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TITLE: Elucidating the Role of Cks Proteins in Breast Cancer by Combining the Disciplines of Molecular Biology, Pathology, and Biophysics.

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14. ABSTRACT Breast cancer often occurs when the proteins that regulate normal epithelial cell division become dysregulated. This proposal examines the role of the cell cycle regulatory proteins, human cyclin-dependent kinase subunits (Cks1 and Cks2) in human breast cancer. The overexpression of Cks genes in breast tumor tissue and the role of Skp2 in tumorigenesis, suggests that Cks and Skp2 levels must be strictly regulated for proper cell cycling. We hypothesize that aberrant Cks protein expression and function contributes to breast carcinogenesis, at least in part, by its ability to interact with Skp2. In year one of this project, we have determined the levels of Cks mRNA and protein in (i) breast cancer cell lines and (ii) normal versus tumor breast tissue. We have also developed breast cancer cell lines that overexpress cks1, cks2, or skp2, and cells that co-overexpress cks1 and skp2. In the coming year, we will focus on characterizing the breast cancer cell lines developed that stably overexpress the aforementioned cell cycle proteins, using both in vitro and in vivo techniques.					
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INTRODUCTION:

A major challenge in the fight against breast cancer remains the ability to treat tumors that can escape currently used therapeutic regimens (i.e. drug resistance). Breast cancer often occurs when the proteins that regulate normal epithelial cell division become dysregulated. In this study, we examined the role of the cell cycle regulatory protein, called human cyclin-dependent kinase subunit 1 (Cks1) in human breast cancer. Specifically, we are investigating whether Cks1 expression alters the growth of breast cancer cells and/or sensitize breast cancer cells to the effects of chemotherapeutic agents.

BODY:

SOW: Determine if Cks1 cooperates with Skp2 in breast epithelial cell transformation. (Months 6-20).

1- Stable transfectants will be analyzed for cell cycle distribution and rate of spontaneous apoptosis.

In year 1, MCF-7 breast cancer cell lines stably expressing pcDNA3.1 vector alone control (termed Pc1, Pc2), and overexpressing Cks1 (termed C1a, C1b, C1c, C1d), Cks2 (termed C2a, C2c, C2e), Skp2, or Cks1+Skp2 were made (Figures 1A - C). In year 2, some of these cell lines were analyzed for growth abnormalities and cell cycle distribution. Growth curves were performed, and the proliferation rates of the Cks1 and Cks2 overexpressing clones were found not to significantly differ from the vector alone transfected/selected stable cell lines (Figure 2A). In year 1, we reported a strong correlation between Cks1 protein expression and disease free survival following adjuvant chemotherapy in patients with breast cancer. In support of this tumor data, we observe that cks1 overexpression in MCF-7 cells sensitizes them to the following chemotherapeutic agents: 5-fluorouracil (5-FU), methotrexate (MTX), and taxol. The expression of Cks1 significantly enhanced each drugs cytotoxicity in MCF-7 cells, as determined using the MTT assay, with the clone expressing cks1 to the highest degree (i.e. clone C1b) responding the best (Figures 2B-D. 1c). This effect seems to be specific to Cks1, since clones overexpressing Cks2 (i.e. clone C2c) did not show an improved response to these same chemotherapeutic drugs.

5-FU is widely used as a treatment for breast cancer but its clinical success is often accompanied by early mortality rates¹. More effective therapeutic strategies are still needed to enhance the clinical effectiveness of 5-FU; therefore we decided to focus subsequent experiments using 5-FU. Flow cytometry profiles of vector control (Pc1), Cks1 (C1a, C1b) or Cks2 (C2e) stable cells lines treated with vehicle alone or 5-FU are shown in Figure 3. MCF-7 stable cell lines treated with 5-FU (see Pc1/5FU) accumulate in S-phase. The percentage of cells in S-phase is further increased when the 5-FU treated cells overexpress cks1 (see C1a/5FU and C1b/5FU). Given the latter results and the fact that 5-FU-induced S-phase arrest has been shown in colorectal cancer to be preceded by apoptosis², we were prompted to look at markers of apoptosis, such as PARP cleavage and fragmented nuclei. In doing so, we found that the amount of cleaved PARP was enhanced in cks1 overexpressing clones as compared to vector control cells (Figure 4A), and that the amount of PARP cleavage was increased further in the 5-FU treated cks1 overexpressing clones as compared to control clones (Figure 4B). DAPI staining was also used to detect an increased number of apoptotic-fragmented nuclei in clone C1B, that expresses the highest amount of cks1, especially when treated with 5-FU (Figure 4C). Together, these data suggest that high levels of cks1 may activate an apoptotic pathway.

Since the major limitation to the clinical use of chemotherapeutic agents for the treatment of breast cancer is acquired or inherent resistance, we next tested whether overexpression of cks1 is sufficient to overcome resistance in breast cancer cell lines. To this end, we obtained 5-FU resistant MCF-7 (termed MCF7-5FU) or 5-FU-sensitive parental control MCF-7 (termed MCF7-WT) cells from the laboratory of Dr. Weiguang Wang. The 5-FU-resistant MCF7-5FU breast cancer cell line was generated by continuously culturing the MCF7-WT parental cell line in medium containing increasing concentrations of 5-FU in a stepwise procedure over 2 years³. Each of the obtained cell lines were infected with cks1 or cks2 adenoviruses and subsequently treated with vehicle or 5-FU and subjected to an MTT assay. We report that infection of MCF7-5FU with cks2 adenovirus has no effect of sensitivity of this cell line to 5-FU, while infection with cks1 adenovirus does overcome resistance, although to a modest degree (Figure 5A). To further test our hypothesis that cks1

sensitizes breast cancer cells to chemotherapeutic agents, MDA-MB-231 breast cancer cell lines stably expressing vector alone control (termed V), and overexpressing Cks1 (termed B and B2) were created. Consistent with the ability of cks1 to overcome resistance, we also find that the MTX-resistant MDA-MB-231 cell line can be resensitized to the effects of MTX by overexpressing cks1 (Figure 5B). The MDA-MB-231 clones overexpressing cks1 seem to have a higher apoptotic index as revealed by the increase in PARP cleavage and increase in numbers of abnormal nuclei in both untreated and MTX-treated cells, as compared to the vector control clone (Figures 5C and 5D).

2- Tumorigenicity of the stable transfectants will be evaluated *in vitro* by growth in soft agar, and *in vivo* using nude mice.

To test whether cks1, cks2, skp2, or cks1+skp2 expression in MCF-7 cells alters their *in vitro* transformation phenotype, defined by the ability of these cells to grow in soft agar, we measured the number of colonies formed in soft agar by the stable transfectants. There was no significant difference in colony number compared to the vector transfectants, thus no loss or gain of anchorage-independent growth of these cks1 overexpressing cells. There are some morphological differences in the colonies formed by the various stable transfectants (Figure 6). The colonies formed by the cells overexpressing Cks1 (clones c1a and c1d) or Cks2 (clones c2c and c2e) look similar to the vector control colonies (clone pc1), but the colonies formed by the cells overexpressing Skp2 are much smaller in size and do not form such well-defined spheres (clones SA and SB). Interestingly, the colonies produced by the cells that co-overexpress cks1 and skp2 (C1SL and C1SG) appear to grow in grape-like formations as opposed to well-shaped spheroid colonies, the potential significance of this is still under investigation.

We are also assessing the growth of cks1 overexpressing clones compared to vector alone control clone in nude mice. Cells from clone c1a and pc1 have been injected into nude mice, and we will assess the growth rate of these cells *in vivo* and also test the chemotherapeutic effects of 5-FU. We expect to complete these animal studies within the next two months.

KEY RESEARCH ACCOMPLISHMENTS:

- 1- Overexpression of Cks1 significantly sensitizes breast cancer cells to chemotherapeutic agents through the induction of apoptosis.
- 2- Cks1 overexpression overcomes resistance to chemotherapeutic agents.
- 3- No gain or loss of anchorage-independent growth by overexpressing cks1, cks2, skp2, or co-overexpressing cks1 and skp2 in MCF-7 cells.
- 4- Morphological differences in the colonies grown in soft agar from cells co-overexpressing cks1 and skp2.

REPORTABLE OUTCOMES:

Manuscripts:

1- del Rincón, S.V., Widschwendter, M., Ellederova, Z., Sun, D., Spruck, CH. Cks1 sensitizes breast cancer cells to chemotherapeutic agents. (in preparation 2007).

Public speaking:

1- November 2006 – I was interviewed by the San Diego Metropolitan Magazine – Feature breast cancer research.

LINK: <http://www.sandiegometro.com/2006/nov/medical3.php>

2- October 1st, 2007 – KUSI News (San Diego) – I was interviewed to speak about my research in breast cancer funded by the DOD-Breast Cancer Research program, and about the volunteer work I do for the Susan G. Komen Breast Cancer Foundation.

Awards received:

1- September 11, 2007 - I was honored as one of San Diego Metropolitan magazines Top 40 under 40 awardees, which recognizes young leaders in San Diego. I was nominated by the Sidney Kimmel Cancer Center for my goal to find a cure for breast cancer by doing research and also volunteering for the Susan G. Komen Breast Cancer Foundation.

LINK: <http://www.sandiegometro.com/2007/sep/40under40/delrincon.php>

CONCLUSION:

This study is the first to report that an elevated level of Cks1 in breast cancer cells sensitizes them to chemotherapeutic agents. Our results indicate that Cks1 is an important mediator of the therapeutic response of breast cancer cells that could potentially be exploited as a broad spectrum molecular target to sensitize breast cancer cells to the effects of chemotherapeutic agents. It will be important to pursue future experiments in year three to better define the molecular mechanism by which cks1 triggers an apoptotic pathway and if any specific apoptotic regulators are being induced by cks1. It is anticipated that the work outlined for year three, i.e. generating the cks1 interacting mutants, will serve as a useful tool to better examine this novel pro-apoptotic role of cks1.

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1. Longley, D.B., Harkin, D.P. & Johnston, P.G. 5-fluorouracil: mechanisms of action and clinical strategies. *Nature reviews* **3**, 330-338 (2003).
2. Yamane, N., Makino, M. & Kaibara, N. S-phase accumulation precedes apoptosis induced by preoperative treatment with 5-fluorouracil in human colorectal carcinoma cells. *Cancer* **85**, 309-317 (1999).
3. Wang, W., Cassidy, J., O'Brien, V., Ryan, K.M. & Collie-Duguid, E. Mechanistic and predictive profiling of 5-Fluorouracil resistance in human cancer cells. *Cancer research* **64**, 8167-8176 (2004).

SUPPORTING DATA:

Please find appended the following documents:

1- FIGURES described in the annual report.

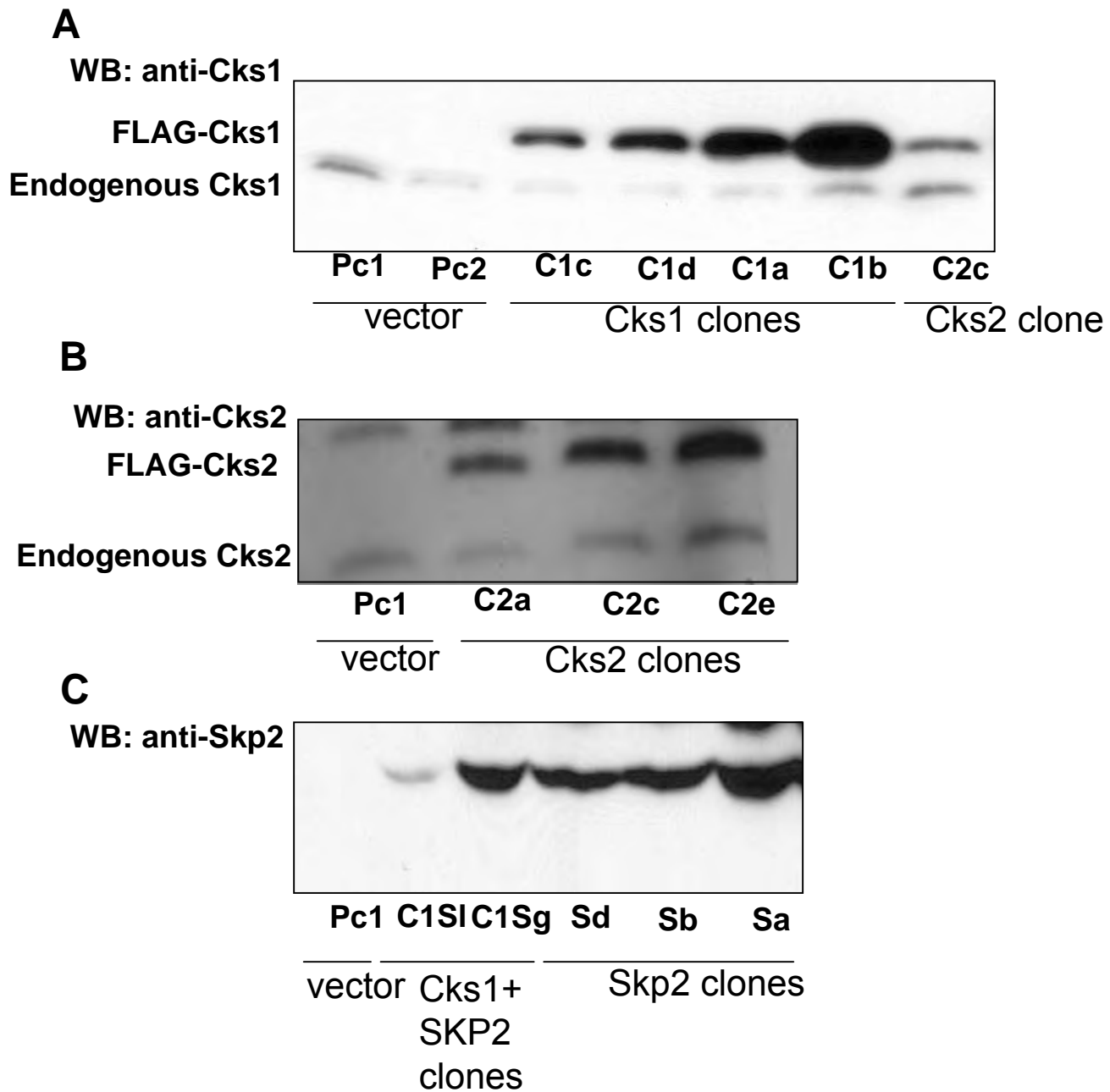


FIGURE 1: MCF-7 cells stably overexpressing cks1, cks2, or skp2.

- A.** Pc1 and Pc2 cells are MCF-7 cells stably expressing the empty vector pcDNA3. MCF-7 cells stably overexpressing cks1 are labelled: C1c, C1d, C1a, and C1b. The WB was probed with a cks1-specific antibody. There is some cross reactivity with cks2 (see C2c clone overexpressing cks2).
- B.** MCF-7 cells stably overexpressing cks2 are labelled: C2a, C2c, and C2e. The WB was probed with a cks2-specific antibody.
- C.** MCF-7 cells stably overexpressing skp2 are labelled: Sd, Sb, and Sa. MCF-7 cells stably overexpressing cks1 and skp2 are labelled: C1Sl, and C1Sg.

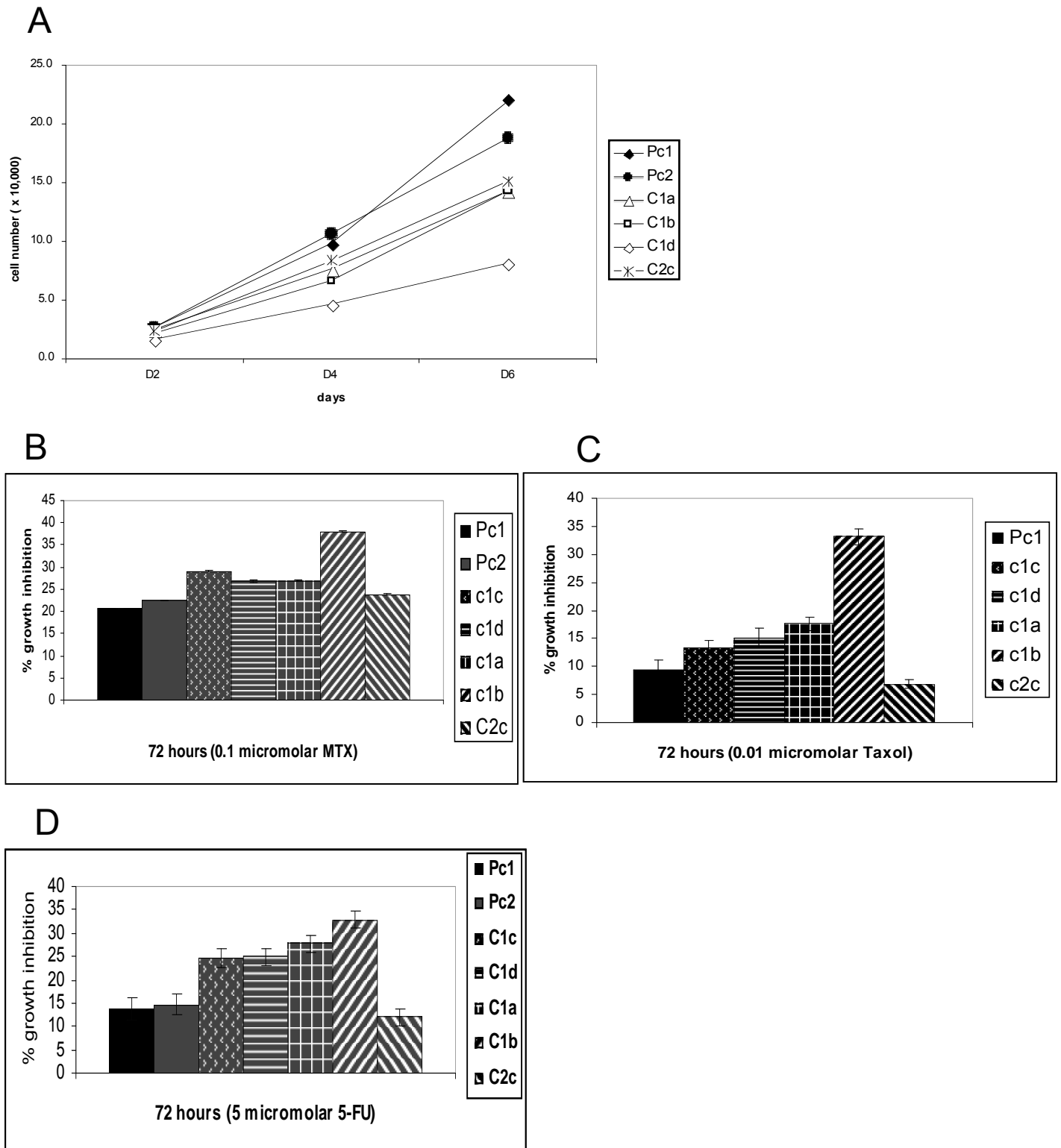


FIGURE 2: MCF-7 cells stably overexpressing cks1 are sensitized to chemotherapeutic agents.

A. Equal numbers of each cell line were seeded and a six day growth curve was done. Pc1 and Pc2 cells are MCF-7 cells stably expressing the empty vector pcDNA3. MCF-7 cells stably overexpressing cks1 are labelled: C1c, C1d, C1a, and C1b.

B-D. Equal numbers of each cell line were seeded and MTT assays were done after 3 day treatments with either 0.1 μ M MTX (**B**), 0.01 μ M Taxol (**C**), or 5 μ M 5-FU to assess the effects of overexpressing cks1 in MCF-7 cells.

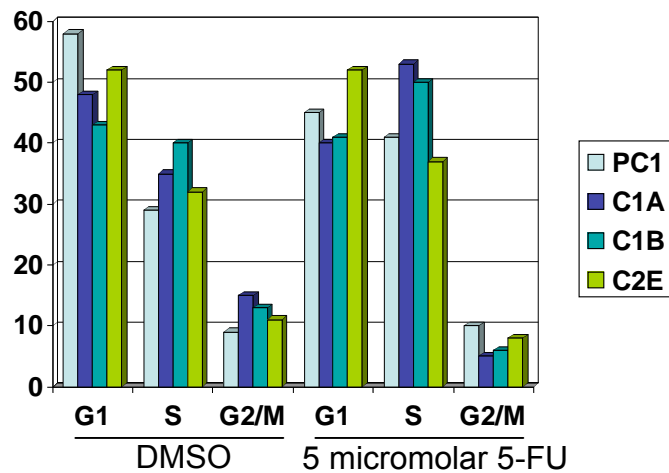
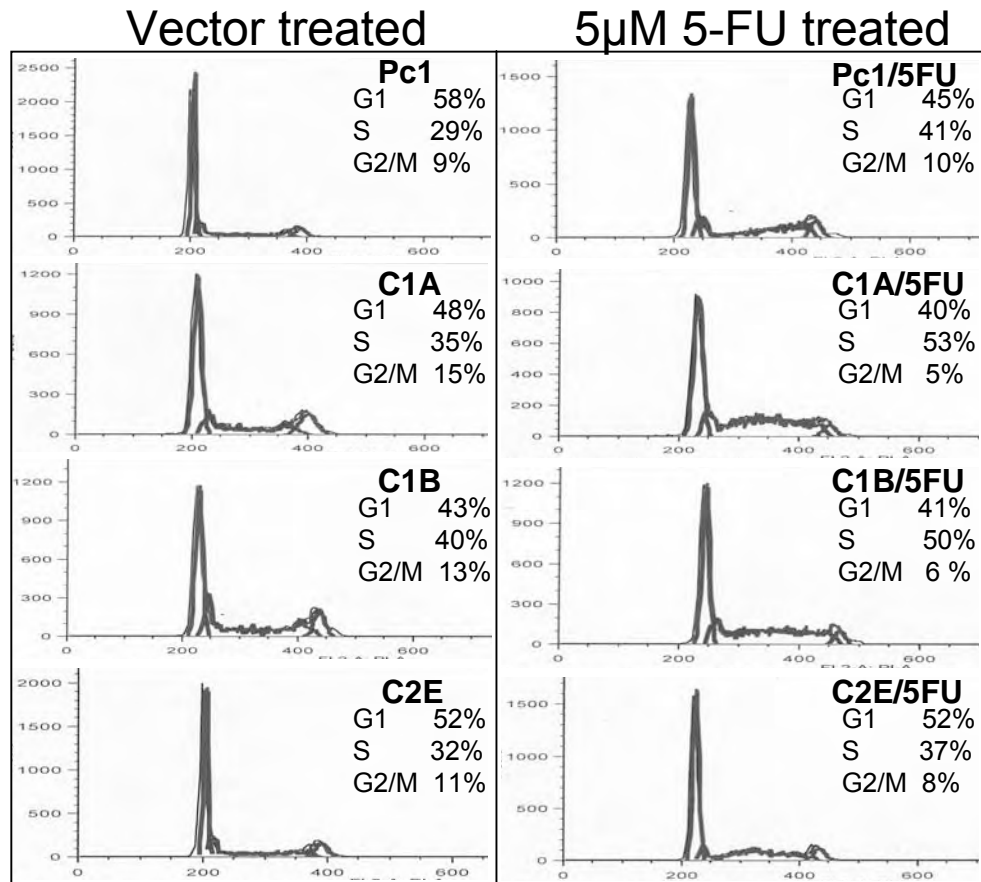


FIGURE 3: MCF-7 cells stably overexpressing cks1 accumulate in S-phase.

A. Equal numbers of each cell line were seeded, and subconfluent cells treated for 48 hours with vehicle or 5µM 5-FU. Cells were then harvested, stained with propidium iodide, and FACS analysis was done.

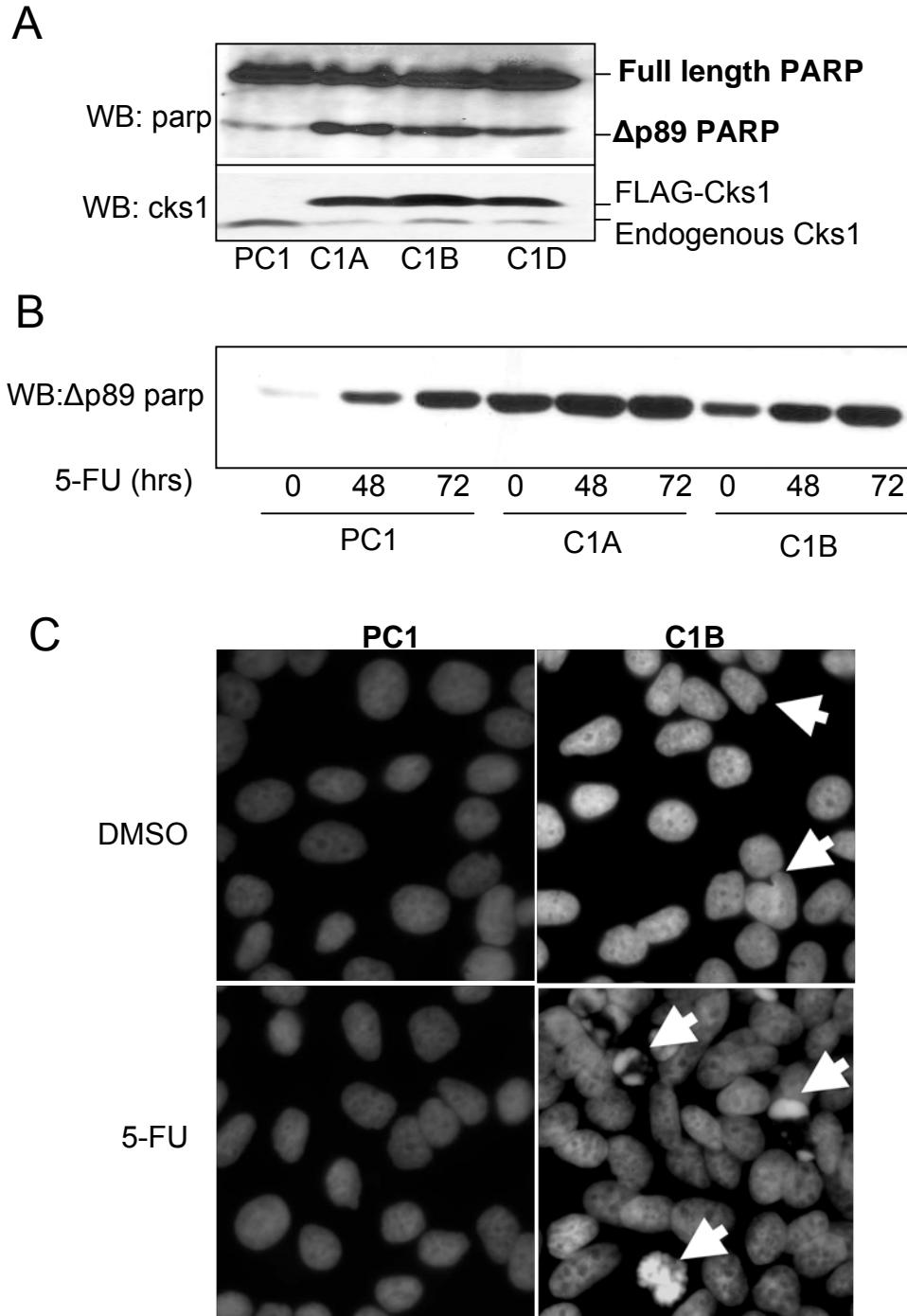


FIGURE 4: MCF-7 cells stably overexpressing cks1 have a higher apoptotic index.

- A. Western blot showing that full length parp is cleaved more in the cell lines overexpressing cks1 (C1A, C1B, C1D) as compared to the vector control cell line (PC1).
- B. Western blot of cleaved PARP showing that there is more cleaved PARP in untreated C1A and C1B clones as compared to the control Pc1 clone. Each cell line was treated with 5 μ M 5-FU for 48 and 72 hours, and more cleaved PARP was again detected in the clones overexpressing cks1.
- C. DAPI staining was done on PC1 and C1B to detect nuclear integrity. Arrow heads depict abnormal nuclei (fragmented, dumbbell-shaped) in untreated C1B and 5-FU-treated C1B.

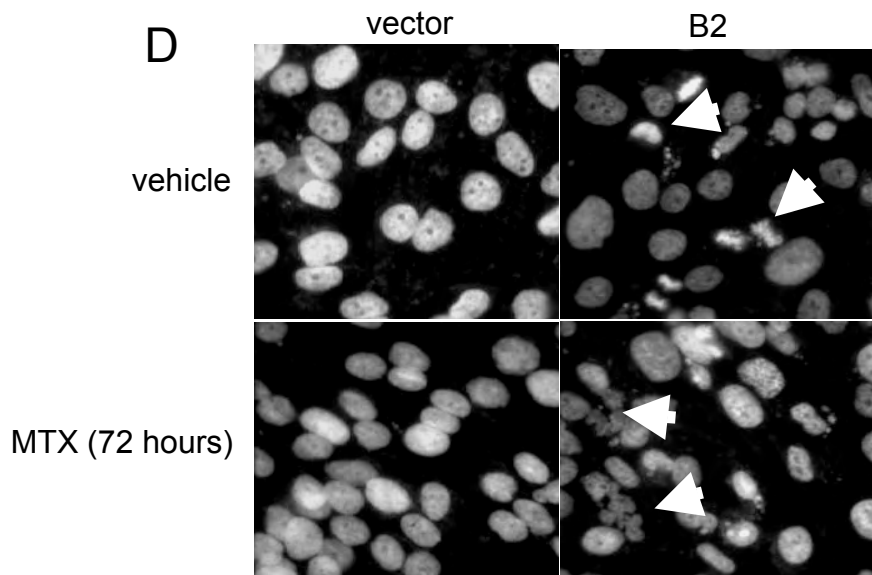
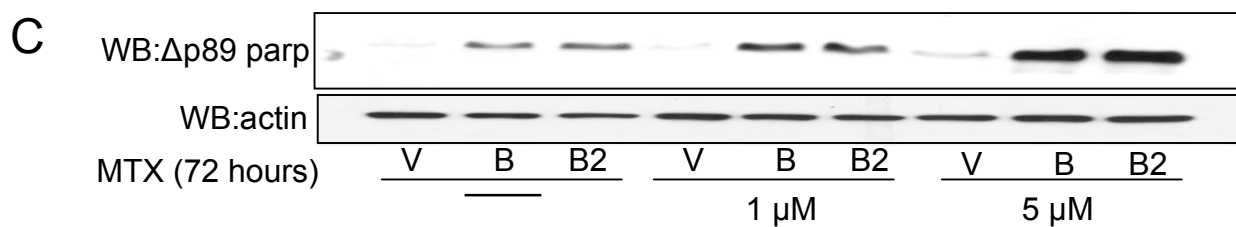
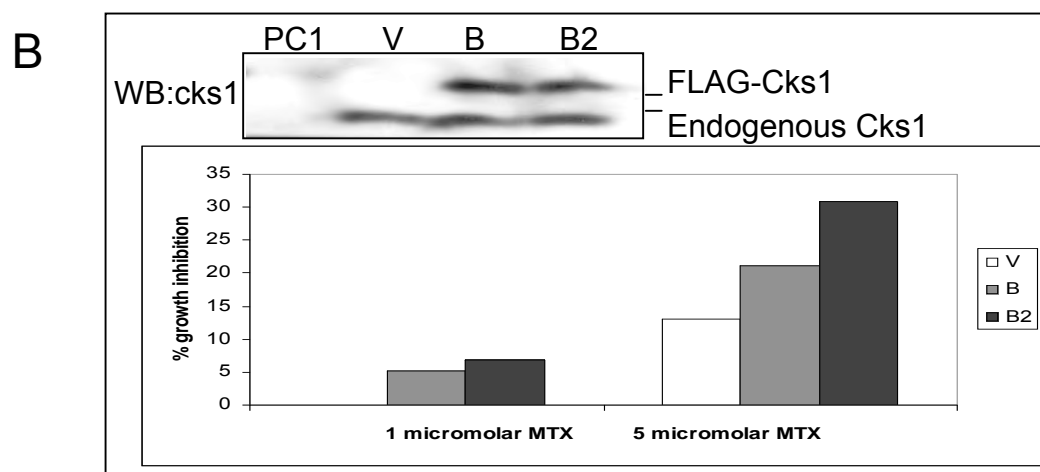
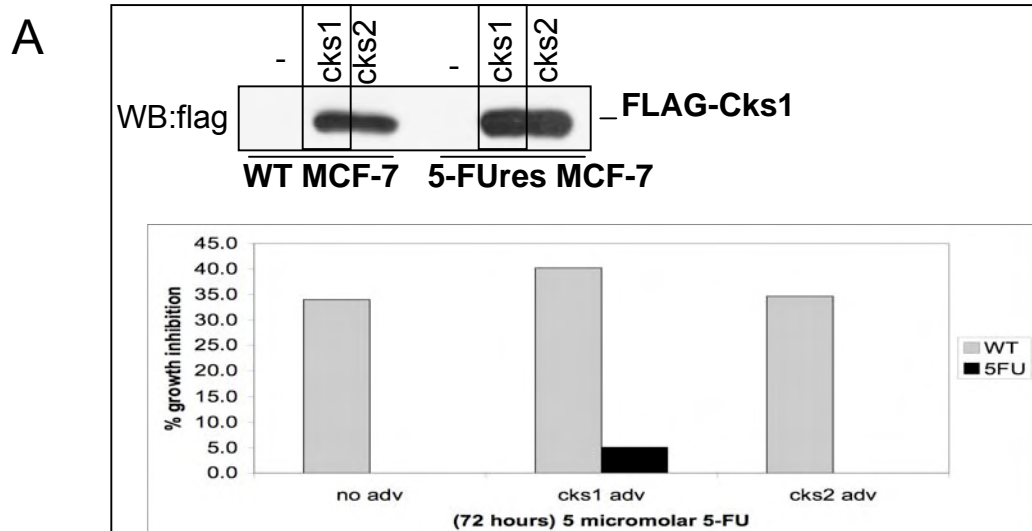
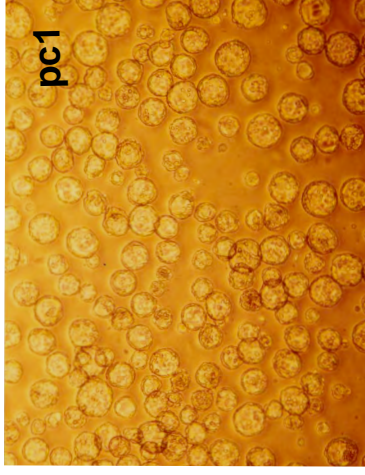


Figure 5

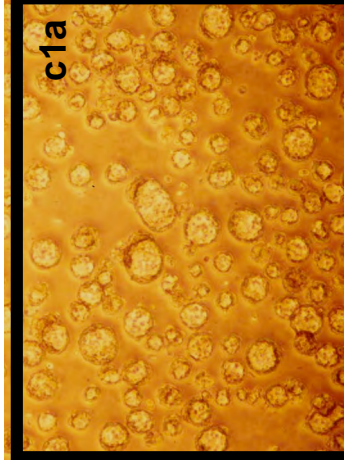
FIGURE 5: MCF-7 cells stably overexpressing cks1 can overcome resistance to chemotherapeutic agents.

- A. Parental and 5-FU-resistant MCF-7 cells were infected with flag-cks1 or flag-cks2 adenovirus. The infected cells were subsequently treated for 72 hours with 5 μ M 5-FU and MTT assays done. Shown at the top is a anti-flag western blot showing the cells have been infected. Shown below is the MTT assay.
- B. Vector control (clone V) or cks1 (clones B and B2) were stably expressed in MDA-MB-231 cells. Shown at the top is a cks1-specific western blot. Shown below is an MTT assay showing that clones B and B2 are responsive to 1 μ M MTX, while clone V is not.
- C. Western blot showing more cleaved PARP in the cks1-expressing MDA-MB-231 clones.
- D. DAPI staining was done on V and B2 to detect nuclear integrity. Arrow heads depict abnormal fragmented nuclei in untreated B2 and MTX-treated B2.

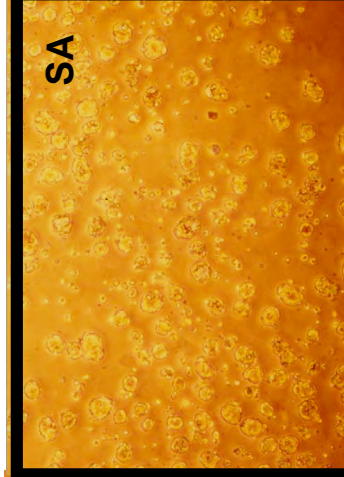
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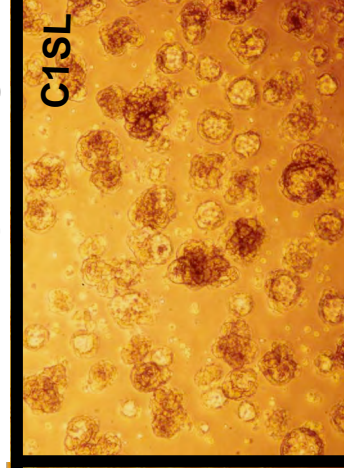
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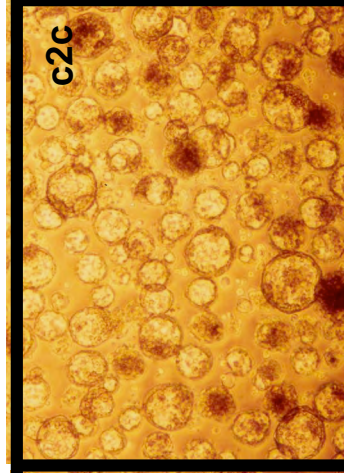
SKP2 overexpressing



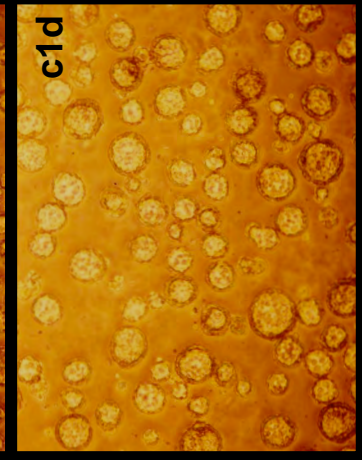
CKS1 + SKP2 overexpressing



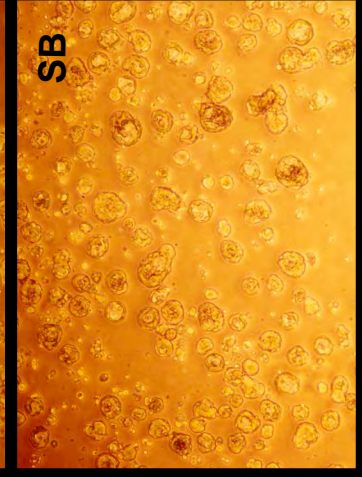
CKS2 overexpressing



c1d



SB



C1SG



c2e

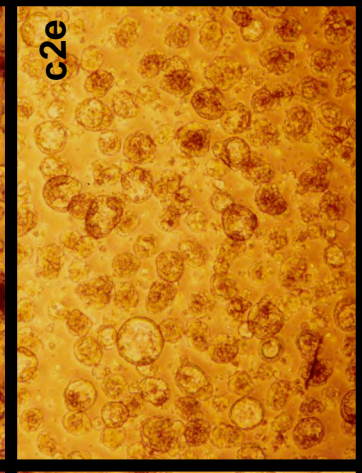


Figure 6

FIGURE 6: Soft agar assays showing morphological differences in the MCF-7 cells stably overexpressing cks1, skp2, or both cks1+skp2.

- A. Pc1 are MCF-7 cells stably expressing the empty vector pcDNA3. MCF-7 cells stably overexpressing cks1 are labelled: C1d, C1a. MCF-7 cells stably overexpressing cks2 are labelled: C2c, C2e. MCF-7 cells stably overexpressing skp2 are labelled: Sb, Sa. MCF-7 cells stably overexpressing cks1 and skp2 are labelled: C1Sl, and C1Sg.