High Cks1 expression in transgenic and carcinogen-initiated mammary tumors is not always accompanied by reduction in $p27^{Kip1}$

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Abstract. Cks1 plays an essential role in SCF^{Skp2}-mediated ubiquitination, and consequently turnover, of the cdk2 inhibitor and tumor supressor $p27^{Kip1}$. High Cks1 expression is associated with aggressive breast tumors and correlates with low $p27^{Kip1}$ levels in some cases, although it is also an independent prognostic marker for survival, and provides predictive information in addition to that provided by $p27^{Kip1}$ alone. In this report we demonstrate that Cks1 protein and mRNA are elevated to very high levels in mammary tumors initiated by erbB2, c-myc and polyoma middle-T (PyMT) in transgenic mice, whereas Cks1 protein is hardly detectable in the normal mammary epithelium. Cks1 is also highly up-regulated in rat mammary tumors initiated by methylnitrosourea (MNU). Despite high levels of Cks1 expression, $p27^{Kip1}$ levels were not reduced, and were in fact slightly higher in mammary tumors initiated by erbB2, PyMT and MNU. In contrast mammary tumors from MMTV-c-myc mice did exhibit low $p27^{Kip1}$ and higher levels of Skp2. Together, these data suggest that deregulated Cks1 expression might play roles in oncogene and carcinogen-initiated mammary tumorigenesis independent of $p27^{Kip1}$ turnover in certain tumors. Stable overexpression of Cks1 in human breast carcinoma MCF-7 cells did not significantly reduce $p27^{Kip1}$ expression, although it conferred resistance to Faslodex (ICI 182780)-mediated inhibition of colony outgrowth in these cells. In contrast, Cks1-depleted MCF-7 cells formed fewer colonies in estrogen-containing medium. Therefore, our studies also suggest that Cks1 levels regulate the responsiveness of ER^+ breast cancers to estrogens and anti-estrogens.

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Introduction

Cyclin kinase subunit 1 (Cks1) is a small protein whose expression is strongly associated with aggressive breast tumors, prostate carcinomas, non-small cell lung cancers, and several other malignancies (1-4). Cks1 was shown to associate with Skp2, a subunit of the SCF^{Skp2} ubiquitin ligase which mediates the ubiquitin-dependent proteolysis of the cdk inhibitor $p27^{Kip1}$ in G_1/S-phase (5,6). Skp2 serves as the specificity component for the SCF^{Skp2} ligase and binds to $p27^{Kip1}$ in a manner that depends on $p27^{Kip1}$ phosphorylation, and on Cks1 (7-9). $p27^{Kip1}$ is also a tumor suppressor protein that works in a dosage-dependent manner (10,11), and analysis of the $Cks^{-/-}$ cells has revealed that depletion of Cks1 increases $p27^{Kip1}$ protein levels (9). Low levels of the cdk2 inhibitor $p27^{Kip1}$ have been demonstrated in a number of studies to be associated with reduced overall and disease-free survival in breast cancer patients (12-14). Interestingly however, other studies show that $p27^{Kip1}$ is overexpressed in a subset of highly proliferative breast carcinomas (15), and this was associated with strong expression of cyclin D1 and ER positivity (16).

Although the role of Cks1 expression in breast cancer, remains to be elucidated, recently Slotky *et al* have demonstrated that Cks1 expression in human breast cancer was associated with aggressive features and inversely correlated with $p27^{Kip1}$ (1). Interestingly, high expression of Cks1 has also been reported in non-small cell lung carcinomas, although it was not inversely related with $p27^{Kip1}$ expression, suggesting that Cks1 may mediate some of its functions via Skp2 and $p27^{Kip1}$-independent mechanisms (3). Recent global expression profiling experiments have also shown high levels of Cks1 mRNA in mammary tumors from the polyoma middle-T (PyMT)-initiated transgenic model (17). Thus, deregulation of Cks1 expression is likely to be an important hallmark of

mammary transformation. However, whether Cks1 protein levels are also induced, and whether $p27^{Kip1}$ levels are also altered in concert, have not been evaluated in these or other models of mammary tumorigenesis. In this report we show that Cks1 protein is highly elevated in mammary carcinomas initiated by the PyMT oncogene in a bitransgenic mouse model. High Cks1 was, however, not associated with decreased $p27^{Kip1}$. Instead $p27^{Kip1}$ levels were higher in these tumors, and were accompanied by decreased Skp2 expression as well. Similarly we also found that Cks1 was also highly elevated in tumors initiated by erbB2, c-Myc, and the carcinogen MNU. However, $p27^{Kip1}$ levels were reduced only in c-Myc initiated tumors. We also assessed the role of Cks1 in estradiol (E_2) dependent pathways in a MCF-7 derived ER^+ breast carcinoma line. We demonstrate that forced ectopic Cks1 expression in these cells confers substantial resistance to the anti-estrogen ICI 182780, although it did not substantially alter $p27^{Kip1}$ levels. In contrast shRNA-mediated depletion of Cks1 in MCF-7 markedly diminished colony formation in estrogen-containing complete medium.

Materials and methods

Materials and chemicals. ICI 182780 (Faslodex®) was a gift from AstraZeneca (Macclesfield, Cheshire, UK) and was dissolved in ethanol prior to use. Improved modified Eagle's medium (IMEM) medium was obtained from Mediatech (Manassas, VA).

Mammary tumors from transgenic mouse models. Murine mammary tumors used in these studies were obtained from three separate models. A bitransgenic mouse model established in our laboratory harbors the tet-transactivator rtTA transgene which is under control of the C3(1) 5' flanking region derived from the C3(1) component of prostatic steroid binding protein (PSBP) (Thottassery JV, *et al*, Proc Am Assoc Cancer Res 42: abs. 913, 2001). In addition these mice have a tetracycline response element (TRE) regulated 1.4 kb sequence encoding the PyMT protein. Mammary tumors were excised from females that were given Dox in the drinking water from 80 to 120 days. MMTV-ErbB2 female mice were obtained from the laboratory of Dr Mike Ruppert (UAB) and mammary tumors, and uninvolved mammary glands, were excised from these mice. MMTV-c-Myc mice have been described before (18). All mice were housed and cared for in AAALAC-accredited facilities in accordance with guidelines established by the NIH.

Mammary tumors from MNU-treated rats. Methylnitrosourea (75 mg/kg BW) was administered via the jugular vein to a female Sprague-Dawley rat at 50 days of age. Mammary tumors were removed at 93 days after carcinogen treatment.

Cks1 shRNA plasmid construction and colony formation studies. To construct a shRNA expression vector targeting Cks1 we used a U6-promoter containing expression plasmid called pSub201-U6.1/Neo previously derived from the plasmid pRNAT-U6.1/Neo (from GenScript, Piscataway, NJ). The vector contains a G418 resistance marker which facilitates enforced expression of the shRNA. Two oligonucleotides with *Bam*HI and *Hin*dIII cohesive ends targeting a sequence in Cks1 were generated. The sequence of the top strand was: 5'-GATCCTCT GATGTCTGAATCTGAATT TTCAAGA GAAATTCA GATTCAGACATCAGA TTTTTA. The sequence incorporates a 21-bp sense and antisense strand flanking a 9-bp hairpin loop (underlined). The top and bottom strand oligonucleotides were annealed, phosphorylated and ligated into the pSub201-U6.1/Neo vector.

Colony formation studies were done with this plasmid, and the parental control plasmid to examine whether depletion of Cks1 in MCF-7 breast carcinoma cells will affect their ability to form colonies in G418-medium containing 10% FBS. We used 10% FBS because it contains the full estrogen complement (estradiol, estriol and estrone). MCF-7 cells ($2x10^5$) were transfected with the pSub201-U6.1/Neo control plasmid or the pSub201-U6.1/Neo-Cks1 shRNA plasmid using Fugene HD (Roche) and were then exposed to G418 (1 mg/ml) in 10% FBS containing medium. Colonies formed were fixed and stained by incubation with 0.05% crystal violet, washed with deionized water and photographed.

Cks1 expression plasmid construction and stable transfections. To test whether overexpressing Cks1 can affect the inhibition of ER^+ breast cancer cell growth by anti-estrogens we constructed an expression plasmid for Cks1 driven by the strong CMV promoter called pIIB-CMV-Cks1. This plasmid contains a synthetic intron and a tandemly linked internal ribosome entry site (IRES) downstream from the full length Cks1 cDNA. This is followed by a blasticidin resistance marker (bsdR). MCF-7 cells ($1.2x10^6$) were plated in 10-cm tissue culture dishes and were transfected the next day either with pIIB-CMV-Cks1 or the empty vector pIIB-CMV. The cells were then exposed to blasticidin (3 μg/ml) and drug-resistant pools were isolated.

Cells ($3x10^4$) of stable polyclonal populations of pIIB-CMV-Cks1 or pIIB-CMV transfectants were plated in 6-well dishes in 5% FBS containing medium and exposed to increasing doses of ICI 182780 for 2 weeks in the presence of blasticidin. The medium was changed every 5 days. Colonies formed were fixed and stained by incubation with 0.05% crystal violet, washed with deionized water and photographed. Colonies were counted using a Scienceware® colony counter (Bel-Art Products, Pequannock, NJ).

Western immunoblotting. For the analysis of Cks1, Skp2, $p27^{Kip1}$ etc., Western immunoblotting was performed on lysates prepared from mammary tumors or MCF-7 transfectant pools. In general, 40 μg of cell lysate was run on Criterion® pre-cast 10% SDS-PAGE gels (Bio-Rad, Hercules, CA). Proteins were transferred onto Protran® filters (Bio-Rad, Hercules, CA) by electroblotting and blots were probed with antibodies. Enhanced chemiluminescence detection with the Supersignal West Pico kit was used (Pierce, Rockford, IL).

QRT-PCR analysis of Cks1 in mammary tumors. To compare the levels of Cks1 mRNA in mammary tumors versus normal mammary tissue from transgenic mice a qRT-PCR approach was used. Total RNA was made from snap-frozen excised tumor fragments and the quality of the RNA was assessed on agarose gels prior to running the RT-PCR reactions. Murine

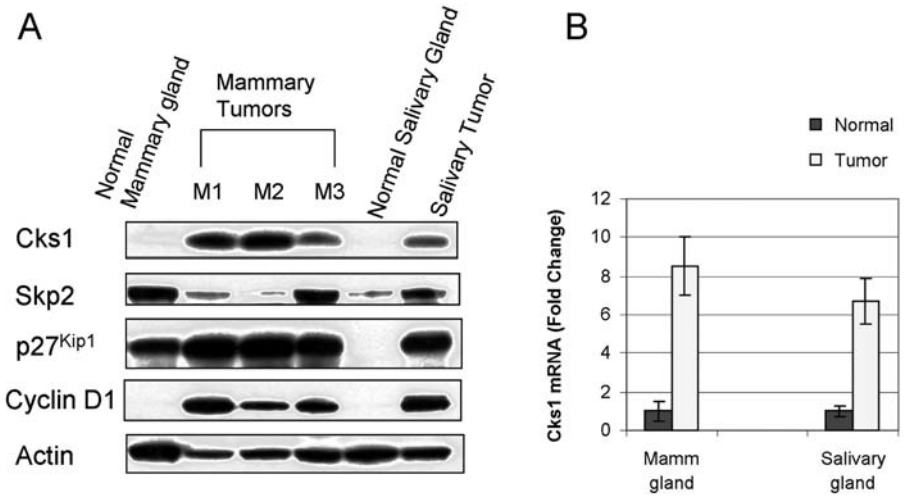

Figure 1. Cks1 is highly expressed in PyMT-initiated mammary adenocarcinomas in bitransgenic mice. A, Mice were given Dox in their drinking water to induce PyMT for 80-120 days and mammary tumors were excised. Protein extracts from mammary and salivary tumors and uninvolved mammary and salivary glands were run on SDS-PAGE gels and were transferred to nitrocellulose membranes and analyzed by immunoblotting with antibodies against Cks1, Skp2, $p27^{Kip1}$, cyclin D1 and actin. B, QRT-PCR analysis of Cks1 specific transcripts in mammary tumors and salivary tumors from bitransgenic mice. Total RNA was made and analyzed by qRT-PCR as described in Materials and methods.

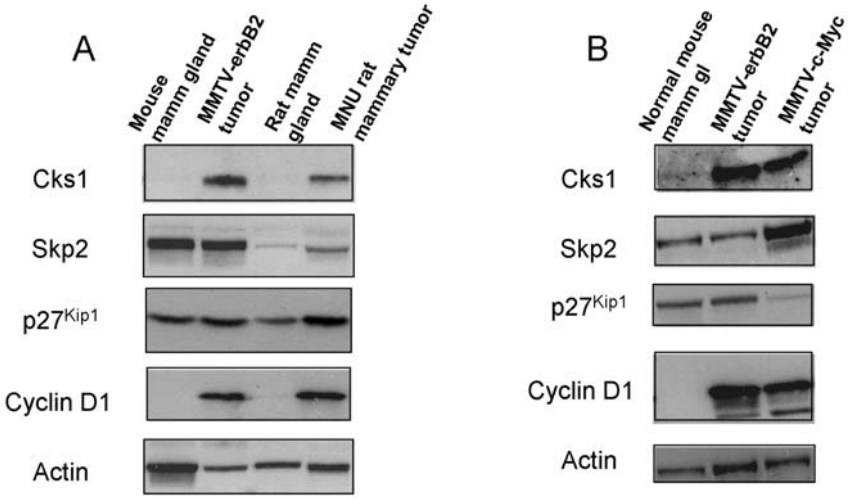

Figure 2. Cks1 is highly expressed in erbB2 and in c-Myc-initiated mammary adenocarcinomas. Cks1 expression is also induced in MNU-initiated rat mammary carcinomas. Protein extracts from mammary tumors and normal glands from MMTV-erbB2 mice and rats treated with MNU (75 mg/kg), A and B, and from MMTV-c-Myc mice; B, were run on SDS-PAGE gels and were transferred to nitrocellulose membranes and analyzed by immunoblotting with antibodies against Cks1, Skp2, $p27^{Kip1}$, cyclin D1 and actin.

Cks1 specific TaqMan primer-probe sets were obtained (Applied Biosystems). Reactions were carried out in an MJ Cycler using the Opticon monitor software. The comparative C_T method was used to represent the relative level of either Cks1 transcripts. The relative expression level is expressed as a unitless number and calculated as $2^{-\Delta\Delta T}$ as previously described (19).

Results

Cks1 protein and mRNA are overexpressed in murine transgenic models of mammary tumorigenesis. We have established a bitransgenic mouse model where the mammary expression of PyMT can be controlled by providing doxycycline (Dox) in the drinking water. These mice harbor two transgenes, a C3(1) promoter driven reverse tet-transactivator (rtTA) and a tetracycline response element (TRE) controlled PyMT. The transforming ability of PyMT is related to its ability to bind and activate signal transduction proteins that have been implicated in human breast cancer (20,21). Importantly, PyMT-initiated mammary tumors have recently been shown to be ER^+ and demonstrate estrogen-dependent growth *in vivo* and also *in vitro* in cells from explants (22,23).

Loss of $p27^{Kip1}$ has been demonstrated to enhance the rate of onset of erbB2 induced mammary tumorigenesis in transgenic mice and reduced $p27^{Kip1}$ expression is frequently detected in many human cancers, including breast cancers (24,25). Since $p27^{Kip1}$ levels are primarily regulated by its ubiquitination by the SCF^{Skp2} ubiquitin ligase and subsequent proteolysis, we assessed the levels of $p27^{Kip1}$, Skp2 and Cks1 in mammary carcinomas initiated by PyMT in our bitransgenic mouse model. Interestingly, we find that $p27^{Kip1}$ levels are not reduced, but are instead induced, reproducibly, in PyMT-initiated tumors (Fig. 1A). We also found that Skp2 levels were reduced in some of these tumors relative to the

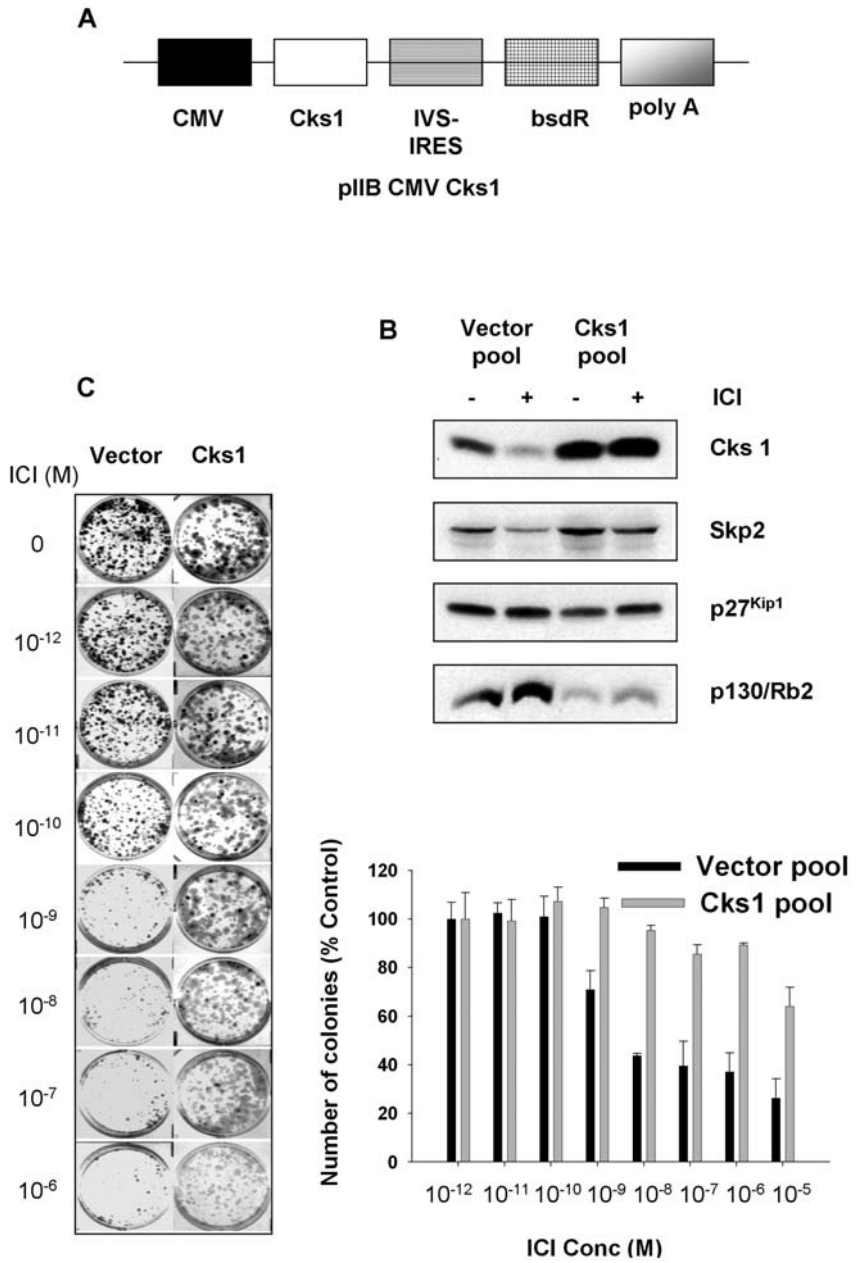

Figure 3. Stable overexpression of Cks1 in MCF-7 breast carcinoma cells decreases p130/Rb2 levels and causes anti-estrogen resistant colony formation. A, Schematic of the expression vector pIIB-CMV-Cks1 that was stably transfected into MCF-7 cells to generate polyclonal pools overexpressing Cks1. B, ICI 182780 mediated up-regulation of p130/Rb2 occurs in vector-transfected pools of MCF-7 cells but is decreased in stably transfected cells overexpressing Cks1. C, Dose response of ICI 182780 effects on colony formation in Cks1 overexpressing pools versus vector-transfected control MCF-7 cells. $3x10^4$ cells from each line were plated in 6-well dishes and subsequently treated over two weeks with the indicated concentrations of ICI 182780. The colonies were the fixed, stained, counted and photographed as described in Materials and methods. Means ± SD of three experiments are graphically represented.

normal mammary gland (Fig. 1A). Cyclin D1 levels were also greatly elevated in mammary tumors. Most remarkably Cks1 levels were markedly enhanced in these tumors, unlike Skp2, suggesting that Cks1 plays a role in mammary oncogenic pathways, possibly through Skp2-independent mechanisms (Fig. 1A). Cks1 protein levels, on the other hand, were minimal in the normal mammary gland unlike Skp2 (Fig. 1A). To assess the relative expression of Cks1 mRNA in tumors versus the normal gland we performed a qRT-PCR assay. As shown in Fig. 1B, the increases in immunodetectable Cks protein in the mammary tumors were accounted for by increases in Cks1 mRNA. The PyMT-transgenic mice also exhibit salivary gland tumors and likewise we also found highly elevated Cks1 protein and mRNA in salivary gland tumors as compared to the normal gland. The normal salivary gland does not have detectable $p27^{Kip1}$ protein, although $p27^{Kip1}$ was greatly elevated in salivary tumors.

We also examined whether the increases in Cks1 exhibited by mammary tumors initiated by the PyMT oncogene could be generalized to other initiating agents by doing Western analyses with lysates obtained from tumors excised out of MMTV-erbB2 and MMTV-c-Myc mice, and also from tumors formed in rats treated with the carcinogen MNU. As shown in Fig. 2, Cks1 protein was elevated in all of these tumors. Once again $p27^{Kip1}$ was slightly elevated in tumors from the MMTV-erbB2 model and the MNU-treated rats (Fig. 2A).

However, $p27^{Kip1}$ levels were lower in tumors from the MMTV-c-Myc mice as compared to the normal gland, and interestingly these mice also had elevated Skp2 levels (Fig. 2B). Cyclin D1 levels were highly upregulated in all three tumor models.

Ectopic Cks1 overexpression causes a decrease in basal and ICI 182780-induced p130/Rb2 levels and also induces ICI 182780-resistant colony formation. To address whether Cks1 overexpression might alter $p27^{Kip1}$ and thereby decrease responsiveness to anti-estrogens in human breast cancer, a MCF-7 line that constitutively overexpresses Cks1 was established. An expression vector that enforces Cks1 expression by use of an IRES-coupled antibiotic resistance marker was employed (Fig. 3A). To avoid effects due to clonal variation, polyclonal populations of cells stably transfected with this expression vector or the empty vector were isolated. Both the vector pool and the Cks1 overexpressing pool were first characterized with respect to Cks1 expression, and also expression of Skp2, $p27^{Kip1}$ and p130/Rb2. As shown in Fig. 3B, ectopic Cks1 expression was high in the Cks1 pool as determined with the anti-Cks1 antibody. A 72-h treatment with ICI 182780 markedly decreases Cks1 in the vector pool but not in the Cks1 transfectants (Fig. 3B and data not shown). Skp2 levels under basal and ICI 182780 treated conditions were slightly higher in the Cks1 pool, and consistently p130/Rb2 levels were markedly decreased in the Cks1 pool under both conditions. However, $p27^{Kip1}$ was only marginally decreased in the Cks1 pool relative to the vector pool. Levels of $p27^{Kip1}$ increased somewhat upon ICI 182780 treatment, although they did not reach the levels in ICI 182780-treated vector-transfected cells (Fig. 3B).

We also performed a clonogenic assay with increasing doses of ICI 182780 to assess the effects of Cks1 overexpression on anti-estrogen effects in long-term growth. As shown in Fig. 3C, ICI 182780 decreased colony formation in both vector transfectants as well as Cks1 transfectants, although higher doses were required for equivalent suppression of clonogenicity in the Cks1 overexpressors. We also found that following a 72-h treatment of the vector-transfected pool with ICI 182780 the proportion of cells in G_1/G_0-phase increased from 52.73±3.19 to 81.06±1.96. On the other hand, a 72-h treatment of the Cks1 overexpressing pool with the anti-estrogen only resulted in an increase in the G_1/G_0-phase from 54±1.7 to 65.2±1.07 ($P<0.05$).

shRNA-mediated Cks1 depletion in MCF-7 breast carcinoma cells abrogates colony formation in estrogen-containing medium. MCF-7 cells are ER^+ breast carcinoma cells that require estrogens in the medium for robust colony formation. Our previously published studies have demonstrated that Cks1 contributes to multiple essential roles during cell cycle progression in MCF-7 cells (26). In the current study we tested whether Cks1 abrogation could affect MCF-7 colony formation in complete medium. Since long-term colony-forming studies cannot be carried out with cells transfected with siRNA duplexes we constructed a Cks1 shRNA expression vector containing a G418 marker. Selection in G418-containing complete medium showed that Cks1 depleted cells formed significantly fewer colonies than the control vector transfected cells (Fig. 4A). We also performed Western analyses with the remaining colonies in the Cks1-shRNA transfected population (Fig. 4B). The results show that remnant colony outgrowth in the Cks1-shRNA transfected population occurs probably because Cks1, although substantially reduced, was not completed depleted in these cells. This could be either because the U6 promoter does not drive expression of Cks1 shRNA strongly, or the expressed sequence was not very efficient in completely silencing Cks1 in these cells.

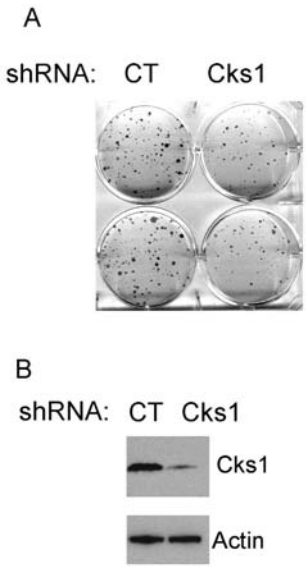

Figure 4. Depletion of Cks1 in MCF-7 breast carcinoma cells abrogates colony formation in estrogen-containing medium. A, MCF-7 breast carcinoma cells were transfected with the pSub201-U6.1/Neo control plasmid or the pSub201-U6.1/Neo-Cks1 shRNA plasmid using Fugene HD (Roche) and were then exposed to G418 (1 mg/ml) in 10% FBS containing medium. Colonies formed were fixed, stained and photographed. B, Protein extracts from G418-resistant colonies were run on SDS-PAGE gels, transferred to nitrocellulose membranes and analyzed by immunoblotting with antibodies against Cks1 and actin.

Discussion

In this report we have shown that Cks1 protein and mRNA are highly elevated in murine mammary tumors initiated by the oncogenes erbB2, c-Myc or PyMT, and in a carcinogen-initiated rat mammary tumor model, suggesting that de-regulated Cks1 expression might play an important role in mammary epithelial transformation by these agents. Cks proteins were originally identified as suppressors of mutations in the yeast cdk1 genes $p34^{cdc2}$ and $p36^{cdc28}$ (27-29). The *S. cerevisiae* Cks1 gene and its *S. pombe* homolog $p13^{Suc1}$ were both shown to be physically associated with cdk1 and with active cyclin-cdk1 complexes (29). However, the biochemical functions of Cks1 had remained elusive until the recent demonstration by different groups that it played an essential role in $p27^{Kip1}$ degradation, by promoting the interaction of Skp2 with $p27^{Kip1}$, and facilitating its ubiquitylation (8,9). Previous studies have implicated $p27^{Kip1}$ as an inhibitor of erbB2 signalling (30). These studies have also suggested that c-Myc functions by abrogating the function of $p27^{Kip1}$ (30). Studies have also implicated that $p27^{Kip1}$ might be a tumor suppressor (31). However, it is rarely mutated in cancers and $p27^{-/-}$ mice do not show enhanced spontaneous tumor onset with the exception of pituitary adenomas (11). The $p27^{+/-}$

mice do exhibit multiple tumors when challenged with radiation or carcinogens. In these tumors $p27^{Kip1}$ is neither mutated nor silenced and therefore it is considered a haploinsufficient tumor suppressor.

Increases in Cks1 expression have been shown in a number of human malignancies (1-4). Increase in Cks1 mRNA has also been shown in PyMT-initiated mammary tumors (17), and in urethane and ENU-initiated lung tumors in rasH2 mice (32). Although it has generally been implied that the role of Cks1 in transformation is related to its essential role in $p27^{Kip1}$ degradation our studies suggest that this is not necessarily the case in all tumors. Indeed, our studies suggest that $p27^{Kip1}$ levels were in fact slightly higher in mammary tumors initiated by erbB2, PyMT and MNU. This apparent discrepancy could be reconciled by recognizing that in addition to its cdk2-inhibitory effects, $p27^{Kip1}$ also functions as an assembly factor for cyclin D/cdk 4/6 complexes, thereby activating cyclin D1 dependent kinases (33). Therefore, the coordinate upregulation of cyclin D1 and $p27^{Kip1}$ seen in these models could play an important role in mammary epithelial transformation by stimulating proliferative signaling induced by cyclin D1 dependent kinases.

What then could be the molecular consequences of Cks1 protein elevations during mammary transformation? Cks proteins also have roles in stimulating the phosphorylations of G_2/M regulators such as cdc2 kinases Wee1 and Myt1 and also the phosphatase Cdc25 (34). Cdc25 mediated dephosphorylation has also been shown to play a crucial role in Cdk2 activation. Thus, it is possible that Cks1 also has other roles in G_1 distinct from its role as an adaptor molecule that facilitates substrate recruitment to the SCF^{Skp2} complex in $p27^{Kip1}$ degradation. In this respect it is also important to note that Deshaies and co-workers have shown that Cks1 plays an essential role in G_1 cyclin Cdk activity in yeast (35). Previously published results from our laboratory have also demonstrated a role for Cks1 in the expression of cdc2 (26). The binding of cyclin B to Cdk1 and cyclin A to Cdk2 has also been shown to enhance Cdk binding to Cks2 suggesting that cyclins and Cks proteins interact cooperatively with Cdks (36). In addition, Cks proteins have stimulatory effects on the Cdc27 component of anaphase promoting complex/cyclosome (APC/C) complex, another ubiquitin ligase that degrades mitotic regulators such as cyclin B and securin (34,37,38). Recently Cks1 was also shown to increase the transcription of Cdc20, a protein which activates APC/C by targeting it to distinct substrates to allow cells to exit from mitosis (39). Therefore Cks1 could likely play important roles in mammary epithelial transformation by regulating any number of important cell cycle processes.

Our previously published results also demonstrated that Cks1 regulates both $p27^{Kip1}$ and p130/Rb2 in MCF-7 cells (26). In this study we assessed whether Cks1 overexpression in these cells would alter their response to anti-estrogens through its effects on these cdk2 inhibitors. We demonstrate that overexpression of Cks1 in these cells induced substantial resistance to Faslodex in a clonogenic assay (Fig. 3). However, the effects of Cks1 overexpression on $p27^{Kip1}$ levels in the MCF-7 line are relatively modest, despite the fact that Cks1 transfectants exhibit decreased G_1/G_0 arrest in Faslodex. Instead we demonstrate that steady-state levels of p130/Rb2 in basal conditions, and also its levels in ICI 182780-treated cells were much lower in Cks1 overexpressors (Fig. 3). This is consistent with the findings of Reed and colleagues who showed that transduction of $Cks1^{-/-}$ fibroblasts with Cks1 expressing recombinant adenovirus decreased p130/Rb2 to wild-type levels (40). In addition, we demonstrate that Cks1 overexpression resulted in a small but reproducible increase in Skp2 levels in these cells. Cks1 has been shown to prevent Skp2 autoubiqitination by the SCF complex, thereby increasing its stability (41). This suggests a scenario wherein up-regulation in Cks1 during G_1/S progression results in co-ordinated increases in Skp2 expression as well, which may be required for sufficient down-regulation of p130/Rb2 and consequent cyclin-cdk2 activation. Skp2 overexpression in the ER^+ line MCF-7 also leads to an acute resistance to the effects of tamoxifen and ICI 182780 on the cell cycle, respectively (42). However, although the role of Skp2 in endocrine therapy failure has not been formally tested, higher Skp2 expression has been demonstrated to be less frequent in ER^+ versus ER^- breast tumors in a microarray study (43).

It is not clear why overexpression of Cks1 causes a selective decrease in the steady state levels of p130/Rb2, without significantly affecting $p27^{Kip1}$ levels in MCF-7 cells. Down-regulation of $p27^{Kip1}$ can also occur by its re-localization to cytoplasm following its prior phosphorylation on Ser10, Thr157 or Thr198 by various signaling cascades (44,45). A recent study comparing several breast cancer lines had concluded that ER^+ lines like MCF-7 and ZR75 express relatively higher levels of $p27^{Kip1}$ (15). Interestingly, these lines also contain relatively high levels of cyclin D1 and cdk4. Therefore, it is likely that the primary model of down-regulation of $p27^{Kip1}$ inhibitory activity towards cyclin-cdk2 is via its relocalization and its sequestration by cyclin D-Cdk4/6 complexes rather than its proteolytic degradation in this system.

In conclusion, our data suggest that Cks1 elevation could play important roles in mammary transformation induced by multiple oncogenic or carcinogenic agents. Our data also suggest that in ER^+ breast cancers Cks1 might play an important role in both estrogen-stimulated and anti-estrogen resistant progression. In addition to being essential in the SCF^{Skp2}-mediated ubiquitination of $p27^{Kip1}$, Cks1 is also essential in the degradation of p130/Rb2, another cdk2 inhibitor whose degradation is also mediated by SCF^{Skp2} (40). In addition to its cdk2 inhibitory function, p130/Rb2 also appears to mediate its anti-proliferative effects by E2F-dependent inhibition of transcription through the formation of suppressor complexes with the E2F factors E2F4 and E2F5 (46). The p130/Rb2 protein is abundant in G_0 and in early G_1 and a distinguishing feature of cell cycle arrest induced by the anti-estrogen ICI 182780 in ER + MCF-7 breast cancer cells is that the cells are arrested in a G_0-like quiescent state characterized by accumulation of p130/Rb2 (47). Thus, in addition to being a potential mammary oncogene Cks1 could also likely promote anti-estrogen resistant progression in breast cancers by abrogating the effects of p130/Rb2.

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