 cells were transfected with SBE2X2-tkLuc with or without a TβRII expression vector. Cells were either exposed or not to sCLU-containing conditioned medium from irradiated MCF-7 cells, containing or lacking excess (1 µg/ml) B5 antibody specific to sCLU and treated with TGFβ1 (10 ng/ml). Cells were then monitored 48 h later for TGFβ1 signaling by SBE2X2-tkLuc. In **D,** sCLU suppresses TGFβ1 signaling. Genetically matched TβRII- HCT116 and TβRII+ HCT116 3-6 cells were treated with sCLU-
containing conditioned medium from irradiated MCF-7 cells and TGFβ1 (10 ng/ml) as in C, but without transfections. TßRII- HCT116 cells were not responsive, and sCLU addition had no affect, whereas HCT116 3-6 cells respond to TGFβ1 by inducing SBE2X2-tkLuc activity that was suppressed by conditioned medium containing sCLU (compare lanes 8 to 9). Addition of B5 antibody restored TGFβ1 signaling (compare lanes 8-10). Data are representative of three separate experiments repeated in triplicate. *p<0.05.

Figure 6. **sCLU prevents TGFβ1-induced apoptosis and lethality in HMEC cells.** *In A,* Primary human mammary epithelial cells (HMECs) were transfected with siRNA-Scr or siRNA-sCLU oligomers to knock down sCLU protein expression as described in Figure 5. sCLU levels were knocked down ~60% in HMECs by siRNA-sCLU, but not significantly altered by siRNA-Scr. Western blots are representative of experiments repeated three times. *In B,* Transiently transfected HMECs from Figure 6A were analyzed for changes in survival before or after various TGFβ1 (ng/ml) exposures as indicated. *In C,* HMECs from Figure 6B were analyzed for TGFβ1-induced apoptosis in the presence or absence of sCLU levels in medium. Treatments with media derived from irradiated MCF-7 cells (MsCLU), cleared or not with polyclonal antibody B5 antibody specific for sCLU (AbB5-sCLU) were as described in Figure 5. Data are means +SE from three separate experiments repeated in triplicate.

**References**


Figure 2

A. hCLUP-Luc Activity (GSV x 10^6) as a function of IR dose (Gy) for p53^-/- and p53^+/+. 

B. hCLUP-Luc Activity (GSV x 10^6) for p53^+/+ MCF-7 cells treated with different agents: VO, PTENCA, AKTKD, wtp53, and E6.

C. Change in Promoter Activity (GSV/TIC) over time (h) after IR/Mock IR for IGF-1p-Luc and hCLUP-Luc under control conditions (UT) and with 0.1 Gy IR treatment.
Figure 4

A. Colon

B. Bone Marrow

C. Colon

D. Bone Marrow

E. Time (h) Post-IR: 24, 72

F. Bone Marrow

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Figure 4
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Figure 6
Oxidative stress, the ATM-IGF-1-sCLU expression pathway, and emergent resistance derived from stress-induced premature senescent (SIPS) cells

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Abstract

Reactive oxygen species (ROS) generated from endogenous oxidative respiration, therapy-based exposure (e.g., ionizing radiation (IR)) and/or the environment are major factors in aging and proneness to cancer. Stress-induced premature stress responses (SIPS) responses are little understood, but likely a major factor in cancer progression and metastatic progression. Cells have developed complicated and sophisticated systems to detoxify ROS, by maintaining homeostatic ROS levels inside the cell and by continuously repairing ROS-generated DNA lesions. ROS causes elevated 8-oxoguanine (8-OG) levels in DNA that are repaired by overlapping DNA mismatch repair (MMR) and base excision repair (BER). ROS-induced DNA damage can cause cellular senescence or death, depending on gene expression responses and extent of DNA damage. Interestingly, altered gene expression responses after transient elevated O₂ exposures have not been examined in detail. Secretory clusterin (sCLU) responses are very sensitive markers of genotoxic stress, elevated by endogenous lesions created in repair-deficient cells. sCLU is induced by ATM activation by low IR doses, transient O₂ exposure, endogenously in genomically unstable cells, and in senescent cells (relicative or SIPS cells) that, in turn, induce IGF-1/MAPK/Egr-1 signaling, resulting in sCLU expression. Elevated 8-OG lesions in DNA are a prime trigger of senescence, a way in which multicellular organisms avoid mutagenic and ultimately carcinogenic cell formation. We theorize that MMR reduces 8-OG levels and mitigates BER-ATM-IGF-1-sCLU expression, and induces cell death avoiding SIPS cell accumulation. These responses mitigate accumulation of IGF-1-sCLU in the microenvironment and thereby decreased the incidence of tumor initiation, progression and therapy-induced resistance.

Keywords: Oxidative stress; senescence; IGF-1; secretory clusterin (sCLU); ATM
Introduction

Aging, Senescence and Changes In The Microenvironment. A long running theory is that aging occurs, or is enhanced by, faulty DNA repair. While this is a contributing factor, we posit that continual oxidative stress from the environment and from metabolism is a major factor in aging, cancer that is associated with aging, and that stress-induced premature stress responses (SIPS) is a major factor in cancer progression and metastatic progression in cancer. Exposure to oxygen (O₂), and in particular to changes in oxidative states is a prevailing stress affecting all life in an oxygenated environment. Elevated O₂ that may occur due to endogenous oxidative respiration, changes in blood supply and ensuing ischemia/reperfusion, and/or alterations in environmental exposure to various radiation sources (such as low doses like those occurring after Chernobyl or after the Fukushima Daiichi) in vivo, result in transient elevated levels of reactive oxygen species (ROS). Cells have developed complicated and sophisticated systems to manage ROS, not only by maintaining homeostatic levels of ROS inside the cell, but also by continuously repairing ROS-generated DNA damage and altering gene expression to cope with changes in oxidative damage to intracellular components. Thus, ROS is a major factor that affects, and is affected by aging and high ROS levels could cause cellular senescence (particularly SIPS) and reduced life span [1-4]. In this paper, we will explore the theory that SIPS and replicative senescence can protect young organisms from cancer formation, but that the accumulation of senescent cells can not only increase the risk of cancer, as outlined by Campisi et al., [5] but also accumulate after repeated exposures to therapy, leading to changes in tissue microenvironments that may enhance cancer initiation and progression. Furthermore, repeated therapeutic treatments of tumors, may cause a rapid accumulation of SIPS, leading to dramatic changes in secretory protein expression that could affect drug and/or radiation resistance and promote cancer progression.

Results and Discussion

Replicative Senescence. Hayflick and Moorhead discovered that normal primary human fibroblasts have a limited proliferative ability, called the ‘Hayflick limit’ [6]. When cells reach the Hayflick limit, they undergo dramatic changes, such as p53-dependent or pRb-dependent irreversible growth-arrest, while maintaining metabolic competence. This is referred to as replicative senescence (RS), a genetically programmed form of senescence. RS is associated with shortening of telomeres, located at the end of chromosomes and consist of repeating telomeric DNA sequences and telomere repeat binding factor (TRF) proteins. Since mammalian cells have linear chromosomal DNA, telomeric DNA must form a telomere-loop with TRF that masks DNA double-stranded break (DSB) ends. One consequence of cell division is that a small amount of DNA is always lost at telomere ends and this loss, in turn, leads to telomere shortening. Telomeric erosion ultimately leads to release of factors, such as TRF2, that otherwise protect telomeres, and protect DNA ends from DNA damage responses (DDRs). Shortening of telomeres also dramatically affects the ability of a cell to proceed with proliferation, since DDRs directly stimulate cell cycle checkpoint responses [7]. Inhibition of telomere elongation by exposure to telomerase inhibitors shortens the Hayflick limit. The resulting telomere-
shortening, in turn, stimulates induction of cellular RS and eventually, exposed telomeric ends induce a DDR involving Ataxia telangiectasia mutant (ATM) kinase activation [8, 9]. ATM activation, in turn, stimulates two redundant signal transduction pathways mediated by the p53 tumor suppressor protein, and the p16-pRb (retinoblastoma) tumor suppressor proteins (reviewed by Campisi et al., [5, 10-12]).

RS responses of normal cells, particularly when an organism is young, are generally thought to be a form of tumor suppression. In this state, cells undergo a genetic program, stimulated by their replication limit, to become ‘reproductively’ eliminated [12-16]. Early on it was noted that senescence could also be initiated by over-expression of oncogenes (e.g., over-expressed oncogenic RAS) [17], as well as by dysfunctional telomeres. The regulatory interactions between p53-mediated and p16/pRb-mediated RS pathways are complex and not completely understood [5, 10, 11]. In general, the p53-dependent pathway is mediated by ATM and involves several downstream p53-responsive genes, such as p21 along with p16\(^{\text{INK4}}\), leading to increased levels of p19\(^{\text{ARF}}\). Modulators of RS include Bmi-1, that represses p16\(^{\text{INK4}}\) and RS [18]. Stimulated MAP kinase (p38) signaling can also modify RS, as recently reviewed [19-21].

**Stress-Induced Premature Senescence.** Unlike RS, which is less affected by oxidative stress since telomeric shortening is mostly a result of DNA replication, senescence can also be induced through extensive DNA damage, inducing a stress-induced premature senescence (SIPS) response. SIPS is most commonly induced by agents that cause extensive oxidative stress and/or DNA damage in the form of altered bases or DNA single (SSB) or double strand breaks (DSBs). Most forms of radiation that damage DNA or chemotherapeutic agents that can elicit extensive DNA lesions can induce SIPS. The overall regulatory pathways that control SIPS remain relatively unknown, but are also thought to be stimulated by pathways involving ATM and p53 [14, 22]. Thus, RS and SIPS have common, as well as different regulatory features. Both senescence pathways appear to involve DNA damage sensing, either from dysfunctional telomeres or extensive DNA damage recognition [23-28], that somehow avoids PARP1 hyperactivation responses. However, unlike RS that is fairly restricted to normal cells, SIPS can be induced in cancer cells as well, apparently re-expressing a signature of proteins that function to induce senescent grow arrest, while inhibiting replication and cell death (i.e., apoptosis and necrosis) [26, 29, 30]. Similar to RS, SIPS responses also result in increases in p16/pRb and p53/p21 levels. However, expression of human telomerase (hTERT) does not appear to suppress SIPS, as it does RS [31, 32]. Furthermore, decreased expression of p21 and p19 appear to be involved in SIPS [32]. Interestingly, in some cell systems damage to cells (e.g., after H\(_2\)O\(_2\) exposure) activates TGF-\(\beta\)1 by some unknown mechanism(s), and exposure to this cytokine can also initiate SIPS [33]. These responses do not appear conserved in all cell systems, since human prostate epithelial cells exposed to H\(_2\)O\(_2\) induce SIPS, whereas, exposure of the same cells to TGF-\(\beta\)1 induced differentiation and increased senescence-associated \(\beta\)-galactosidase (SA-\(\beta\)gal) activity, but cells did not undergo SIPS [34].

**Secreted Proteins Produced During Senescence.** An interesting and novel theory of aging proposed by Campisi et al. [5, 10, 11, 35, 36] is that senescence is
“Antagonistically Pleiotropic” [5]. Simply put, RS and SIPS are tumor suppressive for a multi-cellular organism early in life. However, later in life senescent cells accumulate and increase the secretion of factors, such as vasculature endothelial growth factor (VEGF) [37], that may promote metastasis and tumor growth. Indeed, in a paper by Liu and Hornsby [38], it was strongly suggested that SIPS fibroblasts played a major role to enhance tumor cell growth, presumably by increased metalloproteinases (MMPs) expression. These data supported earlier studies by studies of the Barcellos-Hoff laboratory, in which low dose irradiation of human breast stromal cells augmented the incidence of late-life cancers [39]. In spite of these studies, full elucidation of the secreted proteins that may affect tumor formation and growth remain to be discovered. We hypothesize the possibility that the formation of senescent cells as a direct result of therapy leads to a drug and/or radioresistant microenvironment caused by expression of tumor promoting and/or pro-survival proteins that can affect exposed or non-treated cells in the immediate area of therapy (Fig. 1).

The Senescence Proteome. Over the past several years, evidence for senescence-induced and -related protein secretion has accumulated. Toussaint et al., [24] have used microarray and two-dimensional gel electrophoresis to characterize genes and proteins induced by RS, compared to SIPS. Their data suggest common, as well as unique, patterns of gene expression in cells undergoing RS versus SIPS [26, 40]. Some signature
genes were identified and their functions in carcinogenesis elucidated. Irradiated fibroblasts produced factors that increased the invasiveness of pancreatic cancer cells in culture [41]. Although the exact identity of these factors were not elucidated, their functions in metastasis appear clear. In another study, stromal cell-derived factor 1 (SDF-1), that recruits endothelial progenitor cells and stimulates the formation of new blood vessels or angiogenesis, was induced in RS or SIPS fibroblasts. Co-culture of these RS or SIPS fibroblasts augmented tumor formation and promoted tumor progression [42]. Clearly, senescent fibroblasts can secrete factors that promote cancer progression. Elucidation of these secreted factors, along with the signal transduction pathways required for their production, from RS and/or SIPS cells, which could affect tumor growth and responses to chemotherapy or radiotherapy, are desperately needed. In our studies, we identified insulin-like growth factor-1 (IGF-1) and secretory clusterin (sCLU) expression from RS or SIPS cells. We are exploring how these two factors, which are induced via ATM activation during RS or SIPS, and may promote cancer progression.

**IGF-1 and IGF-1 Binding Proteins.** IGF-1 is a very conserved protein of 70 amino acids. Its function is to stimulate downstream signal transduction pathways, mediated through its receptor, the insulin-like growth factor-1 receptor (IGF-1R). IGF-1R is a highly specific membrane receptor tyrosine kinase. IGF-1 function in the blood (normally at 100-500 ng/ml) is controlled by IGF-1 binding proteins (IGFBPs). Seven such proteins (IGFBP-1 to -7) are produced in human blood. IGFBP-3 binds >90% IGF-1 in the blood, leaving little free IGF-1 to bind IGF-1R. IGFBPs prolong the half-life of IGF-1, allowing IGF-1 to be delivered throughout the body. IGFBPs also modulate the availability of IGF-1 to bind its receptor to mediate proliferative and metabolic/anabolic effects [43-46]. IGFBP-3 is the most common circulating IGFBP and is responsible for binding circulating IGF-1 [46]. IGFBP-3, and the other IGFBPs, is subject to serum proteases that can liberate IGF-1. Regulation of IGF-1 by these proteases is still relatively unknown.

Although IGF-1 over-expression is commonly observed in cancer, its roles in survival and metastasis are unknown. Many cancers, including small cell lung cancers and cancers of the colon, prostate, and breast, have elevated levels of this growth factor [47-54]. The majority of circulating IGF-1 in blood is made in the liver through endocrine regulation by growth hormone (GH). IGF-1 promotes cell growth by acting as a mediator of GH, and can stimulate migration and survival of intestinal and lung fibroblasts [55]. Mice with a liver-specific IGF-1 deletion had a >75% decrease in circulating IGF-1 levels and were less likely to get mammary cancer [56-59]. The remaining ~25% of IGF-1 was produced in other organs, and allowed for normal growth and development of these animals. Therefore, cells and tissues beside the liver can produce IGF-1. Furthermore, regulation of IGF-1 by individual normal tissues can also occur by control of other hormones [60-63].

**Stress-induced IGF-1 Expression.** Changes in IGF-1 expression appear to be an early marker of cancer risk and an indicator of excess reactive oxygen species (ROS)-induced cell stress. It is highly possible that cancer cells can become dependent on IGF-1 for survival, opening a potential therapeutic window. A recent study concluded that IGF-1
expression was seriously imbalanced in patients with gastrointestinal inflammation, regardless of the primary cause. Our own data strongly suggest that ROS formation is a major initiating signal in IGF-1 synthesis in stressed cells, since its induction after ROS generating agents can be prevented by N-acetylcysteine (NAC) treatment [64].

There are few studies that have demonstrated the functional role of IGF-1 in cells and organs after stress. Injection of IGF-1 into the spinal cords or bone marrow of animals can have a major protective effect against IR injury [65, 66]. IGF-1 signaling can suppress Smad activation [67-69], presumably contributing to its role in proliferation. However, the functional outcomes of such regulation remain unknown. IGF-1 administration is also reported to increase homologous recombination and suppress lethality caused by IR [70]. Additional research in vivo on the functions of stress-induced IGF-1 production are needed.

**Stress-Induced Secretory Clusterin.** Secretory clusterin (sCLU) is a stress-inducible, ~80 kDa secreted glycoprotein implicated in various biological processes, including cellular senescence and apoptosis. sCLU over-expression has been linked to Alzheimer's and heart [71, 72] and cancer progression [73], among many other pathologies. Elevated sCLU expression was used as an early marker of carcinogenesis in Adenomatous Polyposis Coli/Min (APC/Min) knockout mice [73] that have dramatic increases in gastrointestinal carcinogenesis [74, 75]. Endogenous over-expression of sCLU in colon, prostate, and breast cancers has been linked to increased aggressiveness and metastatic ability [73]. Although the functional significance of inducible sCLU responses is complex, its role as a multi-functional pro-survival protein is clear. sCLU functions as an extracellular molecular chaperone that binds unfolded stressed proteins to clear cell debris after various cell stresses [76]. Intracellularly, sCLU can suppress Bax-mediated apoptosis [77]. Over-expression of sCLU resulted in resistance to various anti-cancer drugs and IR [78, 79], while down-regulation of sCLU by antisense RNA or small interfering RNA (siRNA) enhanced the radio and/or chemo-sensitivities of human cancer cells [78, 80]. Thus, sCLU is a pro-survival factor made by cells as a countermeasure to cell stress and may be a necessary survival factor for carcinogenic processes. Production of sCLU is cytoprotective, sparing cells from the apoptotic and lethal affects of all chemotherapeutic agents (etoposide, camptothecin, cisplatin, temozolamide, BCNU, H2O2) tested thus far, as well as after IR exposure. Interestingly, after all cell stresses examined, identical kinetics of sCLU induction were noted. CLU promoter activities and sCLU protein levels were induced 2- to 10-fold, from 24 to > 120 hours. Delayed sCLU induction after all damaging agents tested suggested a common upstream signaling pathway.

**Transcriptional Regulation Of sCLU.** Our studies demonstrated that IR induces IGF-1/IGF-1R downstream signaling that regulates sCLU. The roles of Src, MEK-1, and Erk-1/2 in CLU promoter transactivation and overall sCLU protein expression were examined using specific siRNA knockdown, dominant-negative expression and kinase inhibitors [78]. Administration of PP1 (a selective Src inhibitor) or U0126 (a selective MEK-1 inhibitor) blocked CLU promoter and sCLU protein expression [78]. Kinase-dead Src, as well as dominant-negative Erk-1, Erk-2 or Mek-1 inhibited CLU promoter and sCLU
expression. Constitutively active Src (Src CA) activated the CLU promoter, without IR. Thus, IR-induced CLU promoter activity was regulated by delayed expression of IGF-1 [78].

Delayed IGF-1/Src/MAPK activation after IR suggested that CLU promoter activity was regulated by a transcription factor activated late after exposure. Since Egr-1 activation by this pathway was noted and one site in the CLU promoter was found [81-83], we investigated the role of Egr-1 in CLU promoter activation after IR. Indeed, delayed Egr-1 binding to the CLU promoter (by biotinylated 4250 CLU promoter pull-down assays) in MCF-7 cells after IR correlated well with CLU promoter activation and sCLU protein expression. siRNA specifically designed to knockdown Egr-1 prevented CLU promoter induction and sCLU protein expression after IR, while expression of exogenous Egr-1 resulted in higher basal levels of CLU promoter activity. Furthermore, selective Src and Mek-1 inhibitors (PP1 and U0126, respectively) prevented Egr-1 protein binding, as well as IR-induced CLU promoter activity and sCLU expression. Indeed, site-directed mutation of the Egr-1 site within the CLU promoter prevented Egr-1 binding and CLU promoter activity in response to all agents tested (Zou and Boothman et al., Unpublished data).

**p53 Suppresses IGF-1-sCLU.** p53 also negatively regulated sCLU expression, and this regulation was mediated by suppression of IGF-1 and IGF-1R downstream signaling [84, 85]. The IGF-1 promoter contains several NF-Y binding CCAAT boxes. p53 can interact with the transcription factor, NF-YA, to repress the activity of CCAAT containing promoters. By down-regulating IGF-1 production, p53 indirectly suppresses sCLU expression (Fig. 2). However, after DNA damage and in spite of p53 stabilization, p53 was noted to be dissociated from NF-YA in an ATM-dependent mechanism, and released from IGF-1 promoter. Its release relieved p53 repression resulting in IGF-1 induction, and concomitant increased sCLU expression (Fig. 2) [85]. This dynamic regulation, thereby, explains how IGF-1-sCLU expression kinetics are delayed in their overall synthesis in injured cells and tissue (Klokov and Leskov et al., unpublished data).

**Cellular Response To Oxidative Stress.** Recently, we demonstrated that sCLU was a very sensitive marker of genotoxic stress, being induced through ATM activation by low
doses of IR, transient O₂ exposure, or endogenously in genomically unstable cells [85]. ATM functions upstream to control IGF-1 signaling, mediating genotoxic stress-induced sCLU expression [85]. ATM signaling is an important sensor in cellular responses to oxidative stress [86-88]. It can be directly activated by oxidation [86]. ATM is also an important mediator of cellular anti-oxidant defense and the DDR system [89]. ATM plays a control role in cellular response to ROS and is the main DNA damage signaling system for signaling DDRs. As a downstream factor of ATM activation, sCLU appears to be a sensitive indicator of oxidative stress in cells, and also plays a role in the cellular oxidative stress response. Here, the chaperone roles of sCLU in clearing extracellular, unfolded stressed proteins to prevent inflammation responses appear to make sense in the overall injury responses. However, the ability of sCLU to bind to TGFβ1 receptors and potentially affect TGFβ1 signaling is likely to affect the microenvironment, including stimulating tumor promotion.

**Oxidative Stress and Senescence.** Mouse embryonic fibroblasts (MEFs) are very sensitive to oxygen changes in culture, and age faster when cultured under relatively high oxygen content [90, 91]. Recently, we were able to show that the population doubling of IMR-90 normal human lung fibroblasts was significantly decreased when cultured under high oxygen levels (e.g., 20% O₂), inducing senescence faster in comparison to cells cultured under lower oxygen levels (i.e., 2% O₂). As expected, we detected elevated IGF-1-sCLU expression in IMR-90 cells cultured under high oxygen conditions, suggesting that 20% O₂ could cause significant oxidative stress in cells. 8-Oxoguanine (8-OG) is a major DNA lesion caused by ROS [92, 93], including in response to elevated oxygen content. Elevated 8-OG levels stimulate 8-oxo-dGTPase activity, a mammalian MutT Homolog 1 (MTH1) glycosylase that eliminates 8-OG lesions in DNA, and ultimately limits DNA damage under oxidative conditions [94]. Interestingly, MTH1 knockdown using siRNA can stimulate SIPS in cells due to increased DNA lesions that may trigger ATM-dependent SIPS reactions. In addition to glycosylases, cells may also use additional base excision repair (BER) systems to eliminate oxidative DNA lesions. However, these systems can be highly mutagenic,

**Figure 3. Oxidative stress signaling.** Culturing cells in high oxygen (O₂) increases cellular ROS levels that lead to elevated DNA lesions, the most mutagenic being 8-oxoguanine (8-OG) formation. Repair of these lesions by BER activates ATM and stimulates sCLU expression through IGF-1/IGF-1R/MAPK/Egr-1 signaling. In contrast, the high fidelity MMR system eliminates oxidative DNA lesions without ATM activation, lowering BER pathway activation and sCLU expression. The net result would be lowered SIPS cells, increased apoptosis and an avoidance of a carcinogenesis and drug resistance caused by SIPS-ATM-IGF-1-sCLU expression.
particularly if enough 8-OG lesions remain. Luckily, cells also use DNA mismatch repair (MMR) to eliminate 8-OG from DNA, as well as other oxidative DNA lesions. MMR is a high fidelity repair system, known to stimulate cell cycle and growth arrest and provide signaling through c-abl-p73/gadd45a signaling and avoid ATM stimulation. In this manner, 8-OG lesions would be eliminated in a high fidelity manner, avoiding SIPS and eliminating mutagenic 8-OG lesions. Thus, a functioning MMR system would simultaneously prevent SIPS and provide a high fidelity repair pathway for surviving cells. We hypothesize that culturing cells in relatively high oxygen, where elevated 8-OG levels occur, would lead to increased DNA breaks when cells try to repair 8-OG lesions through BER pathways (Fig. 3). This would ultimately cause activation of ATM and SIPS responses. ATM activation, would, in turn, induce dramatic IGF-1-sCLU expression. In contrast, in cells expressing a functional MMR system, 8-OG lesions would be efficaciously removed by MMR, resulting in significantly less ATM-IGF-1-sCLU signaling. Thus, we hypothesize that a functioning MMR system would mediate SIPS, lessening the accumulation of SIPS cells during repeated therapy exposures (Fig. 3). This would lead to significantly less IGF-1-sCLU expression and significantly altering the microenvironment of a tumor under therapy (Fig. 3). The net result would be lessened cancer progression in such a MMR-competent microenvironment, and a reduction in drug and/or radiation resistance. Our preliminary data in MMR+ versus MMR- cells appears to confirm such a diminished IGF-1-sCLU signaling pathway.

**Conclusions and Roles for IGF-1-sCLU In Senescence and Tumor Promotion.** ROS stress can cause premature senescence (SIPS, causing over-expression of IGF-1 and sCLU, which plays a role in drug resistance, tumor development, and cancer progression. Therapeutic agents that target and suppress expression of these two secreted proteins are currently in clinical trials for the treatment of various cancers. We have discovered that replicative and SIPS forms of senescence can cause dramatic elevations of these two secreted proteins, which can act in autocrine/paracrine manners to enhance cell growth and confer cellular resistance to various cell stresses. Thus accumulation of SIPS cells due to repeated therapeutic regimen represents a major resistance factor for the development of secondary tumors, and may represent a major factor in enhancing secondary cancers. SIPS cells and their secreted proteome, which sCLU and IGF-1 are believed to be a major component, act to change the tumor microenvironment, creating a favorable environment for tumor growth and promotion, and conferring drug ad/or radiation resistance. Changes in protein expression within the tumor microenvironment are likely to be mediated by senescent fibroblasts, as well as senescent cancer cells. Our results indicated that IGF-1 and sCLU are two major candidate factors expressed by SIPS fibroblasts and cancer cells, and promote tumor development by altering the tumor microenvironment. Interestingly, MMR and BER repair systems appear to play opposite roles in IGF-1-sCLU expression, and MMR could allow a multi-cellular organism avoid SIPS cell accumulation. Further experiments are needed to elucidate the functions of IGF-1 and sCLU in the tumor microenvironment affecting drug resistance and tumor progression.
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Stress-induced Premature Senescence Induces Tumor Growth Promoting Factors
IGF-1 and Secretory Clusterin

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INTRODUCTION

Recent evidences suggested that the causes of cancer development are not limited to mutations within cancer cells, but also involve alterations of cancer microenvironment. Senescent cells are inevitably growth arrested, but remain metabolically active. Senescent cells, especially senescent fibroblasts in the stroma, may provide a beneficial environment for tumor growth through secretion of certain factors. Accumulation of senescent cells in stroma could be an important factor that causes alteration of microenvironment that benefits tumor development. It is known that multiple and long-term exposure of low-dose ionizing radiation (IR) induces stress-induced premature senescence (SIPS) in both stromal and tumor cells. Thus low-dose IR exposure might promote cancer growth due to microenvironment change through factors secreted by induced SIPS cells. We hypothesize that accumulation of senescent fibroblasts due to low-dose IR exposure would lead to alteration of microenvironment that could promote cancer growth and progression.

RESULTS

1. Induction of senescence in normal human fibroblast.

A

SA-β-gal staining

B

IMR-90

p-Ser15-p53

p16

100%

(%) e >30 f <50

Figure 1. Induction of replicative senescence in normal human fibroblast, IMR-90. A. Normal human fibroblast, IMR-90 were allowed to replicate, and population doubling times were recorded (circles) along with senescence activated p53 and p16. B. Representative image of SA-β-gal staining showing senescent fibroblasts had increased p53 and p16 activity.

2. sCLU level is increased during senescence.

A

IMR-90

HE40

p-CLU

100%

(%) 3 36 81 3 60

Figure 2. Expression of secretory clusterin (sCLU) is increased during senescence. A. sCLU was induced in normal human fibroblasts in IMR-90 cell lines by continuous culturing. Expression of p-CLU and β-actin were detected by western blot analysis. The percentage of SA-β-gal-positive cells indicates the stage of senescence. B. sCLU promoter reporter construct was introduced into young, middle aged, and senescent IMR-90 cells. sCLU promoter was activated during senescence as indicated by relative luciferase activity.

3. IGF-1 level is increased during senescence.

A

IMR-90

Scf

sCLU

100%

(%) 6 84 0 42 55

Figure 3. Expression of insulin-like growth factor 1 (IGF-1) is increased during senescence. IGF-1 in the medium from young (Y) or senescent (S) cells was measured by ELISA.

4. sCLU induction during senescence is mediated through IGF-1 signaling pathway.

A

p-Src

Scr

p-Erk

Egr-1

β-actin

C

AG1024

PsCLU

sCLU

β-actin

Dox

Figure 4. IGF-1 mediates sCLU expression through IGF-1/Src/MAPK/Erk-1 signaling pathway in senescent fibroblasts. A. Src and Erk kinase were activated in IMR-90 cells with or without sCLU knockdown. B. IMR-90 cells underwent senescence faster after sCLU knockdown as indicated by relative luciferase activity. C. Representative images of SA-β-gal staining showing senescent IMR-90 cells with or without sCLU knockdown. D. IMR-90 cells with sCLU knockdown contains more SA-β-gal staining positive cells than cells without knockdown. Some experiments were performed using a vector containing shRNA against sCLU. No difference of population doubling and SA-β-gal positive cells was found when culture with or without doxycycline.

5. ATM is required for sCLU induction during senescence.

A

Y M PS S

B

- Dox

psCLU

+ Dox

Figure 5. ATM is required for sCLU induction during senescence. A. Knockdown of ATM in IMR-90 cells with or without sCLU knockdown. B. IMR-90 cells with sCLU knockdown contains more SA-β-gal staining positive cells than cells without knockdown. Some experiments were performed using a vector containing shRNA against sCLU. No difference of population doubling and SA-β-gal positive cells was found when culture with or without doxycycline.

6. Knockdown of sCLU promotes IMR-90 cell senescence.

A

B

C

D

Figure 6. Knockdown of sCLU promotes IMR-90 cell senescence. A. Knockdown of sCLU in IMR-90 cells with or without sCLU knockdown. B. IMR-90 cells undergone senescence faster after sCLU knockdown as indicated by decreasing of population doubling. C. Representative images of SA-β-gal staining showing senescent IMR-90 cells with or without sCLU knockdown. D. IMR-90 cells with sCLU knockdown contains more SA-β-gal staining positive cells than cells without knockdown. Some experiments were performed using a vector containing shRNA against sCLU. No difference of population doubling and SA-β-gal positive cells was found when culture with or without doxycycline.

CONCLUSION

1. Both sCLU and IGF-1 levels are elevated in senescence fibroblasts.
2. Senescent induced sCLU expression is mediated through IGF-1/Src/MAPK/Erk-1 signaling pathway.
3. sCLU induction during senescence requires ATM.
4. Down-regulation of sCLU promotes cellular senescence.

FUTURE STUDIES

1. Elucidate signaling pathways that mediate IGF-1 expression during senescence.
2. Investigate the role of sCLU in cellular senescence.
3. Investigate the role of senescent fibroblasts secreted sCLU and IGF-1 in prostate tumor growth and progression.

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