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Over-activation of positive cell cycle regulators and inactivation of cyclin-dependent kinase inhibitors (ckis) play a significant role in oncogenesis. We have shown previously that the cki p27 is a substrate of the ubiquitin-dependent proteolytic pathway. Importantly, we have found that aggressive human carcinomas contain high p27-specific proteolytic activity and that the absence of p27 is a powerful prognostic marker for poor survival in patients with breast carcinomas. These data suggest that aggressive tumors may obtain a growth advantage by selecting clone(s) that express low p27 because of an increase in its degradation. Our initial concept is that specific ubiquitinylating enzymes responsible for recognizing and driving these proteins into the proteolytic pathway may themselves be targets of oncogenic events. This would consequently affect the timed-degradation and the cellular abundance of their substrates. If our predictions are correct, ubiquitinylating enzymes might turn out to be important prognostic markers in human cancer and, hopefully, novel therapeutic targets. We have found that the F-box protein Skp2 is a rate-limiting component of the machinery that ubiquitinylates and degrades p27. The goal of this project is to study the signals regulating Skp2 activity in breast epithelium and how these events are deregulated in breast cancer cells.

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Introduction

Human Skp1 and the F-box protein Skp2 were originally identified as two proteins physically interacting with cyclin A and therefore designated as S-phase kinase-associated proteins (1). We and others have demonstrated that Skp2 is required for the ubiquitination and consequent degradation of the cdk-inhibitor p27 both in vivo (2, 3) and in vitro (2, 4). A decrease in the level of p27 protein in the absence of alteration of the p27 gene is commonly seen in many human cancers including epithelial cancers, brain tumors and lymphoproliferative neoplasms (reviewed in (5)). Importantly, reduced levels of p27 correlate with poor prognosis. In colon cancers (6), non-small cell lung cancers (7), oral squamous cell carcinomas (8), lymphomas (9) and astrocytic brain tumors (10) the decrease in p27 protein levels has been shown to be due to an increase in its ubiquitin-proteasome mediated proteolysis. One possible reason for this enhanced degradation might lie in the presence of high levels of Skp2. Accordingly, our lab and others have shown that Skp2 has oncogenic potential (11, 12) and is overexpressed in colorectal carcinomas (13) and lymphomas (12, 14). Because p27 is downregulated in aggressive human breast cancers (15, 16) and because of the role of Skp2 in p27 degradation, we have assessed the levels of Skp2 and p27 in human breast cancer samples and performed experiments to evaluate the role of Skp2 in breast cancer both in cell lines and transgenic mice.

Body

MMTV-*SKP2* mice were generated by placing the human *SKP2* cDNA under the control of an MMTV LTR promoter (mouse mammary tumor virus long terminal repeat) located upstream of the first ATG of this cDNA. This construct also contains an SV40 polyadenylation signal. Transgenic mice have been produced with the assistance of the Transgenic Facility at NYU Medical Center. Screening of founder animals and their offspring was performed using tail genomic DNA by PCR analysis. We have obtained four founders (all transmitting the transgene to their progeny). Using immunohistochemistry and immunoblot, we have determined that the transgenic protein is indeed in the breast epithelium. We have analyzed the effects of Skp2 expression in mammary development. We have examined the mammary glands of ten MMTV-*SKP2* and ten wild type virgin mice during puberty, both at 4 and 10 weeks. We found that at 10 weeks, the time of maximal hormonal stimulation, breast epithelium of the MMTV-*SKP2* mice proliferated more than the epithelium of wild type mice, as detected by both whole mount preparation and in vivo BrdU incorporation. Skp2 expression resulted in an increase in lateral ductal branching and extensive arrays of lobules. At higher magnification, the ducts were shorter, wider and covered with small lobular protuberances. This effect is probably due to an accelerated degradation p27 that, in turn, enhances cell proliferation.

We evaluated Skp2 expression by oligonucleotide microarray analysis (in a series of 90 human breast cancers) and by immunohistochemistry (in a series of 84 human breast cancers). We determined that high levels of Skp2 are present more frequently in ER negative tumors (67 % by immunohistochemistry and 61 % by microarray analysis) than in ER positive cases (15 % by immunohistochemistry and 24 % by microarray). Interestingly, the subset of ER negative breast carcinomas overexpressing Skp2 are also characterized by high tumor grade, negativity for Her-2, basal-like phenotype, high expression of certain cell cycle regulatory genes and low levels of p27 protein. We also found that Skp2 expression is cell adhesion-dependent in normal human mammary epithelial cells but not in breast cancer cells and that an inhibition of Skp2 induces a decrease of adhesion-independent growth in both ER positive and ER negative cancer cells. Interestingly, expression of Skp2 was regulated by estrogens and forced expression of Skp2 abolished anti-estrogens effects, suggesting that deregulated Skp2 expression might play a role in the development of resistance to antiestrogens. Our data support an oncogenic role for Skp2 and suggest it may be a therapeutic target in a subset of ER negative, Her-2 negative human breast cancers with a basal-like phenotype for which no specific therapy (other than standard chemotherapy) is currently available.

Key Research Accomplishments:

- Generation and characterization of MMTV-*SKP2* transgenic mice.

- Total of 90 primary untreated breast carcinoma frozen tissue samples analyzed by oligonucleotide microarray analysis.

- Total of 84 primary untreated breast carcinoma frozen tissue samples analyzed by immunohistochemistry.

Reportable Outcomes

1) Manuscript submitted for publication (attached)
2) Presentation at the "First International Meeting on Ubiquitin, Ubiquitin-Like Proteins, and Cancer", MD Anderson Cancer Center, Houston, TX. January 25-27, 2002.
3) Presentation at the Cdk inhibitors symposium part of the "93rd annual AACR meeting". San Francisco, CA. April 6 – 10, 2002.

Conclusions

Abnormal degradation of cell cycle regulatory proteins may result in deregulated proliferation typical of cancer cells. Indeed, an increased stability of positive regulators of proliferation (e.g., cyclins, ß-catenin) can be achieved by lowering the activity or the levels of the specific enzymes necessary for their degradation. Thus, the ubiquitinating enzymes specific for positive regulators of proliferation could act as tumor suppressors. Accordingly, other ubiquitinating enzymes could be oncogenic if their specific function is to ubiquitinate tumor suppressors (e.g., p27 and p53). Overall our analysis in human tumors, together with the results present in the literature, demonstrate the existence of a subtype of high grade, highly proliferating, ER/Her-2 negative breast carcinomas with a basal-like phenotype characterized by high Skp2 and low p27 levels. These observations lead to the hypothesis that Skp2 overexpression plays a relevant role in the pathogenesis of this subset of tumors by providing a growth advantage, likely by promoting p27 degradation. We investigated the oncogenic potential of Skp2 and found that in contrast to normal epithelial cells, breast cancer cells express Skp2 independently of adhesion to the cell matrix. This loss of adhesion-dependent control suggests a role for Skp2 in the adhesion-independent ability of tumor cells to grow and, in fact, inhibition of Skp2 activity reduces the adhesion-independent growth ability of cancer cells, as measured by the number of colonies in soft agar. The effects produced by Skp2 inhibition are independent of the presence of the estrogen receptor since they are observed both in ER positive (MCF-7) and ER negative cells (MDM-MB-435, which also express very low levels of Her-2). Interestingly, while a specific therapy with anti-Her-2 monoclonal antibodies (Herceptin) is currently utilized in ER negative breast cancer that overexpress Her-2, no specific therapy has been identified in those tumors that are both ER and Her-2 negative. Since Skp2 expression is associated with lack of both ER and Her-2, and Skp2 inhibition affects the in vitro growth of ER/Her-2 negative breast cancer cells, our study identify Skp2 as a potential specific therapeutic target in this subset of aggressive breast cancers. Finally, we show that Skp2 levels are positively regulated by estrogens and that enforced expression of Skp2 induces resistance to anti-estrogens. Albeit much less than in ER negative tumors, where Skp2 is highly expressed in 67% of cases, Skp2 is overexpressed also in 15% of the ER positive tumors. Thus, it is tempting to speculate that Skp2 overexpression may be one of the factors implicated in the lack of response to hormonal therapy, which occurs in roughly one third of tumors expressing ER. Similarly, it is possible to infer that deregulated Spk2 expression in ER positive tumors treated with anti-hormonal therapy may play an important role in the development of resistance to antiestrogens. In summary, our results support the notion that Skp2 is the product of a proto-oncogene and indicate that an alteration of Skp2 function and expression might contribute to the malignant behavior of breast tumors.

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Oncogenic role of the ubiquitin ligase subunit Skp2 in human breast cancer

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ABSTRACT

Estrogen receptor (ER) expression and Her-2 amplification define specific subsets of breast tumors for which specific therapies exist. The S-phase kinase-associated protein Skp2 is required for the ubiquitin-mediated degradation of the cdk-inhibitor p27 and is a bona fide proto-oncogene. We determined that high levels of Skp2 are present more frequently in ER negative tumors (67 % by immunohistochemistry and 61 % by microarray analysis) than in ER positive cases (15 % by immunohistochemistry and 24 % by microarray). Interestingly, a subset of ER negative breast carcinomas overexpressing Skp2 are also characterized by high tumor grade, negativity for Her-2, basal-like phenotype, high expression of certain cell cycle regulatory genes and low levels of p27 protein. We also found that Skp2 expression is cell adhesion-dependent in normal human mammary epithelial cells but not in breast cancer cells and that an inhibition of Skp2 induces a decrease of adhesion-independent growth in both ER positive and ER negative cancer cells. Interestingly, expression of Skp2 was regulated by estrogens and forced expression of Skp2 abolished anti-estrogens effects, suggesting that deregulated Skp2 expression might play a role in the development of resistance to antiestrogens. We conclude that Skp2 has oncogenic potentials in breast epithelial cells and is overexpressed in a subset of breast carcinomas (ER and Her-2 negative) for which Skp2 inhibitors may represent a valid therapeutic option.

INTRODUCTION

Patients with breast carcinomas can display a different response to therapy and clinical outcome despite similar stage and grade of disease. Gene expression profiling utilizing microarray technology has recently allowed the identification of subgroups of breast carcinomas with distinct molecular signatures and biologic behavior. Targeted therapy with Tamoxifen [1] and Herceptin [2] is available for estrogen receptor (ER) positive and Her-2 overexpressing breast tumors, respectively. ER negative breast carcinomas have been further classified by molecular profiling into at least two biologically different subtypes. These include Her-2 positive as well as Her-2 negative tumors with a basal-like phenotype [3,4]. This latter group of ER negative, Her-2 negative tumors expressing genes associated with a basal-like phenotype lacks specific therapeutic options and is characterized by aggressive biologic behavior.

F-box proteins (Fbps) are characterized by an approximately 40 amino acid domain called F-box as it was first identified in cyclin F [5]. Studies involving several organisms have shown that Fbps play a crucial role in the ubiquitin-mediated degradation of cellular regulatory proteins (e.g., cyclins, cdk-inhibitors, ß-catenin, IKB, etc.) (reviewed by[6-8]. Indeed, many Fbps are subunits of ubiquitin ligases named SCFs because they are formed by the following basic subunits: Skp1; Cul1; Roc1/Rbx1 and one of many Fbps. Since the substrate specificity of SCF ligases is dictated by different F-box proteins that act as substrate targeting subunits, a large number of Fbps is necessary to ensure highly specific substrate recognition. In fact, large families of Fbps are present in all eukaryotes (e.g., 11 members in yeast, 29 members in flies and at least 54 in mammals [9-11]; and our unpublished results).

Human Skp1 and the F-box protein Skp2 were originally identified as two proteins physically interacting with cyclin A and therefore designated as S-phase kinase-associated proteins [12]. We and others have demonstrated that Skp2 is required for the ubiquitination and consequent degradation of the cdk-inhibitor p27 both in vivo [13,14] and in vitro [13,15]. Skp2 deficient mice grow slower than littermate controls and show smaller organs with all tissues containing decreased numbers of cells [16]. Skp2-/- cells show high levels of p27, polyploidy and centrosome overduplication. This phenotype underscores the importance of Skp2 in positively regulating cell proliferation. All cellular and histopathological abnormalities observed in Skp2 deficient mice are abolished in Skp2/p27 double knock-out mice, indicating that p27 is a primary substrate of Skp2 (Nakayama K., personal communication).

A decrease in the level of p27 protein in the absence of alteration of the p27 gene is commonly seen in many human cancers including epithelial cancers, brain tumors and lymphoproliferative neoplasms (reviewed by [17]. Importantly, reduced levels of p27 correlate with poor prognosis. In colon cancers [18], non-small cell lung cancers [19], oral squamous cell carcinomas [20], lymphomas [21] and astrocytic brain tumors [22], this decrease in p27 protein levels has been shown to be due to an increase in its ubiquitin-proteasome mediated proteolysis. One possible reason for this enhanced degradation might lie in the presence of high levels of Skp2. Accordingly, it has been shown that unregulated Skp2 expression induces p27 degradation and entry into S-phase both in the presence of low serum concentration [14] and in the absence of cell adhesion [23]. Indeed, it has recently been shown that Skp2 has oncogenic potential [24,25] and is overexpressed in oral epithelial [20,24], colorectal carcinomas [26] and lymphomas [25,27].

Because p27 is downregulated in aggressive human breast cancers [28-30] and because of the role of Skp2 in p27 degradation, we have assessed the levels of Skp2 and p27 in human breast cancer samples and performed experiments to evaluate the role of Skp2 in breast cancer cell lines. Our data support an oncogenic role for Skp2 and suggest it may be a therapeutic target in a subset of ER negative, Her-2 negative human breast cancers with a basal-like phenotype for which no specific therapy (other than standard chemotherapy) is currently available.

RESULTS

Skp2 message is highly expressed in a subgroup of ER-negative and Her-2-negative tumors by oligonucleotide microarray analysis

The 89 arrayed primary breast tumor samples (sample set A) were first analyzed by hierarchical clustering using a filtered set of 2130 variably expressed genes (Figure 1A). The 89 tumors are clustered across the top and the genes are clustered down the side based on similar patterns of expression. The gene cluster that includes Skp2 is highlighted in blue in the horizontal dendrogram. The tumor cluster that shows the highest levels of expression of Skp 2 is highlighted in blue in the vertical dendrogram. The histologic tumor grade and the results of the immunohistochemistry analysis [estrogen and progesterone receptors (ER/PR) and Her-2 proteins] are represented by the blue, green, and purple color bars, respectively. First, high levels of Skp2 protein were more frequently observed in ER negative tumors (11/18 or 61%) than in ER positive cases (17/71 or 24%). Second, as shown in Figure 1A, the highest Skp2 expression levels were in a small subgroup of 5 tumors that were all high grade, negative for estrogen and progesterone receptors, and negative for Her-2 over-expression. When compared to the other samples, the tumor subset with high Skp2 expression was noted to have higher relative expression of other proliferation associated genes and a number of genes previously described to characterize a basal-like subset of breast tumors [3,4]. In order to better visualize the correlation between the expression of some of these genes, we performed cluster analysis of the 89 tumors in a more limited gene space of 41 genes (Figure 1B). The 41 genes were selected to include Skp2, other cell cycle regulatory genes, hormone receptor genes, ER-regulated and ER-associated genes, genes from the Her-2 amplicon, keratins, and representative genes associated with the previously described basal-like tumor subgroup. In this more limited and selected gene space, there were now 6 tumors in the cluster characterized by high Skp2 expression (cluster highlighted in blue). The high Skp2 cluster included the 5 ER, PR and Her-2 negative, high grade cases that previously clustered together in the larger gene space, plus an additional ER/PR negative, Her-2 negative, high grade tumor. As shown in Figure 1B, the high-Skp2 tumor cluster was characterized by high expression of other genes encoding proteins known to physically interact with Skp2: Cyclin A2, Cyclin E1 and Cdk2. In addition, markers of S-phase, such as PCNA, and G2/M, such as cyclin B1 and Cks2, were also high in this subgroup. Not all cyclins, however, clustered with

Skp2. For example, Cyclin D1 and Cyclin D2, two cyclins known to play an important role for the proliferation of breast epithelium, were not highly expressed in this subset. In contrast, Cyclin D3 and Skp2 showed similar expression pattern. Finally, the tumor subset with high Skp2 levels also showed expression of genes characteristic of the basal-like breast cancer subgroup described by Sorlie, et.al. [4], including keratin 5, keratin 17, UDP-N-Ac-galactosamine, chitinase 3-like 2, and P-cadherin.

High levels of Skp2 protein are associated with ER negativity, low expression of p27, high proliferation rate and poor survival

We then compared the results of the microarray analysis with an immunohistochemical study in a separate series of 84 breast carcinomas (sample set B), for which follow-up data were also available. We analyzed levels of Skp2, p27, Ki67 and ER proteins. Skp2-positive cells ranged from 0% to 35% (median 4%) in the breast carcinoma samples (Figure 2B), whereas normal breast epithelium was consistently negative for Skp2 expression (Figure 2A, panels ii, iv and vi). Expression levels ranged from 0% to 90% (median 40%) for p27, 0% to 70% (median 15%) for Ki67, and 0% to 90% for ER. When cutoff points were used to categorize high and low expressors, breast carcinoma cases showed three major patterns of Skp2/p27 expression (Figure 2B). Thirty tumors (36%) were characterized by high p27 and low Skp2 levels. Twenty-one tumors (25%) expressed low p27 and high Skp2 levels. In thirty-two tumors (38%), both low p27 and low Skp2 levels were observed. Only one tumor expressed both high p27 and high Skp2 levels. Fisher's exact test showed that the percentage of high Skp2 expressors was significantly greater in the group with low p27 expression (37%) than in the group with high p27 expression (3%) ($p < 0.001$). In addition, as for the mRNA levels, we found that high levels of Skp2 protein were more frequently observed in ER negative tumors (12/18 or 67%) than in ER positive cases (7/47 or 15%) (Fisher's exact test, $p<0.0001$) and in tumors with high proliferation index (Ki67 positive) (Figure 3A). Simple regression analysis demonstrated a direct linear association between Skp2 and Ki67 expression levels ($R^2=0.84$) (Figure 3B). We further investigated whether Skp2 and Ki67 proteins were expressed in the same subset of tumor cells. To this end, double immunofluorescence experiments for Skp2 and Ki67 were performed both in breast cancer MCF-7 cells and in paraffin-embedded tumor samples. In both cases, Skp2 protein was detected only in cells expressing Ki67 (Figure 3C). Both Ki67 and Skp2 were localized in the nucleus. A subset of cells that expressed Ki67 protein was negative for Skp2 expression, indicating that as already shown for lymphomas [27] levels of Skp2 might define the fraction of Ki67 cells that are in S-phase.

Finally, we looked at the relationship between Skp2 and survival. Table 1 shows the distribution of age, stage and grade in patients with low or high Skp2. There was a statistically significant association between Skp2 expression levels and grade. The Cox regression model showed that high expression levels of Skp2 were associated with poor survival (calculated from the time of the first recurrence) in the univariate model [hazard ratio=2.83, 95% CI=(1.48-5.44), p=0.02]. When grade was added to this model, Skp2 still showed a trend towards discrimination of biologic behavior [(hazard ratio=1.71, 95% CI=(0.83-3.50)], although this was not statistically significant. As expected, high values of p27 expression were associated with better survival [hazard ratio=0.37, 95% CI=(0.16-0.84), p=0.018] in the univariate analysis. When Stage, grade or Skp2 were added into the model, one at a time, similarly to Skp2, p27 also showed a trend in predicting survival [hazard ratio=0.5] but was no longer statistically significant. These results indicate that larger series of patients, with more statistical power, need to be interrogated in order to establish if Skp2 is an independent predictor of biologic behavior.

Skp2 is required for anchorage-independence in breast cancer cells

The results in human breast cancers suggest that high Skp2 expression might contribute to oncogenesis in breast epithelium. We have recently shown that in primary diploid fibroblasts Skp2 expression requires cell adhesion to the extracellular matrix [[23] and Figure 4B, lanes 1-3]. We determined whether the requirement of cell adhesion for the expression of Skp2 is also present in breast epithelial cells. Monolayer cultures of normal human breast epithelial cells (HBEC) were trypsinized and re-plated in suspension. Under these conditions, the overall amount of Skp2 dramatically decreased by 12 hours (Figure 4A, lanes 1 - 3), which corresponded to an accumulation of p27. In contrast, MCF-7 cells (ER-positive), two ER-negative breast cell lines (HBL-100 and MDM-MB-435) and other transformed cell lines expressed the same levels of Skp2 and p27 regardless of their adhesion conditions (Figure 4A and 4B). Thus, transformed cell lines have lost an adhesion-dependent control of Skp2 that is still present in normal cells. To determine if this deregulation of Skp2 expression is causally linked to the transformed phenotype of cancer cells, we infected MCF-7 and MDM-MB-435 cells with a retroviral vector expressing either Skp2 or a Skp2 dominant negative mutant missing the F-box [Skp2(ΔF)] [13]. Infected cells were then monitored for their ability to grow in an anchorage independent manner by forming colonies in soft agar. While Skp2 expression did not have any significant effect, the expression of Skp2(ΔF) mutant strongly inhibited the growth in soft agar of these two cell lines (Figure 4C). These results show that the inhibition of Skp2, in both ER positive and ER negative cells, results in a suppression of tumor growth and suggest that Skp2 is a target of therapies aimed at inhibiting the tumor metastatic potential of breast carcinomas.

Skp2 expression abrogates antiestrogen-mediated cell cycle arrest in hormone-dependent breast epithelial cancer cells

Although most ER negative tumors display high levels of Skp2 (67 % by immunohistochemistry and 61 % by microarray), a fraction of ER positive tumors also have high Skp2 expression (15 % by immunohistochemistry and 24 % by microarray). This finding prompted us to study the relationship between estrogens and Skp2 expression. Thus, we assessed the cell cycle regulation of Skp2 by estrogens in the well-characterized human breast MCF-7cell line, which is estrogen sensitive. Asynchronous MCF-7 cells were arrested in G1 by treatment with tamoxifen combined with estrogen deprivation, as monitored by cyclin A downregulation (Figure 5, bottom panel, lanes 1 - 3). As for cyclin A, the total abundance of Skp2 significantly decreased during cell cycle exit in parallel with an increase in p27 abundance (Figure 5, top two panels, lanes 1 - 3). When cells were re-stimulated to enter the cell cycle by the sole addition of 17-ß estradiol, Skp2 protein abundance increased in parallel with a decrease in p27 levels (Figure 5, top two panels, lanes 4 -6). In contrast, levels of cyclin D1 (Figure 5, third panel from the top) and cyclin E (data not shown) did not change significantly in tamoxifen and estradiol treated cells. Thus, Skp2 levels are positively regulated by estrogens. We then studied the phenotype of MCF-7 cells constitutively expressing Skp2 and/or G1 cyclins from exogenous cDNAs. To express these proteins in MCF-7 cells, we used retroviral expression vectors and found that Skp2, cyclin D1 and cyclin E were all expressed in an estradiol- and tamoxifen-independent manner at levels two-to-four fold higher than those of the respective endogenous proteins (data not shown). Infected cells were either left asynchronous or were synchronized in G1 by tamoxifen treatment and estrogen deprivation. Percentage of cells in S-phase was then analyzed by BrdU incorporation (Figure 5B). No significant differences in BrdU incorporation were observed in untreated cells infected with either Skp2 or G1 cyclins. In contrast, significant differences were observed in tamoxifen-treated cells. In fact, compared to control cells, a significant increase in BrdU incorporation was observed in Skp2-expressing cells. In agreement with previous published results, a significant but minor effect was also observed in cyclin D1- but not in cyclin E-expressing cells. However, co-expression of Skp2 and cyclin D1 did not induce a further increase in cells entering S phase. These results show that the enforced expression of Skp2 in hormone-dependent breast cancer cells allows cell proliferation in the presence of an anti-mitogenic stimulus given by antiestrogens. Thus, in ER positive breast carcinomas treated with antiestrogens, deregulated Skp2 expression might play a relevant role in the development of resistance to these drugs.

DISCUSSION

This study provides insights into the oncogenic role of Skp2 in human breast cancer. Using microarray hybridization of RNA from 89 breast carcinomas (sample set A), we found that Skp2 message is overexpressed in a subset of high grade ductal carcinomas which are negative for ER expression and do not overexpress Her-2 (Figure 1). The inverse correlation between Skp2 and ER protein levels as well as the direct correlation between Skp2 levels and tumor grade was confirmed by immunohistochemistry in a second series of 84 breast cancers (sample set B) (Figure 2). Although the percentage of Her-2 positive cases was low overall in this series of breast cancers (8%), we found that among ER negative cases Her-2 overexpression was twice as frequent in the subset of low Skp2 expressors as in the high Skp2 expressors (not shown). Interestingly, our gene expression analysis also demonstrates that tumors overexpressing Skp2 are negative for keratins characteristic of epithelial luminal cells but show the expression of epithelial basal cell markers, including Keratins 5 and 17 (Figure 1). A similar subtype of ER/Her-2 negative breast tumors with basal-like phenotype has been recently identified and found to be associated with poorer outcome as compared to the ER positive subgroups of tumors with luminal phenotypes [3,4]. Accordingly, when we assessed the relationship between Skp2 and survival in sample set B, we found that high levels of Skp2 were associated with poor survival by univariate analysis. Larger series of tumors will have to be analyzed to determine the independent prognostic significance of Skp2 in breast cancer.

Reduced protein levels of the cell cycle inhibitor p27 are frequently observed in a variety of human tumors and are associated with poor prognosis (reviewed by [17]. In breast carcinomas we and other investigators have previously shown that loss of p27 expression in tumor cells is an independent predictor of both overall survival and disease free survival [28-30]. In agreement with the role of Skp2 in p27 degradation, we found that Skp2 levels inversely correlate with p27 expression (Figure 2). An interesting finding was the fact that in a subset of breast cancers, Skp2 was expressed at low levels despite low expression of p27. This could be due to low levels but hyperactive Skp2 (e.g., because of mutations, overexpression of the Skp2-cofactor Cks1, etc.). Alternatively, in these tumors p27 downregulation could be due to other, as yet unknown, mechanisms. We also found that breast tumors expressing high Skp2 are characterized by high expression of markers of both S-phase and G2/M at both RNA and protein levels. In addition, we show that in both human cancers and breast cancer cells in culture, Skp2 protein is selectively expressed in a subset of proliferating cells (Figure 3). Remarkably, among the three different D-type cyclins, only Cyclin D3 clustered with Skp2. These data confirm prior data showing positive correlation between Cyclin D3 and high tumor grade [31]. As expected, Cyclin D1 was predominantly expressed in ER positive, low grade cancers [32]. Interestingly, a recent study of gene expression profiling in breast cancer has identified genes regulating cell cycle to be one of the defining clusters of the molecular signature of poor prognosis [33].

Overall our analysis in human tumors, together with the results present in the literature, demonstrate the existence of a subtype of high grade, highly proliferating, ER/Her-2 negative breast carcinomas with a basal-like phenotype characterized by high Skp2 and low p27 levels. These observations lead to the hypothesis that Skp2 overexpression plays a relevant role in the pathogenesis of this subset of tumors by providing a growth advantage, perhaps by promoting p27 degradation. We investigated the oncogenic potential of Skp2 and found that in contrast to normal epithelial cells, breast cancer cells express Skp2 independently of adhesion to the cell matrix (Figure 4A). This loss of adhesion-dependent control suggests a role for Skp2 in the adhesion-independent ability of tumor cells to grow and, in fact, inhibition of Skp2 activity reduces the adhesion-independent growth ability of cancer cells, as measured by the number of colonies in soft agar (Figure 4B). The effects produced by Skp2 inhibition are independent of the presence of the estrogen receptor since they are observed both in ER positive (MCF-7) and ER negative cells [MDM-MB-435, which also express very low levels of Her-2 [34]]. Interestingly, while a specific therapy with anti-Her-2 monoclonal antibodies (Herceptin) is currently utilized in ER negative breast cancer that overexpress Her-2 [2], no specific therapy has been identified in those tumors that are both ER and Her-2 negative. Since Skp2 expression is associated with lack of both ER and Her-2, and Skp2 inhibition affects the in vitro growth of ER/Her-2 negative breast cancer cells, our study identify Skp2 as a potential specific therapeutic target in this subset of aggressive breast cancers. Finally, we show that Skp2 levels are positively regulated by estrogens (Figure 5A) and that enforced expression of Skp2 induces resistance to anti-estrogens (Figure 5B). Albeit much less than in ER negative tumors, where Skp2 is highly expressed in 61 - 67% of cases, Skp2 is overexpressed also in 15% (as detected by immunohistochemistry) - 24 % (as detected by microarray) of the ER positive tumors. Thus, it is tempting to speculate that Skp2 overexpression may be one of the factors implicated in the lack of response to hormonal therapy, which occurs in roughly one third of tumors expressing ER. Similarly, it is possible to infer that deregulated Spk2 expression in ER positive tumors treated with anti-hormonal therapy may play an important role in the development of resistance to antiestrogens.

In summary, our results support the notion that Skp2 is the product of a proto-oncogene and indicate that an alteration of Skp2 function and expression might contribute to the malignant behavior of breast tumors.

MATERIALS AND METHODS

Patient populations

Sample set A (oligonucleotide microarrays analysis): A total of 90 primary untreated breast carcinoma frozen tissue samples were selected from a repository held by the Dana-Farber/Harvard Specialized Program of Research Excellence (SPORE) in Breast Cancer. Histologic sections of tumor were blindly reviewed and scored for modified Bloom-Richardson grade. Hormone receptor and Her-2 receptor immunohistochemical sections (performed for clinical assessment) were reviewed and scored as positive or negative. Histologic sections of tumor were blindly reviewed by a pathologist and scored for modified Bloom-Richardson grade, and histologic tumor type.

Sample set B (immuhistochemistry and immunofluorecence analysis): Breast cancer specimens from the years 1987-1996 were retrieved from the files of the Department of Oncology AUSL Rimini, Italy. 84 patients with breast carcinoma were selected for the study. All the patients developed either local recurrences or metastatic disease (nodal or distant) during the follow-up period. Paraffin-embedded tissue samples from the primary tumor were utilized in the immunohistochemistry and immunofluorescence experiments. Stage and grade were available in all cases. The median follow-up for survivor patients was 40 months.

Oligonucleotide microarrays analysis

The frozen bulk tumor samples used for RNA extraction from the 90 tumors from sample set A were confirmed to contain at least 70% viable tumor by area on histologic thin sections. Total RNA extracted from frozen tissue was used to generate biotinylated cRNA target and subsequently hybridized to Affymetrix U95A oligonucleotide probe arrays (Affymetrix, Santa Clara, CA) according to standard protocols [35]. These arrays contain approximately 12,600 human probe sets, representing at least 8,000 individual genes. Raw expression values were obtained using GENECHIP software from Affymetrix, and then further analyzed using the DNA-Chip Analyzer (dChip) custom software. Of the 90 arrayed samples, one array was excluded for a gross defect (scratch), leaving 89 tumors for analysis. Hierarchical clustering was performed using the dChip software which clusters the tumor samples in a dendrogram across the top and clusters the genes in a dendrogram down the side. Samples and genes with more similar expression patterns are grouped together within branches of the dendrograms. The gene expression data is presented in the form of a colorgram (red: highest relative gene expression, blue: lowest relative gene expression) with the columns representing the tumor specimens and rows representing the genes. The clustering diagram in Figure 1A was performed using a subset of 2130 genes filtered for those showing a variation of expression across the samples. The filtering criteria included genes with a coefficient of variation (standard deviation/mean) between 0.65 and 10.0 and with a mean intensity value of greater than 50 units in at least 5% of the tumor samples. The smaller clustering diagram in Figure 1B was performed using a smaller set of 41 genes selected to show the correlation between the expression of Skp2, cell cycle regulatory genes, hormone receptors and related genes, Her-2 amplicon genes, keratin genes, and several markers of the basal cell phenotype.

Immunohistochemistry

Both p27 and Skp2 expression was assessed in all 84 tumor samples (sample set B), while Ki67 expression was investigated in a subset of 37 tumors. Immunohistochemistry experiments were performed as previously described [36]. The following primary antibodies were utilized: anti-Skp2 (Zymed Inc.)

at 1:100 dilution as described [25], anti-p27 (Transduction Laboratories, Lexington, KY) at 1:200 dilution, anti-Ki67 (Immunotech, Westbrook, ME) at 1:50 dilution, anti-ER (Dako, Corp., Carpinteria, CA) at 1:100 dilution, and anti-Her-2 (AO485; Dako) at 1:200 dilution.

Scoring of slides immunostained for p27 and Ki67 was done according to the percentage of tumor cells exhibiting nuclear staining (500 cells counted). Skp2 immunostains were scored according to the percentage of tumor cells showing either nuclear or cytoplasmic staining (500 cells counted). In order to define high and low p27 expressors we utilized a cutoff of $\geq$ 50% positive tumor cells, which is the cutoff utilized in the majority of the previous studies. An arbitrary cutoff of $\geq$10% was utilized to define high and low Skp2 expressors. A cutoff of $\geq$10% was utilized to define ER positive cases. Fisher's exact test was used to assess the association between Skp2 and p27 expression levels (utilizing cutoff points to define high and low expressors). Linear regression analysis was used to assess the association between Skp2 and Ki67 expression levels as continuous variables. Stage and grade of the tumors were compared with Skp2 and p27 dichotomized expression levels using Fisher's exact test. The proportional hazards model was used to assess the association of Skp2 and p27 with survival time, controlling for stage and grade of the tumor.

Immunofluorescence

Both the breast carcinoma cell line MCF-7 (American Type Culture Collection, Rockville, MD) and five paraffin-embedded breast carcinoma samples from sample set B were submitted to double immunofluorescence staining for Skp2/Ki67. MCF-7 cells were grown on Plus Superfrost microscope slides (Fisher Scientific, Pittsburgh, PA) in Dulbecco's modified essential medium (Gibco, BRL; Gaithersburg, MD) supplemented with 10% Fetal Bovine Serum (Gibco, BRL). Cells were subsequently fixed in 4% paraformaldehyde (PFA) with 0.2% Triton. For breast carcinomas analysis, five micron tissue sections were deparaffinized, rehydrated and subjected to microwaving as described in the immunohistochemistry section. Double immunofluorescence experiments were performed as previously described [37].

Cell cultures, cell synchronization and cell cycle analysis

Human breast cancer cell lines (MCF-7 and MDA-MB-435), human breast cell line HBL-100 (expressing SV-40 Large T protein), human fibrosarcoma cell line HS-913-T, and human embryonic kidney adenovirus- and SV-40 Large T-transformed epithelial cell line 293 were obtained from the American Type Culture Collection (ATCC) and cultured in Dulbecco's-Modified Eagle's Medium (DMEM) supplemented with 10% fetal calf serum (FCS), 2 mM glutamine, 100 U/ml penicillin and 1 μg/ml streptomycin. Normal human breast ductal epithelial (HBEC) cells were obtained from Clonetics/BioWhittaker Inc. and cultured in the growth medium recommended by the manufacturer as described [38]. Human normal diploid fibroblasts (HF, also called IMR-90) and SV-40 Large T-transformed IMR-90 (IDH-4) were grown as described previously [38].

To test the effects of lack of adhesion to the extracellular matrix, cells were trypsinized and reseeded on Petri dishes coated with 1% agarose (suspension) using 0.5 - 1 x 10^6 cells per 10 cm dish. Colony formation in soft agar was done as described [23]. For cell cycle analyses, adherent cells were grown on glass coverslips, labeled for 5 hours with 10 μm BrdU, rinsed with PBS and fixed for 10 minutes in -20°C methanol-acetone (1:1). Fixed cells were rehydrated in PBS at room temperature and processed for cell staining. Cells were stained for BrdU as described previously [39] and counterstained with Hoechst to identify all nuclei. The percentage of BrdU labeled cells (BrdU positive cells/Hoechst positive cells x 100) was quantified using a fluorescence microscope. At least 300 cells were counted for each sample and each experiment was performed at least 3-4 times. To test the effects of lack of adhesion to the extracellular matrix, cells were trypsinized and then reseeded on tissue culture coated Petri dishes (adherent) or Petri dishes coated with 1% agarose (suspension) using 0.5 - 1 x 10^6 cells per 10 cm dish.

Retroviral-mediated gene transfer

Packaging GP-293 cells (Clontech) were transfected with retroviral plasmids according to the manufacturer's instructions. Forty-eight hours after transfection, the virus-containing medium was collected and supplemented with 8 μg/ml polybrene (Sigma). Then, the culture medium of the target cells was replaced with this viral supernatant for 24 hours. After a 12 hour recovery in normal medium, the infection process was repeated a second time with Skp2, cyclin D1 and cyclin E viruses, and three - four times with Skp2(ΔF) virus. The percentage of infected cells was quantified by flow cytometry or immunofluorescence. In all cases, greater than 85% of the cells were infected. Multiple genes were introduced sequentially.

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Table 1. Characteristics of the patients by Skp2 expression level (sample set B)

Patients' characteristics	**Skp2**		p-value
	Low (< 10) n (%)	High (≥ 10) n (%)	
Age			0.78
< 50	15 (24)	6 (27)	
≥ 50	47 (76)	16 (73)	
Grade			< 0.001
1	9 (15)	0 (0)	
2	29 (47)	3 (14)	
3	24 (39)	19 (86)	
Stage			0.07
T1	29 (47)	4 (18)	
T2	24 (39)	12 (55)	
T3	2 (3)	2 (9)	
T4	7 (11)	4 (18)	
ER-	6 (33)	12 (67)	p<0.0001
ER+	40 (85)	7 (15)	

FIGURE LEGENDS

Figure 1. Hierarchical clustering of 89 breast tumor samples (sample set A)

Each column represents a tumor sample and each row represents a different gene probe set. The relative expression level of the genes in the samples is indicated by a gradient of color with blue representing expression levels below the mean and red representing expression levels above the mean. The tumors are clustered in the dendrogram at the top and the genes are clustered in the dendrogram down the side. Tumors and genes appearing next to each other are more similar in expression pattern. The pathologic data for each tumor sample is represented by the color bars below the tumor dendrograms: dark blue, modified Bloom-Richardson grade III; light blue, modified Bloom-Richardson grade I or II; dark green, estrogen and progesterone receptor positive; light green, estrogen and progesterone receptor negative; dark purple, Her-2 over-expressed (3+); light purple, Her-2 non-over-expressed. The tumor clusters showing the highest levels of Skp 2 expression and the gene clusters which include the Skp 2 gene are highlighted in blue. **A:** Hierarchical clustering of 89 tumors for 2130 filtered genes. **B:** Clustering of 89 tumors to demonstrate relative expression of 41 selected genes including cell cycle regulatory genes (e.g. cyclins, CDKs, yc, Cks2, PCNA, p16), keratins, hormone receptors (e.g. ER alpha, ER beta, ERR alpha, androgen receptor), estrogen receptor associated genes (e.g. HNF3, TFF3, GATA3, LIV-1), Her-2 amplicon genes (e.g. Her-2, PPAR binding protein, MLN64, GRB7), and genes associated with the basal-like tumor subgroup (e.g. UDP-N-Ac-galactosamine, chitinase 3-like 2, P-cadherin, AC133/prominin-like 1, HDGF, p53-regulated DDA3).

Figure 2. Relationship between Skp2 and p27 expression in 84 human breast carcinomas (sample set B)

A: Immunohistochemical stains for p27 (i, iii and v) and Skp2 (ii, iv and vi) of three breast carcinomas representative of the three patterns of Skp2/p27 expression. The carcinoma in the top row shows high p27 expression in both the residual normal duct in the center and in the infiltrating tumor surrounding it (i). Only one Skp2 positive cell is seen in the corresponding section in ii. The tumor in the middle panel shows a p27 negative tumor (with positive residual normal ducts) (iii). Strongly positive Skp2 tumor cells are seen in panel iv. The bottom left panel shows a tumor which is negative for p27 (positive infiltrating lymphocytes are seen on the right) (v). This tumor is also negative for Skp2 (vi). **B:** Graphic representation of the expression of Skp2 and p27 in the 84 tumors. Utilizing the cutoffs (50% for p27 and 10% for skp2) to define high and low expressors, tumors fall into three categories: high p27/low Skp2; low p27/high Skp2 and low p27/low Skp2.

Figure 3. Correlation between Skp2 and proliferative index in sample set B

A: Immunohistochemical stains of two representative breast carcinomas with anti Skp2 (i and iii) and anti-Ki67 (ii and iv) antibodies. The tumor in the top row shows high Skp2/high Ki-67 levels. The one in the bottom row displays low Skp2/low Ki67 levels. **B:** Graphic representation of the direct correlation between Skp2 expression and proliferative index in the 37 breast carcinomas. **C:** Dual immunofluorescence of Skp2 with Ki67 in the breast cancer cell line MCF7 and in breast carcinomas. In MCF-7 cells (i, ii and iii) as well as in breast carcinoma samples (iv, v and vi), Skp2 protein

(Texas Red) was detected only in cells expressing Ki67 (FITC). Both Ki67 and Skp2 are localized in the nucleus. A subset of cells that expressed Ki67 protein was negative for Skp2 expression (iii and vi).

Figure 4. Expression of Skp2 is required for anchorage-independence in breast epithelial cancer cells

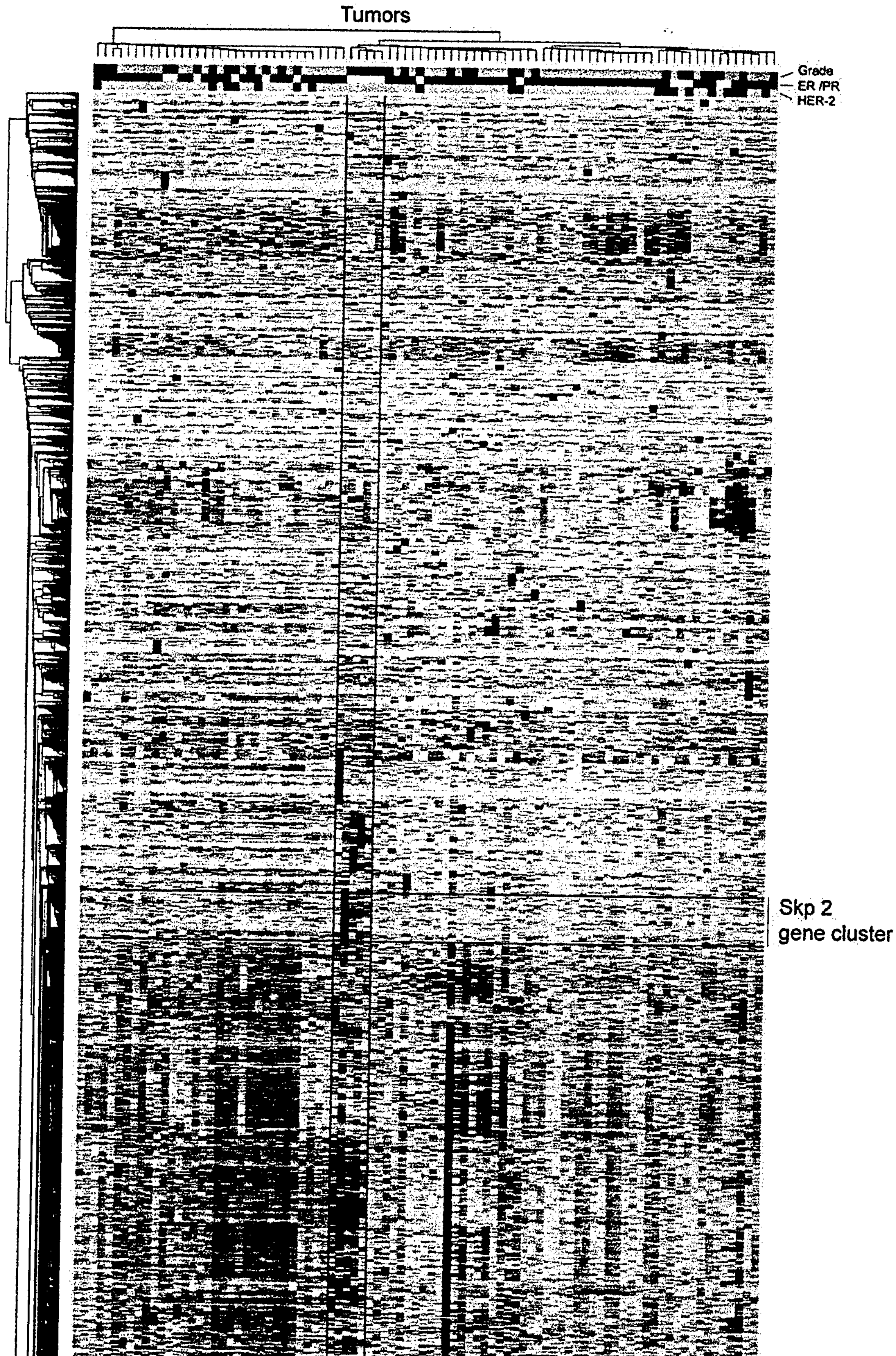
Proliferating normal human breast epithelial cells (HBEC, panel **A**, lanes 1-3), three different breast cell lines (panel **A**, lanes 4-12), normal human fibroblasts (panel **B**, lanes 1-3) and others transformed cell lines (panel **B**, lanes 4-12) were trypsinized and re-plated in suspension. Cells were collected at the indicated times and protein extracts were analyzed by immunoblotting with antibodies to the indicated proteins. **C**: Comparative analysis of the number of colonies larger than 200 μM in diameter obtained growing breast cancer cells infected with various retroviruses in soft-agar. A total of 1 x 10^5 MCF-7 cells or 5 x 10^3 MDM-MB-435 cells were seeded in 0.3% agar supplemented in 5% CS. Colonies were scored after 3 weeks of growth. The number of colonies scored represented ≥ independent experiments +/- the standard deviation of the mean.

Figure 5. Enforced expression of Skp2 abrogates anti-estrogen-mediated cell cycle arrest in MCF-7 cells

A: MCF-7 breast cancer cells were synchronized in G1 phase by treatment with 1 μM tamoxifen for the indicated times (lanes 1-3). The cells were then restimulated to enter the cell cycle by addition of 500 nM estradiol for the indicated times (lanes 4 - 6). Cell extracts were analyzed by immunoblotting with antibodies to the indicated proteins. **B**: MCF-7 cells infected with retroviruses expressing the indicated proteins were either left asynchronous or were synchronized in G1 by depletion of estradiol and treated with tamoxifen for 48 hours. The percentage of MCF-7 cells in S-phase was then analyzed by BrdU incorporation. The results shown are the mean percentage obtained from at least three independent experiments.

Figure 1

A



B

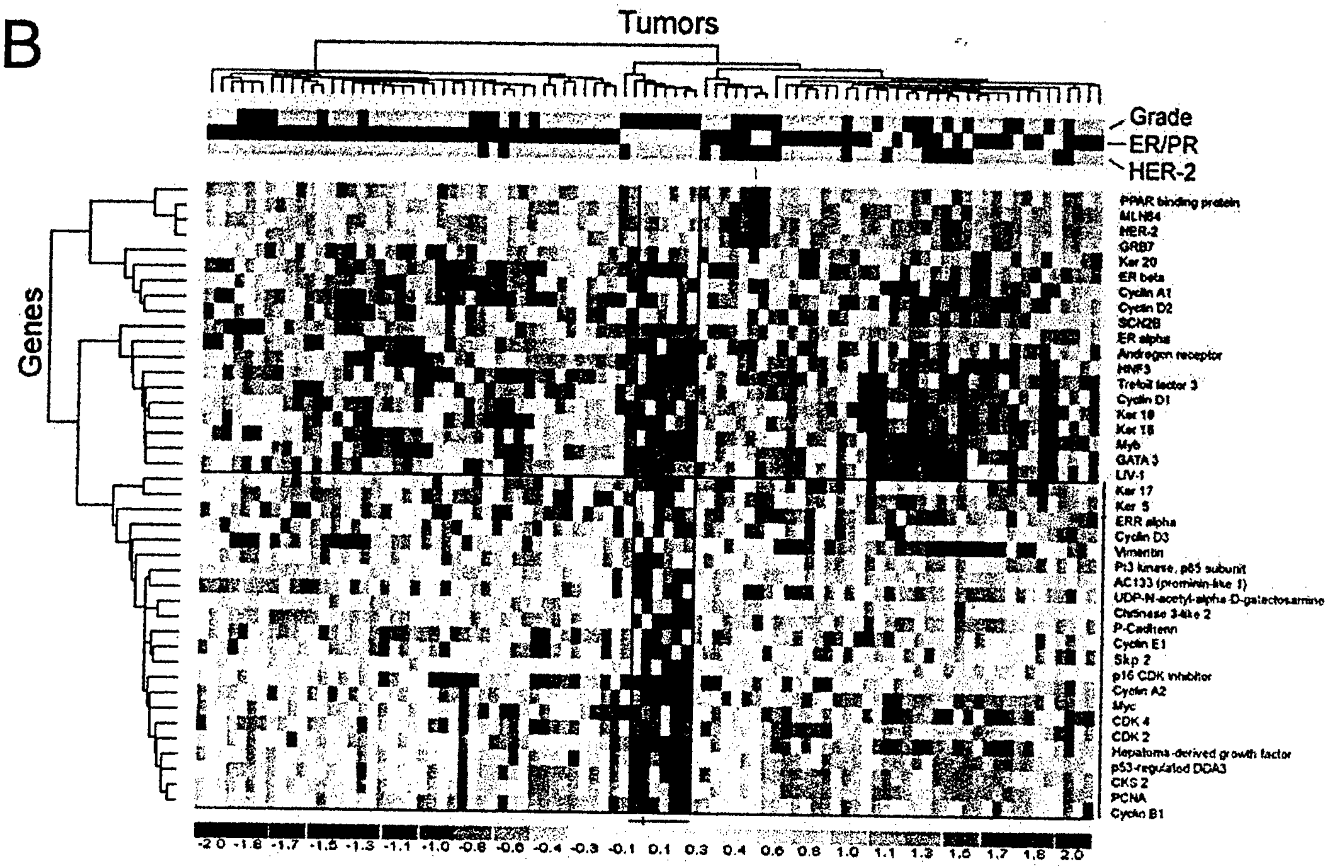

Tumors
Grade
ER/PR
HER-2
Genes
PPAR binding protein
MLN64
HER-2
GRB7
Ker 20
ER beta
Cyclin A1
Cyclin D2
SCN2B
ER alpha
Androgen receptor
HNF3
Trefoil factor 3
Cyclin D1
Ker 19
Ker 18
Myb
GATA 3
LIV-1
Ker 17
Ker 5
ERR alpha
Cyclin D3
Vimentin
PI3 kinase, p85 subunit
AC133 (prominin-like 1)
UDP-N-acetyl-alpha-D-galactosamine
Chitinase 3-like 2
P-Cadherin
Cyclin E1
Skp 2
p16 CDK inhibitor
Cyclin A2
Myc
CDK 4
CDK 2
Hepatoma-derived growth factor
p53-regulated DDA3
CKS 2
PCNA
Cyclin B1
-2.0 -1.8 -1.7 -1.5 -1.3 -1.1 -1.0 -0.8 -0.6 -0.4 -0.3 -0.1 0.1 0.3 0.4 0.6 0.8 1.0 1.1 1.3 1.5 1.7 1.8 2.0

Figure 2

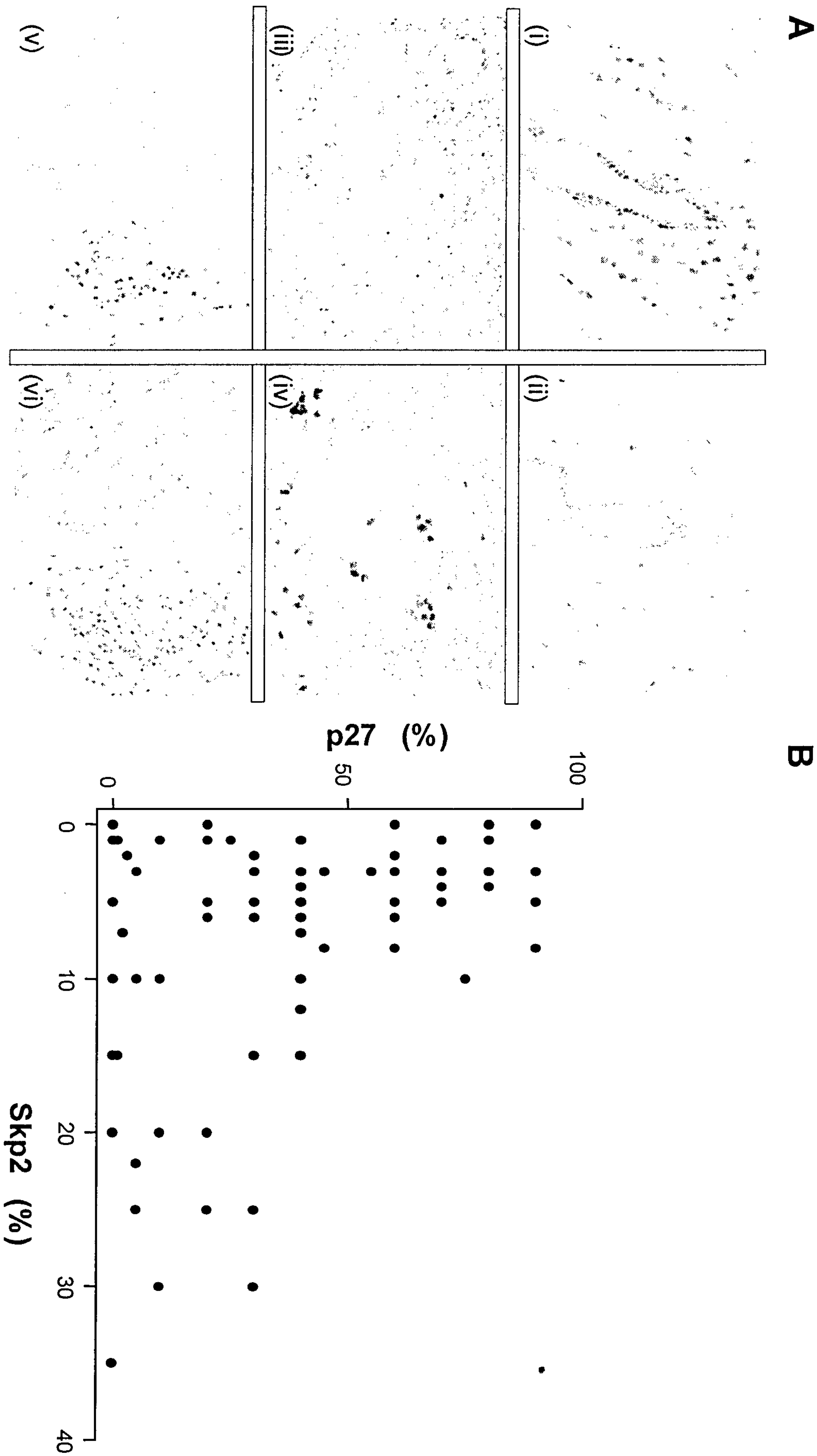
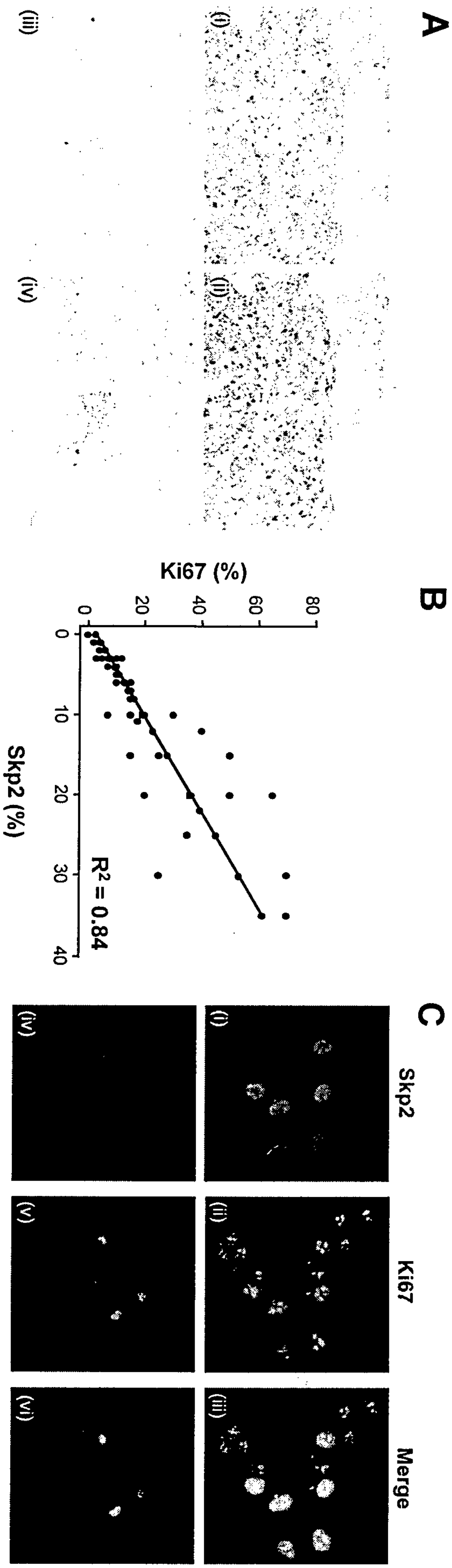


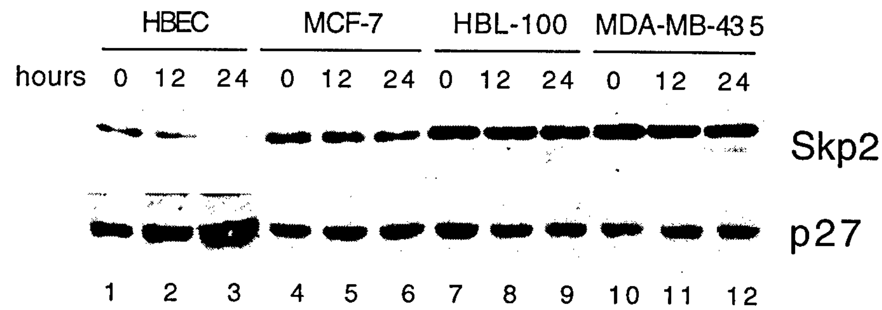
Figure 3



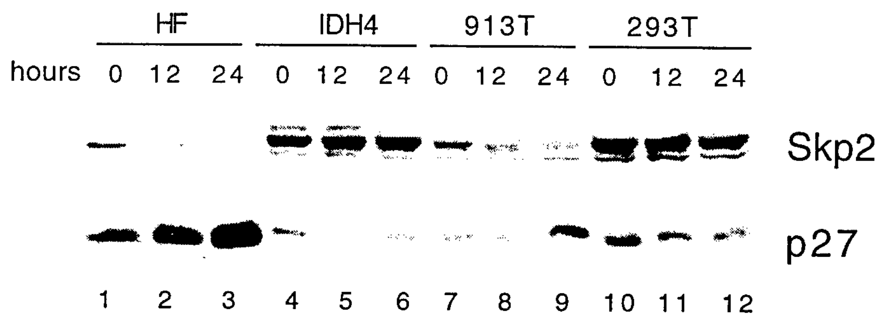
A
(i)
(ii)
(iii)
(iv)
B
Ki67 (%)
Skp2 (%)
$R^2 = 0.84$
0
20
40
60
80
0
10
20
30
40
C
Skp2
Ki67
Merge
(i)
(ii)
(iii)
(iv)
(v)
(vi)

Figure 4

A


HBEC
MCF-7
HBL-100
MDA-MB-435
hours 0 12 24 0 12 24 0 12 24 0 12 24
Skp2
p27
1 2 3 4 5 6 7 8 9 10 11 12

B


HF
IDH4
913T
293T
hours 0 12 24 0 12 24 0 12 24 0 12 24
Skp2
p27
1 2 3 4 5 6 7 8 9 10 11 12

C

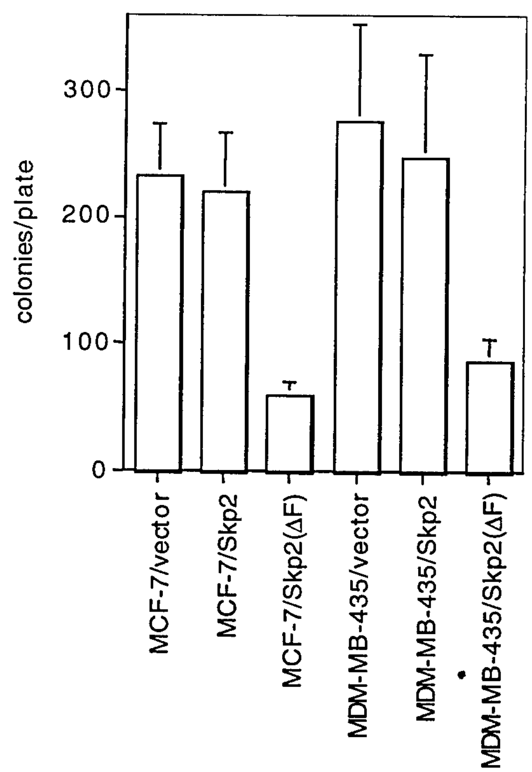
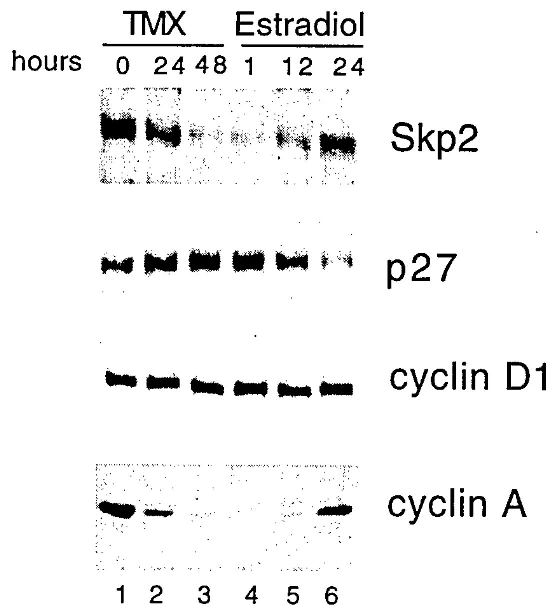

colonies/plate
0
100
200
300
MCF-7/vector
MCF-7/Skp2
MCF-7/Skp2(ΔF)
MDM-MB-435/vector
MDM-MB-435/Skp2
MDM-MB-435/Skp2(ΔF)

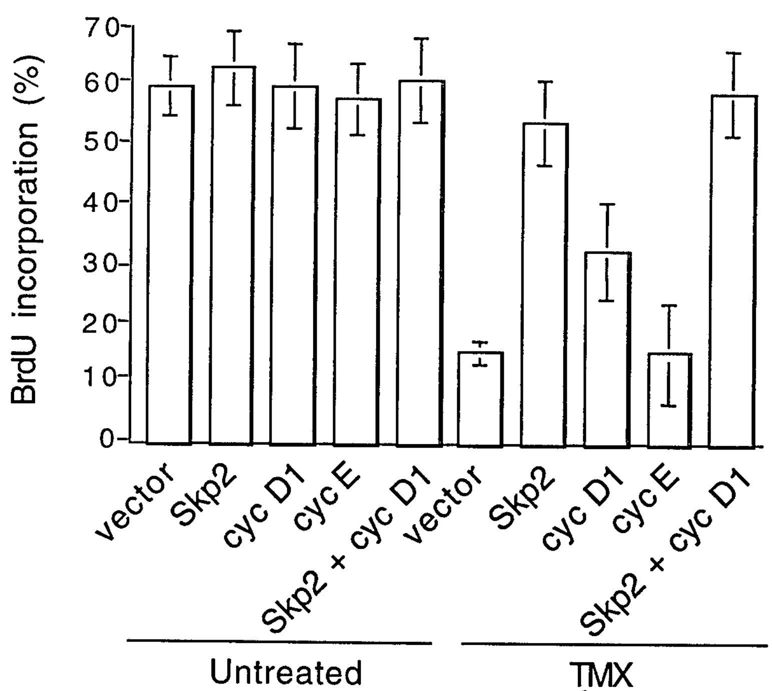
Figure 5

A



TMX
Estradiol
hours
0 24 48 1 12 24
Skp2
p27
cyclin D1
cyclin A
1 2 3 4 5 6

B



BrdU incorporation (%)
70
60
50
40
30
20
10
0
vector
Skp2
cyc D1
cyc E
Skp2 + cyc D1
vector
Skp2
cyc D1
cyc E
Skp2 + cyc D1
Untreated
TMX