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SKP2 Overexpression Is Associated With Increased Serine 10 Phosphorylation of p27 (pSer10p27) in Triple-Negative Breast Cancer

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S-phase kinase-associated protein 2 (SKP2) is an important cell cycle regulator, targeting the cyclin-dependent kinase (CDK) inhibitor p27 for degradation, and is frequently overexpressed in breast cancer. p27 regulates G_1/S transition by abrogating the activity of cyclin/CDK complexes. p27 can undergo phosphorylation at serine 10 (pSer10p27). This phosphorylation event is associated with increased cell proliferation and poor prognosis in patients with glioma. The relationship between SKP2 and pSer10p27 in breast cancer has not been previously investigated. Immunohistochemistry (IHC) of SKP2, p27, pSer10p27, and other genes involved in this pathway, was analyzed in 188 breast tumors and 50 benign reduction mammoplasty samples. IHC showed SKP2 to be more highly expressed in estrogen receptor α (ERα)-negative breast cancers and demonstrated that triple-negative tumors were more likely to have high expression of SKP2 than were non-triple negative, ERα-negative tumors. A significant positive relationship was discovered for SKP2 and pSer10p27. High levels of SKP2 and pSer10p27 were observed significantly more often in ERα-negative and triple-negative than in ERα-positive breast cancers. Use of the triple-negative TMX2-28 breast cancer cell line to address the role of SKP2 in cell cycle progression confirmed that SKP2 contributes to a more rapid cell cycle progression and may regulates pSer10p27 levels. Together, the results indicate that presence of high SKP2 plus high pSer10p27 levels in triple-negative breast cancers is associated with aggressive growth, and highlight the validity of using SKP2 inhibitors as a therapeutic approach for treating this subset of breast cancers.

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Breast cancer tops the list of new cancer diagnoses and is the second leading cause of cancer related mortality in women. An estimated 227,000 women will be diagnosed with breast cancer in 2012 and approximately 40,000 will die as a result of their breast cancer (American Cancer Society, 2012). Breast cancer patients with disease of similar stage and grade often respond differently to therapy resulting in disparate clinical outcomes.

Estrogen receptor α (ERα), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2) are accepted as predictive factors and are used to dictate a patient's therapeutic regime (Bast et al., 2000). Approximately 70% of breast cancers express ERα and are termed ERα-positive (Lin et al., 2010). For these patients, targeted endocrine therapy with anti-estrogens is available and their tumors are often responsive. The remaining 30% of breast cancers are classified as ERα-negative (Lin et al., 2010) and are often more aggressive. Pathologically, all tumors that are negative for all three receptors, ERα, PR, and HER2, are referred to as triple negative. Basal-like tumors are typically triple-negative, but are a distinct molecular subclass that has positive basal cytokeratin (CK) 5, 14, and/or 17 expression pattern (Sorlie et al., 2001; Cheang et al., 2008; Rakha et al., 2009). Triple-negative and basal-like tumors tend to be highly aggressive and generally have poor patient outcome, as patients with these types of tumors will not be responsive to anti-estrogen or anti-HER2 therapies (Dickson and Lippman, 1995; Perou et al., 2000; van de Rijn et al., 2002). Design of individualized treatment for specific disease subgroups is necessary and requires targeting genes or pathways actively engaged in the pathophysiology of breast cancer.

Dysregulation of the cell cycle results in uncontrolled cell proliferation. In particular, the G_1/S transition, and aberrant regulation of the proteins involved in this process are pivotally

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involved in cancer development and progression (Hanahan and Weinberg, 2000; Malumbres and Barbacid, 2001). S-phase kinase-associated protein 2 (SKP2) plays an important role in cell cycle regulation. SKP2 in normal cells promotes progression into the S phase of the cell cycle through targeted degradation of the cyclin dependent kinase inhibitor p27 (Carrano et al., 1999; Sutterluty et al., 1999). During the transition from G_1 to S phase, p27 undergoes phosphorylation modifications at three major sites, Serine 10 (Ser10), Threonine 198 (Thr198), and Threonine 187 (Thr187; Vervoorts and Luscher, 2008). Phosphorylation of p27 mediates the stability of the protein as well as its activity. Phosphorylation of Thr187 is catalyzed by SKP2 and promotes the ubiquitin ligase-mediated degradation of p27 (Zhang et al., 1995; Carrano et al., 1999; Sutterluty et al., 1999). Phosphorylation at Ser10 (pSer10p27) has a dual role depending on the status of the cell. In quiescent cells phosphorylation of this residue appears to promote quiescence; however, in cancer cells it can promote cell cycle progression (Boehm et al., 2002; Deng et al., 2004; Besson et al., 2006; Morishita et al., 2008). Thr198 phosphorylation is catalyzed by ROCK I kinase, only when Ser10 is already phosphorylated, and results in the inactivation of both free and cyclin-dependent kinase (CDK) bound p27 (Arakawa-Takeuchi et al., 2010; Park et al., 2011). SKP2 is not known to facilitate phosphorylation of p27 at Ser10 or at Thr198.

SKP2 has oncogenic potential and its overexpression has been found in several cancers, including breast, thyroid, non-small cell lung carcinoma, melanoma, and Kaposi's sarcomas (Gstaiger et al., 2001; Penin et al., 2002; Osoegawa et al., 2004; Katagiri et al., 2006; Chiappetta et al., 2007; Fujita et al., 2008). Emerging evidence has demonstrated that SKP2 overexpression significantly promotes the transition from G_1 to S phase of the cell cycle, resulting in accelerated proliferation, making this process critically involved in the pathogenesis of breast cancer (Fujita et al., 2008; Voduc et al., 2008; Chan et al., 2010). With respect to breast cancer, overexpression of SKP2 has been associated with poor prognosis on its own (Signoretti et al., 2002) and in combination with high Cyclin E expression (Voduc et al., 2008) as well as other unfavorable prognostic factors including increased tumor grade, lack of expression of ERα and PR, and HER2 overexpression (Davidovich et al., 2008; Ravaioli et al., 2008). High SKP2 expression has been shown to be a frequent characteristic in triple-negative, basal-like tumors (Foulkes et al., 2010).

The relationship between SKP2 and p27 nuclear protein expression in breast cancer is equivocal. To date, eight studies have assessed this relationship statistically, and of these, six observed a significant inverse relationship (Signoretti et al., 2002; Slotky et al., 2005; Zheng et al., 2005; Sonoda et al., 2006; Davidovich et al., 2008; Ravaioli et al., 2008) while two reported no association between expression of SKP2 and p27 proteins (Traub et al., 2006; Voduc et al., 2008). Even among the three largest studies the results are ambiguous. Voduc et al. (2008) examined SKP2 and p27 in 438 breast cancers and found no correlation between SKP2 and p27 protein levels. Likewise, Traub et al. (2006) reported no association between SKP2 and p27 among 338 breast cancers; however, they did find that high SKP2 and low p27 was associated with poorer outcome. The largest study to date, published by the International Breast Cancer Study Group in 2008, examined 1,598 breast cancers and reported a significant inverse relationship between SKP2 and p27 but found no predictive or prognostic value (Ravaioli et al., 2008).

In this study, we sought to further our understanding of how SKP2 expression affects cell cycle, p27 protein expression and post-translational modification, in triple-negative breast cancers. We evaluated the relationships between SKP2, p27, and Ser10 phosphorylated p27 (pSer10p27) using both breast cancer tissue, and the triple-negative breast cancer cell line, TMX2-28; which naturally has high expression of SKP2 (Gozgit et al., 2007; Fagan-Solis et al., 2013). Together, results from cell culture and human cancer tissues indicate that high levels of SKP2 and pSer10p27 in triple-negative breast cancers are associated with aggressive growth, and highlight the need of SKP2 inhibitors for treating this subset of breast cancers.

Materials and Methods

Human tissue

Institutional Review Board approval was obtained from Baystate Medical Center and the University of Massachusetts at Amherst. An independent party, prior to our acquirement of tissue, de-identified samples by assigning them numerical codes (to maintain patient anonymity) and thus individual consent was not needed. For the mRNA analysis of SKP2 and cytokeratins, 30 frozen breast tumor samples were retrieved from Baystate Medical Center, Department of Surgical Pathology as previously described (Gozgit et al., 2006, 2007; Turk et al., 2012). For the immunohistochemistry (IHC) of SKP2 and related proteins, 159 tumors and 40 reduction mammoplasty (RM) tissues were stained. These tissues were obtained as follows: six sections were cut from each of five tissue microarrays (TMAs) containing a total of 101 tumor and 40 RM cases represented in triplicate as previously described (Turk et al., 2012), and six slides from each of 58 formalin-fixed paraffin-embedded (FFPE) breast tumors were purchased from University of Massachusetts Medical School Cancer Tissue Bank, Worcester, MA. An additional 29 tumor cases and 10 RM blocks from TMAs and tissue block sections previously described were stained and scored (Gozgit et al., 2007) for SKP2 only as previously described, for a total of 188 cases and 50 RM samples (238 total breast tissue samples). Clinical pathology reports accompanied all cases providing data on age, tissue type, histological grade, and in most cases ERα, PR, and HER2 status. Table 1 provides an overview of the clinical and pathological characteristics of the RM and breast carcinoma patients.

Immunohistochemistry (IHC)

Immunohistochemical study was performed using the Dakocytomation LSAB2 System-HRP kit (Dako, Carpinteria, CA) as previously described (Turk et al., 2012). Primary antibodies were as follows: SKP2 (1:25; sc-7164), Cyclin D1 (1:200; sc-753), Cyclin E (1:200; sc198), p27 (1:200; sc-528) (polyclonal; Santa Cruz Biotechnology, Santa Cruz, CA); CDK2 (1:1,000; ab6538; polyclonal) and pSer10p27 (1:50; ab62364; monoclonal; Abcam, Cambridge, MA), with secondary antibody anti-rabbit from Dako (Dako polymer K4003; Dako). Slides were stained with diaminobenzidine (DAB) chromogen for 10 min. The slides were then counterstained for 15 sec in Mayer's hematoxylin, transferred to glacial acetic acid water for 15 sec, and then to ammonia water to blue. Finally, slides were dehydrated in ethanol and xylene before manual coverslipping.

The performance of each antibody was optimized through the use of negative (no primary antibody) and positive (tonsil, skeletal muscle, or breast cancer tissues with known protein positivity) controls and by testing a series of dilutions bracketing the manufacturer's suggested dilutions. Antigen retrieval methods (sodium citrate, pH, EDTA) were also optimized. Western immunoblotting was performed to confirm protein specificity based on protein size.

Review of IHC sample staining and pathology

Scoring was conducted by one anatomic pathologist (CNO) without knowledge of the hormone receptor status of samples. TMAs and tissue slides were scored for immunoreactivity of the six antigens, assigning values of negative, weak, moderate, or

TABLE 1. Clinical and pathological characteristics of patients

Characteristic	Median (range)	N (%)
Histology		
DCIS		37 (20)
IDC		138 (73)
Other		13 (7)
Tumor grade		
G_1		23 (12)
G_2		48 (26)
G_3		105 (56)
N/A		12 (6)
Woman's age (in years)		
All tumors	55 (25–89)	188 (100)
ERα+	55 (25–89)	93 (49)
ERα−	54 (29–87)	95 (51)
NTN[a]	58 (32–85)	24 (25)
TN[b]	53 (29–87)	49 (52)
N/A[c]	59.5 (38–86)	22 (23)
Reduction mammoplasty	25.5 (15–55)	40 (80)[d]

Note: All data, including ERα/PR/Her2 status, were obtained from accompanying pathology reports.
[a]NTN: non-triple negative, ERα-negative but either PR or HER2 positive.
[b]TN: triple negative.
[c]ERα-negative tumors for which HER2 status was not available.
[d]Age was not available for 10 reduction mammoplasty samples.

strong for the intensity of staining (Hammond et al., 2010). Tissues in which immunoreactivity was scored as weak, moderate, or strong were further evaluated for the distribution of the staining and received descriptors of focal or diffuse for low or high fractions of cells stained respectively. The number of cells showing immunoreactivity was not counted, but tissue was scored as diffuse if greater than 50% of the cells were estimated as stained. Tissue was scored as focal if less than 50% of the cells were estimated as stained, and in general these tissues had less than 10% of the cells stained. Tissues scored as negative were not evaluated for focal versus diffuse distribution. Scoring occurred over 12 sessions during which a single observer (KDF-S) recorded all evaluations. Periodically, previously evaluated slides were included in the blinded scoring session resulting in a total of 10 different tumors being independently scored three times (three tumors for SKP2, two for p27, two for pSer10p27, and one each for Cyclin E, CDK2, and Cyclin D1). Of these 30 repeat scores, there was only one discrepancy: a tumor was scored once as moderate/diffuse and twice as strong/diffuse for p27.

Cell culture

TMX2-28 and MCF-7 cells were maintained as previously described (Fagan-Solis et al., 2013). Puromycin (2.5 μg/ml) was added to media of TMX2-28-NC, TMX2-28-S2, and TMX2-28-MC cell cultures. MDA-MB-231 cells were maintained in L-15 medium supplemented with 10% fetal bovine serum (FBS) at 37°C and 0% CO_2. SKBR-3 cells were maintained in McCoy's 5A medium supplemented with FBS at 37°C and 5% CO_2. All cell cultures were passaged when near 80% confluence.

RNA and protein isolation

Total RNA (n = 3 biological samples) was isolated with Tri-Reagent (Molecular Research Center, Cincinnati, OH) as previously described (Fagan-Solis et al., 2013). RNA was treated with Turbo DNA-Free (Ambion, Austin, TX); RNA quality was assessed by 260/280 nm spectrophotometer readings (Nanodrop 8000; Thermo Scientific, Wilmington, DE). Protein-containing cell lysates were isolated from cell cultures (n = 3 biological samples) with pre-chilled SDS lysis buffer (1% SDS, 0.06 M Tris–HCL, and 10% glycerol) according to our standard laboratory protocols (Fagan-Solis et al., 2013).

Western immunoblotting

Protein lysates were mixed with NuPage sample buffer and reducing agent (Invitrogen, Carlsbad, CA), heated at 70°C for 10 min, separated on Mini-PROTEAN TGX gels (Bio-Rad, Hercules, CA) and then transferred to an Immuno-Blot polyvinylidene difluoride membrane (Bio-Rad) according to manufacturer's protocols. Membranes were incubated in blocking buffer (5% nonfat dry milk/Tris buffered saline and 0.1% Tween 20) for 1 h at room temperature with gentle shaking. Membranes were then incubated with the same primary antibodies used for IHC but with Western-specific dilutions [SKP2 (1:200), Cyclin D1 (1:200), Cyclin E (1:200), p27 (1:200), CDK2 (1:500), and pSer10p27 (1:10,000)], overnight at 4°C, followed by incubation with the secondary antibody, anti-rabbit IgG linked to horseradish peroxidase (1:1,000; 7074S; Cell Signaling Technology, Danvers, MA), for 1 h at room temperature. Chemiluminescent signals were detected with SuperSignal West Pico Kit (Pierce, Rockford, IL), and imaged using the G.BOX Chemi HR-16 (Syngene, Fredrick, MD). Membranes were stripped using Restore stripping buffer (Thermo Scientific, Rockford, IL) and re-probed for glyceraldehyde-3-phosphate (GAPDH; 1:10,000; 5174; Cell Signaling Technology).

Quantitative real-time reverse transcriptase PCR (qRT-PCR)

RNA samples (n = 3 biological samples) were reverse transcribed and amplified using the One Step RT-PCR kit (Qiagen, Valencia, CA) in the Roche LightCycler (Roche, Indianapolis, IN). Total RNA (75 ng) was incubated with Qiagen RT-PCR master mix including primers (250 nM each) and SYBR Green I nucleic acid stain (2X; S7563, Invitrogen) in pre-cooled capillaries (Roche) and reversed transcribed (50°C for 30 min). Samples were then heated to 95°C for 15 min. PCR was monitored over 45 cycles (denaturation: 95°C for 15 sec; annealing: 60°C for 15 sec; extension: 72°C for 30 sec) by fluorescence of intercalating SYBR Green. Relative mRNA levels were normalized to hypoxanthine ribosyltransferase (HPRT). Gene-specific primers (Supp. Table 1) spanning exon–exon junctions were designed using Primer3 software (http://frodo.wi.mit.edu/), and purchased from Integrated DNA Technologies, Inc. (Coralville, IA).

RNA interference

pGIPz lentiviral shRNAmir (Open Biosystems, Lafayette, CO) targeting SKP2 (Source ID: V2LHS_199552) or a negative control (random sequence with no significant homology to any endogenous human, mouse or rat gene) pGIPz lentiviral shRNAmir were transfected into TMX2-28 cells using Superfect Transfection Reagent (Qiagen) per manufacturer's instructions. Forty-eight hours post-transfection, cells were treated with selection medium (DMEM supplemented with 2.5 μg/ml puromycin). Five days following initial selection, cells transfected with the negative control shRNAmir were seeded at low density into 100 mm culture dishes. Cloning cylinders (BellCo Glass, Inc., Vineland, NJ) were used to isolate small colonies (<50 cells) generating the TMX2-28-NC (*N*egative *C*ontrol) cell line. Cells transfected with the shRNAmir targeting SKP2 were split into two populations. One population of cells was kept and termed TMX2-28-MC (*M*ass *C*ulture). The other population was seeded at low density and cloning cylinders were used to isolate colonies, one of which was the TMX2-28-S2 (*S*KP2 knockdown) cell line.

Cell cycle analysis

Adherent cells cultures were harvested by trypsinization, resuspended in phosphate-buffered saline (PBS), and fixed with 95% ethanol (ETOH) at 4°C overnight. Fixed cells were collected

by centrifugation, resuspended in staining solution (PBS, 2 mM $MgCl_2$, 10 μg/ml RNase A, 100 μg/ml propidium iodide) and incubated at 37°C for 20 min. Single cell populations (1×10^6 cells/ml) were analyzed using FACSDiva 4.1 and cell cycle analysis was performed using BD FACSDiva software (Becton-Dickinson Bioscience, Franklin Lakes, NJ).

Statistical analyses

STATA 11.2 (Statacorp LP, College Station, TX) was used for analysis of IHC data. Cell cycle and mRNA expression data were analyzed and graphed with GraphPad Prism Version 3.02 (GraphPad Software, Inc., San Diego, CA). For ANOVA and post hoc two-tailed comparisons, significance was set at $P < 0.05$. A Bonferonni correction was used to adjust the *P*-value whenever multiple *t*-tests were conducted.

For analysis of the IHC results, the intensity scores of negative, weak, moderate, and strong were converted to two metrics: positive (including weak, moderate, and strong) versus negative (including negative only) and high (including moderate and strong) versus low (including negative and weak). For each case in the TMAs, the highest score given to any of the three punches was used for analysis. For the slides, scores were based on evaluation of all tumor or RM tissue. For all tissues that were not scored as negative, analysis also was conducted on the descriptors of diffuse and focal, as well as the original scores of negative, weak, moderate, and strong, providing a total of four analyzed metrics. Relationships between antigen staining and hormone status, tumor grade, and tumor type were assessed with two-tailed Fisher exact tests and *P*-values are presented. For tumor type and grade the original evaluations of negative, weak, moderate, and strong were used in the analysis. Relationships between immunostaining and age were assessed with regression analysis on the original evaluations of negative, weak, moderate, and strong.

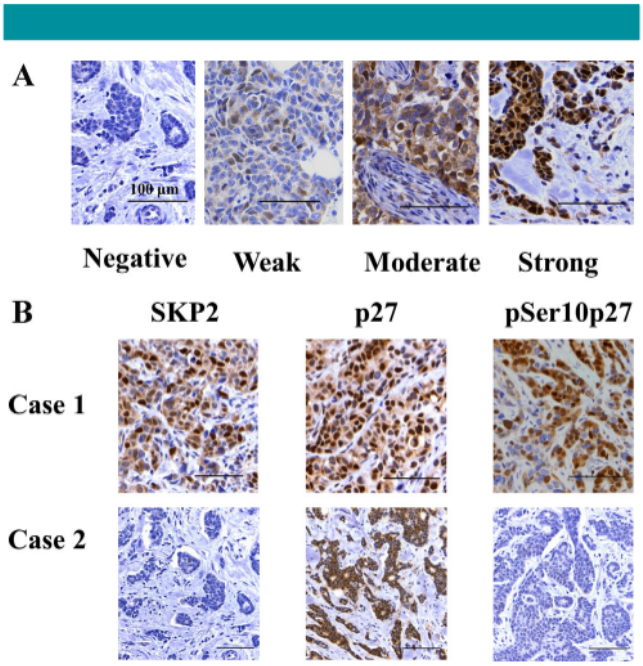

Fig. 1. Expression of SKP2 in breast cancer tumors is highly correlated with pSer10p27. A: Intensity of immunoreactivity in epithelial cells was used to assign scores of negative, weak, moderate, and strong for each protein; immunoreactivity to SKP2 is shown. B: Breast tumor TMAs and tissue slides were prepared, stained, and scored as described in the Materials and Methods Section. Representative images of tumor tissue from two triple-negative cases demonstrate the relationship among SKP2, p27, and pSer10p27 expression typically observed in the present study. Scale bar = 100 μm.

Results

SKP2 and pSer10p27 are overexpressed in triple-negative breast cancers

SKP2, p27, pSer10p27, CDK2, Cyclin E, and Cyclin D1 expression was examined immunohistochemically using tissue sections and microarrays. We evaluated nuclear protein staining characteristics of benign RM epithelial cells and of tumor epithelial cells. Figure 1A provides examples of breast cancer cases that received scores of negative, weak, moderate, or strong based on staining intensity for SKP2.

Table 2 provides a summary of the protein expression in human breast tissue. Immunohistochemical analysis of 238 breast tissue specimens showed that SKP2 protein is predominantly expressed in breast cancer (59%; 111 out of 188) as compared to RM tissue (8%; 4 out of 50; $P < 0.001$). Of the tumors evaluated for distribution of SKP2 immunoreactivity, 21% (21 out of 98) were scored as diffuse.

SKP2 was expressed significantly more often in ERα-negative tumors than in ERα-positive tumors: 84% of ERα-negative tumors were positive for SKP2 while only 33% of ERα-positive tumors were positive for SKP2 ($P < 0.001$). Similar results were obtained when immunoreactivity was analyzed by high/low: 63% of ERα-negative tumors had high SKP2 expression while only 16% of ERα-positive tumors had high SKP2 ($P < 0.001$). Additionally, of the tumors scored for staining distribution, 28% of ERα-negative tumors had diffuse staining whereas only 6% of ERα-positive tumors were scored diffuse (Table 2).

Among ERα-negative tumors, triple-negative tumors were more likely to be positive for SKP2 than were non-triple-negative tumors (ERα-negative tumors that were positive for either PR or HER2): 98% versus 75%, respectively ($P = 0.002$), and the staining intensity was more frequently scored as high in triple-negative tumors than it was in non-triple negative tumors (80% vs. 58%, respectively; $P = 0.056$). Furthermore, triple-negative tumors were significantly more likely to be scored as having a high percent of cells with immunoreactivity for SKP2: 40% of triple-negative tumors were scored as having diffuse SKP2 expression, while no non-triple negative samples were scored as having diffuse SKP2 expression ($P = 0.003$).

p27 expression has been inversely correlated with SKP2 levels in subsets of breast cancer with poor prognosis (Signoretti et al., 2002; Slotky et al., 2005; Zheng et al., 2005; Sonoda et al., 2006; Davidovich et al., 2008). We observed higher levels of SKP2 in ERα-negative and triple-negative tumors, therefore we expected these groups of tumors to have low p27 immunoreactivity. Contrary to expectations, no significant relationship was found between p27 and SKP2 immunoreactivity, regardless of hormone receptor status or the scoring metric used in the analysis. p27 immunoreactivity was detected in most tumors: 92%, 69%, and 61% were scored positive, high and diffuse respectively for p27 immunoreactivity. In contrast to SKP2, p27 protein was common in RM tissue but still at significantly lower levels than in cancer when scored as either positive/negative or as high/low ($P = 0.040$ and $P < 0.001$, respectively; Table 2).

Next, we assessed the levels of pSer10p27 in breast cancer. Analysis in 192 breast tissue specimens showed that pSer10p27 protein is more frequently expressed in breast cancer (50%) as opposed to RM tissue (24%; $P = 0.006$). Of the 80 tumors scored positive for pSer10p27, 34% had diffuse staining. pSer10p27 expression was significantly greater in ERα-negative than in ERα-positive tumors when assessed by either positive/negative or high/low immunoreactivity (Table 2: $P < 0.001$ for

TABLE 2. Expression of SKP2, p27, and pSer10p27 in breast cancers and reduction mammoplasty (RM)

	Positive % (N)	P	High % (N)	P	Diffuse[a] % (N)	P
SKP2						
Tissue type						
RM	8% (4 of 50)	<0.001	[b]	<0.001	[c]	—
Cancer	59% (111 of 188)		40% (75 of 188)		21% (21of 98)[c]	
Hormone receptor status in breast cancers						
ERα+	33% (31 of 93)	<0.001	16% (15 of 93)	<0.001	6% (2 of 31)	0.016
ERα−	845 (80 of 95)		63% (60 of 95)		28% (19 of 67)[c]	
NTN[d]	75% (18 of 24)	0.002	58% (14 of 24)	0.056	0% (0 of 14)[c]	0.003
TN[e]	98% (48 of 49)		80% (39 of 49)		40% (19 of 48)	
p27						
Tissue type						
RM	79% (23 of 29)	0.040	21% (6 of 29)	<0.001	4% (1 of 23)	<0.001
Cancer	92% (146 of 159)		69% (109 of 159)		61 (95 of 146)	
Hormone receptor status in breast cancers						
ERα+	93% (78 of 84)	0.615	69% (58 of 84)	0.887	71% (55 of 78)	0.165
ERα−	91% (68 of 75)		68% (51 of 75)		59% (40 of 68)	
NTN[d]	100% (18 of 18)	0.120	67% (12 of 18)	0.917	56% (10 of 18)	1.0
TN[e]	88 (43 of 49)		65 (32 of 49)		58% (25 of 43)	
pSer10p27						
Tissue type						
RM	24% (8 of 33)	0.006	12% (4 of 33)	0.015	12% (1 of 8)	0.427
Cancer	50% (80 of 159)		33 (53 of 159)		34% (27 of 80)	
Hormone receptor status in breast cancers						
ERα+	36% (30 of 84)	<0.001	15% (13 of 84)	<0.001	33% (10 of 30)	1.0
ERα−	67% (50 of 75)		53% (40 of 75)		34% (17 of 50)	
NTN[d]	56% (10 of 18)	0.372	56% (10 of 18)	0.856	30% (3 of 10)	0.711
TN[e]	67% (33 of 49)		53% (26 of 49)		36% (12 of 33)	

[a]Diffuse and focal staining was evaluated only for tissues scored positive.
[b]RM tissues were scored as positive/negative only.
[c]Thirteen tumors with SKP2 reactivity were not evaluated for distribution of staining, therefore the numbers for diffuse and focal are reduced.
[d]NTN: non-triple-negative, tumors scored from pathology reports as negative for ERα, but positive for PR and/or HER2.
[e]TN: triple-negative, tumors scored from pathology reports as negative for ERα, PR, and HER2.

both comparisons). However, staining distribution (diffuse vs. focal) did not differ with ERα status. When categorized by triple-negative status, there were no significant differences in pSer10p27 expression or staining distribution.

All three of the remaining cell cycle genes, Cyclin E, CDK2, and Cyclin D1, were expressed primarily in cancer as opposed to RM tissue (data not shown). Despite the higher levels in cancer, when evaluated as high/low, most tumors, regardless of hormone status, were scored as low for Cyclin E. Likewise, the majority of tumors were scored low for CDK2. In contrast, Cyclin D1 was scored as positive and high more frequently in ERα-positive cancers, and was more likely to be scored as low in triple-negative cancers (data not shown).

Expression of SKP2 and Ser10 phosphorylated p27 is highly correlated

Relationships between SKP2 and other proteins are presented in Table 3 in which the total number of tumors staining positive or negative, and high or low for SKP2 are presented, along with the percentage and number of those tumors that were scored positive (left side of table) or high (right side of the table) for each of the cell cycle genes. Analysis of 159 tumors for which data were available for both SKP2 and p27 revealed no significant relationship between the two proteins (samples images are shown in Fig. 1B), when the analysis was conducted on either positive/negative or high/low scores. In contrast, pSer10p27 levels were positively associated with SKP2 expression. Assessing the relationship between SKP2 and pSer10p27 for high/low staining: 67% of the SKP2-positive tumors (66 of 98) were also positive for pSer10p27. In contrast, 23% of the SKP2-negative tumors (14 of 61) were positive for pSer10p27.

Similarly, 83% of tumors positive for pSer10p27 (66 of 80) were positive for SKP2. The positive relationship between SKP2 and pSer10p27 immunostaining was highly significant ($P < 0.001$; $n = 159$) as was the relationship when data were analyzed using SKP2 high/low scores ($P < 0.001$; $n = 159$).

Furthermore, the positive relationship between SKP2 and pSer10p27 was more frequent in ERα-negative and triple-negative tumors. Among the 188 tumors with known ERα status, 38 tumors were scored high for both SKP2 and pSer10p27, and of these, 35 were ERα-negative while only 3 were ERα-positive (Fisher's Exact $P = 0.0001$; $n = 188$). Likewise, among the 158 tumors with known triple-negative status, 37 were scored high for both SKP2 and pSer10p27, and of these, 25 were triple-negative and 12 were non-triple negative (Fisher's exact $P = 0.0001$; $n = 158$). Analysis of SKP2 and Cyclin E expression demonstrated a positive association, for both metrics analyzed ($P = 0.035$ for positive/negative and $P = 0.04$ for high/low). CDK2 on the other hand, was more likely to be scored high in tumors that were scored low for SKP2 ($P = 0.031$). There was no relationship between SKP2 and Cyclin D1 expression.

Previous research suggests that high levels of SKP2 and Cyclin E together with low levels of p27 occurs more frequently in triple-negative tumors and may be a biomarker for poor prognosis (Voduc et al., 2008). While we did not observe a relationship between p27 and SKP2, it remained feasible that the combined expression pattern could be an indicator of prognosis. Therefore, we examined the extent to which high levels of SKP2 and Cyclin E together with low levels of p27 occurred in breast cancers. Despite small sample sizes, we found that the pattern of high SKP2 and Cyclin E with low p27 was significantly more common in triple-negative tumors than in ERα-negative tumors that were not triple-negative (data not shown; Fisher's exact = 0.008).

SKP2 is more highly expressed in ERα-negative and triple-negative tumors from younger women while pSer10p27, Cyclin D1, p27, and CDK2 are more highly expressed in tumors from older women

The relationship between strong protein expression and age is presented in Supplementary Table 2. SKP2 expression in tumors was inversely correlated with age; tumors from

TABLE 3. Relationships of SKP2 and cell cycle gene expression in breast cancer

	SKP2 expression % (N)			SKP2 expression % (N)		
Cell cycle gene	Positive	Negative	*P*	High	Low	*P*
p27 positive	94% (92 of 98)	89% (54 of 61)	0.231	74% (51 of 69)	64% (58 of 90)	0.202
pSer10p27 positive	67% (66 of 98)	23% (14 of 61)	<0.001	55% (38 of 69)	17% (15 of 90)	<0.001
CDK2 positive	35% (33 of 94)	42% (25 of 59)	0.367	7% (5 of 67)	20% (17 of 86)	0.031
Cyclin E positive	56% (54 of 97)	40% (23 of 57)	0.035	26% (18 of 68)	13% (12 of 89)	0.040
Cyclin D1 positive	78% (76 of 97)	84% (51 of 61)	0.418	65% (44 of 68)	59% (53 of 90)	0.457

P-values reflect results of Fisher exact test of the relationship between expression of SKP2 and one other cell cycle gene.

younger women were more likely to be scored strong for SKP2 ($F(1, 157)$; $P = 0.005$; $n = 159$). When tumors were categorized by ERα status, SKP2 expression was inversely correlated with age in ERα-negative tumors only ($F(1, 73)$; $P = 0.014$; $n = 75$). Further stratification of ERα-negative tumors showed that SKP2 immunoreactivity was more frequently scored as strong in younger women with triple-negative tumors ($F(1, 27)$; $P = 0.029$; $n = 49$), but not in younger women with non-triple negative tumors ($F(1, 16)$; $P = 0.84$; $n = 18$). While expression of SKP2 was generally stronger in younger women, age explained little of the variability in SKP2 expression, either when the analysis was conducted on all tumors ($R^2 = 0.05$), ERα-negative tumors ($R^2 = 0.08$), or triple-negative tumors ($R^2 = 0.1$).

Immunoreactivity of p27, pSer10p27, CDK2, and Cyclin D1, in contrast to SKP2, was positively correlated with age. Analyses on the total tumor set revealed a trend for stronger staining in older women for pSer10p27 ($F(1, 157)$; $P = 0.003$; $n = 159$) and Cyclin D1 ($F(1, 156)$; $P = 0.012$; $n = 158$), this trend was also significant in ERα-negative tumors (pSer10p27: $F(1, 73)$; $P = 0.004$; $n = 75$ and Cyclin D1: $F(1, 72)$; $P = 0.006$; $n = 74$), and for Cyclin D1 in triple-negative ($F(1, 47)$; $P = 0.017$; $n = 49$). Strong immunoreactivity of p27 was positively correlated with age among non-triple negative tumors only ($F(1, 16)$; $P = 0.018$; $n = 18$), while the association between strong CDK2 immunoreactivity and age was significant among ERα-negative tumors only ($F(1, 69)$; $P = 0.032$; $n = 71$). There was no significant relationship between Cyclin E expression and age.

SKP2 is more highly expressed in invasive and high grade tumors

The relationship between tumor type or grade and protein immunoreactivity is presented in Supplementary Tables 3 and 4, respectively. Invasive ductal carcinomas (IDC) were more likely to have strong SKP2 immunoreactivity than were ductal carcinomas in situ (DCIS; $P = 0.018$; $n = 188$). Furthermore, strong SKP2 expression was more frequently associated with grade three tumors than with lower grade tumors ($P = 0.010$; $n = 188$). The only other association between cell cycle protein expression and tumor type or grade was for CDK2, which was more likely to be strongly expressed in DCIS than in IDC tumors ($P < 0.001$; $n = 159$).

SKP2 is overexpressed in the triple-negative breast cancer cell line, TMX2-28

Given the high levels of SKP2 in triple-negative tumors we asked whether the triple-negative, tamoxifen-selected TMX2-28 cell line (Fagan-Solis et al., 2013) had increased levels of SKP2. Both SKP2 mRNA and protein levels were compared among three breast cancer cell lines, TMX2-28, MCF-7, and MDA-MB-231. The mRNA and Western immunoblot analyses demonstrated that SKP2 levels were significantly higher in TMX2-28 cells (Fig. 2A). SKP2 mRNA levels in TMX2-28 cells were roughly six times greater than in the parent cell line MCF-7 and the triple-negative MDA-MB-231 cell line. These data suggest TMX2-28 may be a good cell model for studying mechanisms underlying the growth of triple-negative breast cancers that also express high levels of SKP2.

In addition to being triple-negative, the TMX2-28 cell line has acquired a more basal-like protein expression pattern as compared to its parent MCF-7 cell line (Gozgit et al., 2007; Fagan-Solis et al., 2013), prompting us to ask whether SKP2 expression is higher in basal-like human breast cancers. Therefore we examined SKP2 mRNA expression in 30 frozen breast carcinoma samples by qRT-PCR. Eighteen ERα-positive and 12 ERα-negative breast cancers were assessed for mRNA expression of SKP2 and basal cytokeratins 5 and 17 (Fig. 3). Tumors expressing CK 5 and/or 17 ($n = 11$) expressed significantly higher SKP2 mRNA levels than tumors that did not express basal cytokeratins ($n = 19$; $P = 0.038$). Additionally, significantly higher SKP2 expression was found in ERα-negative tumors expressing basal cytokeratins 5 and 17 ($n = 6$, basal-like tumors) than in all other tumors ($n = 24$; $P = 0.043$). There was a marginally significant trend toward higher SKP2 expression in ERα-negative tumors ($n = 12$) over ERα-positive ($n = 18$; $P = 0.067$).

SKP2 levels regulate expression of its pathway genes in TMX2-28

The mRNA and protein levels of p27, CDK2, and Cyclin E, were assessed in TMX2-28 cells using qRT-PCR and Western immunoblotting to investigate the role of other genes involved in the SKP2 pathway. TMX2-28 cells had significantly higher p27 mRNA ($P < 0.05$) and protein levels than did either MCF-7 or MDA-MB-231. Interestingly, the targets of p27 inhibition, Cyclin E and CDK2, also were overexpressed in TMX2-28 as compared to MCF-7 cells ($P < 0.05$ and $P < 0.01$, respectively; Fig. 4B,C). The levels of the non-SKP2 pathway gene, Cyclin D1, were similar among all cell lines ($P > 0.05$; Fig. 4D). Additionally, levels of pSer10p27 are substantially higher in TMX2-28 than in either MCF-7 or MDA-MB-231 (Fig. 4A).

To determine whether a high expression of SKP2 controls the expression of its target S-phase transition pathway genes we assessed the effects of suppressing SKP2 expression in TMX2-28 cells. For this study, TMX2-28 cells were stably transfected with either a negative control shRNA or an shRNA targeted against SKP2. The resulting lines were termed TMX2-28-NC (negative control), TMX2-28-S2 (single clone SKP2 knockdown), and TMX2-28-MC (mass culture SKP2 knockdown). As shown in Figure 2B, SKP2 mRNA levels were reduced by approximately 83% in both knockdown lines; suppression was confirmed by Western immunoblotting. Expression of p27, pSer10p27, CDK2, Cyclin E, and Cyclin D1 was determined in the three knockdown cell lines. Cells with shRNA to SKP2 showed a significant ($P < 0.001$) increase of p27 RNA expression that was associated with an increase in protein expression (Fig. 4A). Although not significant, there was a slight increase in Cyclin E RNA expression that was

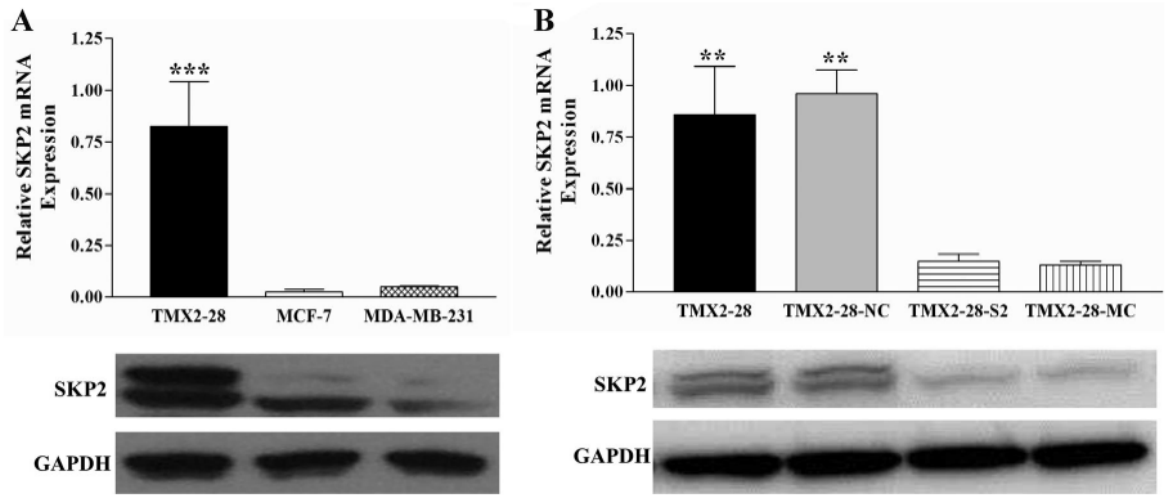

Fig. 2. SKP2 is overexpressed in TMX2-28 cells. A: Relative SKP2 mRNA expression in TMX2-28, MCF-7, and MDA-MB-231 cells was determined by real time qRT-PCR. SKP2 protein expression was determined by Western immunoblotting (GAPDH as loading control). Differences among cell lines were analyzed by ANOVA with Bonferroni's correction (*$P < 0.001$). B: TMX2-28 cells were stably transfected with either a negative control pGIPZ shRNAmir (TMX2-28-NC), or a pGIPZ shRNAmir targeted against SKP2 (TMX2-28-S2). Relative SKP2 mRNA expression in TMX2-28-NC and TMX2-28-S2 cells was determined by real time qRT-PCR and normalized to HPRT. Protein expression of SKP2 was determined by Western immunoblotting (GAPDH as loading control). Differences among cell lines were analyzed by ANOVA with Bonferroni's correction (**$P < 0.01$).**

reflected at the protein level (Fig. 4B). SKP2 knockdown in TMX2-28 cells also resulted in a significant increase in CDK2 gene and protein expression and as expected, RNA and protein expression of Cyclin D1 remained consistent ($P > 0.05$; Fig. 4C, D) TMX2-28-MC cells mirrored TMX2-28-S2 cells in their expression profiles. Lastly, suppressing SKP2 expression in the TMX2-28-S2 cells reduced the detectable phosphorylation at serine 10 of p27, despite the increase in p27.

Knockdown of SKP2 alters cell cycle in TMX2-28

TMX2-28-NC, TMX2-28-S2, and TMX2-28-MC cells were subjected to cell cycle analysis by flow cytometry to further investigate the role played by SKP2 overexpression in the control of cell cycle in breast cancer. Suppression of SKP2 in both the clonally selected and mass culture cell lines resulted in a noticeable shift of the cell cycle toward G_0/G_1 (Fig. 5). We found a significant increase in the percentage of cells in the G_0/G_1 phase and a significant decrease in the percentage of cells in the S phase of the cell cycle. We did not, however, see a significant change in the percentage of cells in the G_2/M phase of the cell cycle. The results from this experiment point to a prominent role of SKP2 in the proliferative potential of TMX2-28 cells.

Discussion

Patients with triple-negative and basal-like tumors face poor prognosis, as their tumors are not responsive to anti-estrogen or

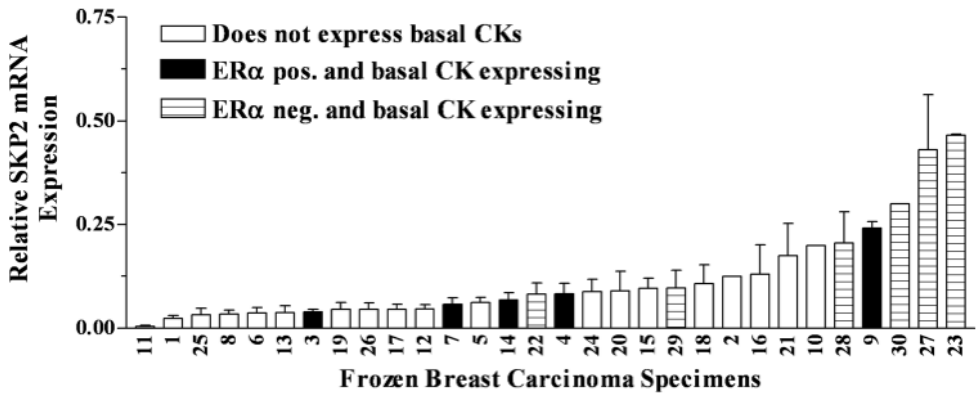

Fig. 3. SKP2 is overexpressed in ERα-negative breast tumors that also express basal cytokeratins 5 and/or 17. Relative SKP2 mRNA expression was determined in 30 frozen breast carcinoma samples using real time qRT-PCR normalized to HPRT. Numbers 1–18 indicates ERα-positive tumors, while numbers 19–30 indicates ERα-negative tumors. White bars represent tumors (regardless of ERα status) that did not have expression of CK5 and/or 17. Black bars represent ERα-positive tumors that were positive for CK5 and/or CK17. Striped bars represent ERα-negative tumors that had expression of CK5 and/or 17.

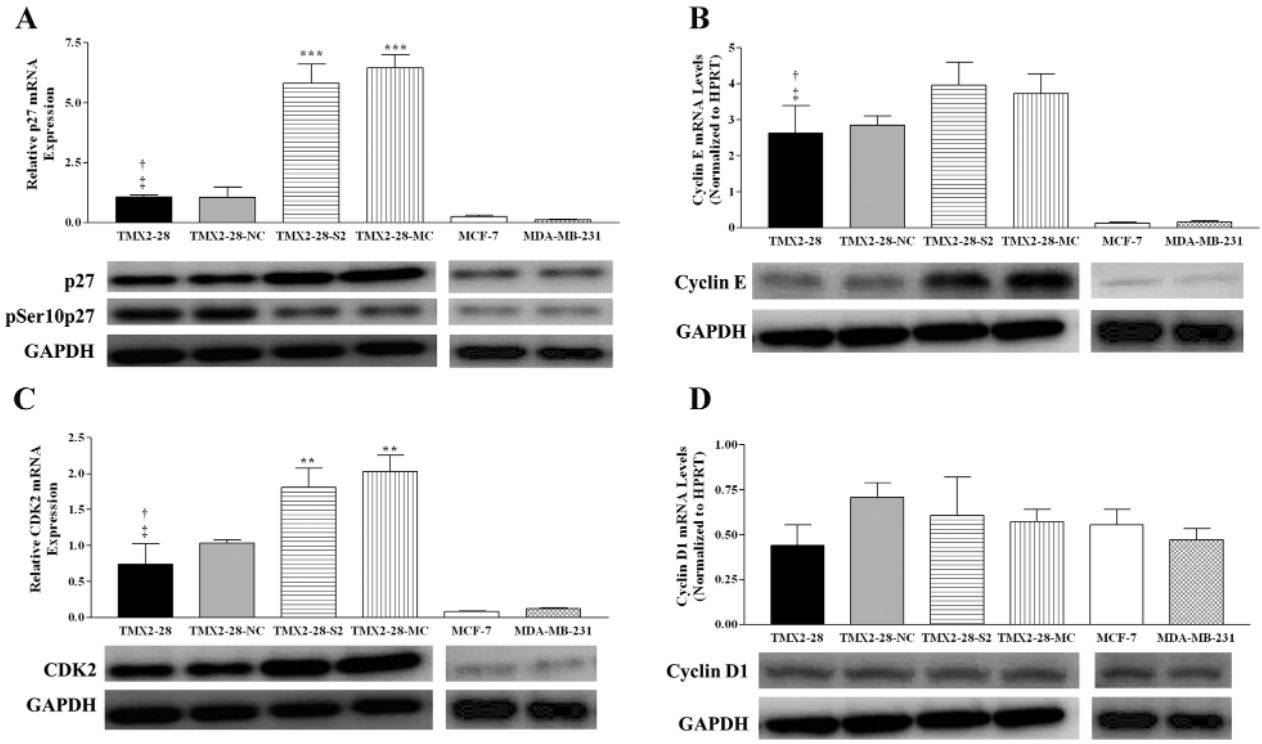

Fig. 4. Expression of SKP2 pathway genes in TMX2-28 cells. Relative mRNA expression of (A) p27, (B) Cyclin E, (C) CDK2, and (D) Cyclin D1 were determined by real-time qRT-PCR and normalized to HPRT. Differences in mRNA levels among cell lines were analyzed using one-way ANOVA and post hoc t-tests with a Bonferroni correction for comparisons between TMX2-28 cells and MCF-7 cells. $^{\dagger}P < 0.005$; for comparisons between TMX2-28 cells and MDA-MB-231 cells. $P < 0.05$; for comparisons between TMX2-28-NC and TMX2-28-S2 or TMX2-28-MC cells $^{**}P < 0.01$, $^{***}P < 0.001$. Protein expression of (A) p27, (B) Cyclin E, (C) CDK2, and (D) Cyclin D1 were determined by Western immunoblotting (GAPDH as loading control).

anti-HER2 therapies (Dickson and Lippman, 1995; van de Rijn et al., 2002). The progression of these hormone receptor negative breast cancers may be significantly impacted by dysregulation of cell cycle regulatory proteins. SKP2 is a component of the E3 ligase SCF^{SKP2} complex, which governs the specificity of the complex to target CDK inhibitors, like p27, for proteasome degradation and cell cycle progression (Toyoshima and Hunter, 1994; Zhang et al., 1995; Carrano et al., 1999; Sutterluty et al., 1999). High expression of SKP2 has been associated with cancer aggressiveness (Signoretti et al., 2002; Davidovich et al., 2008; Ravaioli et al., 2008; Foulkes et al., 2010).

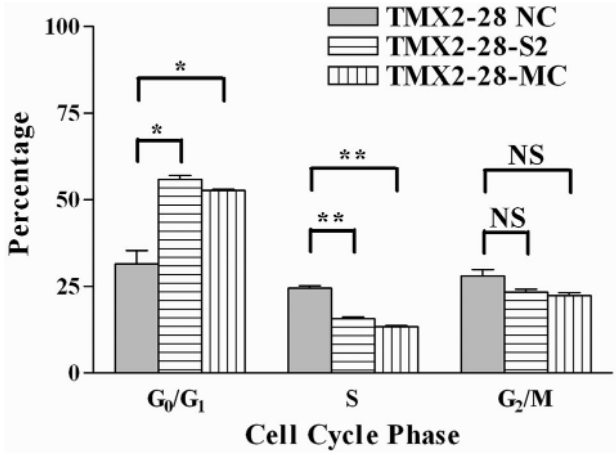

Fig. 5. SKP2 knockdown decreased the percentage of cells in the S-phase of the cell cycle. TMX2-28-NC, TMX2-28-S2, and TMX2-28-MC cells were fixed and stained with propidium iodide. Cell cycle analysis was assessed by flow cytometry ($^{*}P < 0.05$; $^{**}P < 0.01$).

In agreement with the studies by Voduc et al. (2008) and Traub et al. (2006; discussed in the Introduction Section), we did not find a significant correlation between SKP2 and p27 protein expression. Our novel finding was that SKP2 expression was significantly positively correlated with pSer10p27 in human breast cancers. Furthermore, the combination of high levels of SKP2 and high pSer10p27 were observed significantly more often in ERα-negative and triple-negative breast tumors than in ERα-positive breast cancers. While the significant relationship between high SKP2 and high pSer10p27 was observed across ages, younger women were more likely to be scored high for SKP2 and older women were more likely to be scored high for pSer10p27. The age differences may be important in identifying patients most likely to benefit from tailored treatments and requires further study.

To validate and further our understanding of the results seen in tumor tissue, we utilized the triple-negative TMX2-28 breast cancer cell line for a number of in vitro studies. We demonstrated that, unlike the parent MCF-7 cell line, or the triple-negative, aggressive MDA-MB-231 cell line, TMX2-28 express high levels of SKP2, befitting of a triple-negative and basal-like breast tumor model. To our knowledge, TMX2-28 is the only breast cancer cell line that is triple-negative and

naturally expresses high levels of SKP2, a hallmark of basal-like phenotype (Signoretti et al., 2002). Additionally, we found that on both the mRNA and protein levels, p27 was significantly higher in TMX2-28 cells than in either of the cell lines with low SKP2 expression.

In line with our IHC data, significantly higher levels of pSer10p27 were detected in TMX2-28 as compared to other cell lines. This finding led us to ask whether SKP2 was altering p27 post-translational modification in TMX2-28 cells. Suppression of SKP2 resulted in a significant decrease in phosphorylation at serine 10 of p27. Reduction of SKP2 also resulted in a slowing of cell cycle progression as evidenced by an increase in the percentage of cells in the G_1/G_0 and a decrease in the percentage of cells in S phase of the cell cycle. Recent studies show that phosphorylation of p27 at Serine 10 results in translocation of pSer10p27 to the cytoplasm where it is unavailable to inhibit CDK2, leading to cell cycle progression (He et al., 2012) and support our findings; however, the mechanisms by which SKP2 alters p27 phosphorylation at Serine 10 remain to be determined.

Interestingly, suppression of SKP2 also resulted in an increase in both mRNA and protein levels of p27. While elevation of p27 protein as a consequence of SKP2 suppression is agreement with current paradigms describing SKP2 as acting on p27 at post-translational levels, the increase in mRNA was initially surprising. However, as discussed above suppression of SKP2 expression in TMX2-28 cells resulted in an increase in the percentage of cells in G_1/G_0 and a decrease in the percentage of cells in S phase of the cell cycle. During G_1 p27 levels increase and inhibit the S-phase transition that normally occurs when SKP2 sends p27 for destruction (Malumbres and Barbacid, 2001). It appears that suppressing SKP2 and the subsequent G_1 arrest triggered the cells to up-regulate p27 as they normally would during this phase.

The results from the present IHC analysis identified a subset of tumors, based on their molecular phenotype of increased levels of SKP2 and pSer10p27, with poor prognosis and limited available therapy, which could benefit from tailored treatments with inhibitors of SKP2. From the cell culture data we learned that suppressing SKP2 in our triple-negative breast cancer model TMX2-28 reduced the phosphorylation of p27 at Ser10 and slowed the cell cycle, while the level of p27 was high both before and after SKP2 knockdown. Together, these data indicate that levels of p27 protein may not be the most sensitive biomarker of SKP2-dependent activity.

The combination of high levels of SKP2 and pSer10p27 may be a better indicator of whether inhibiting SKP2 will result in slowing cell cycle and proliferation and that inhibiting SKP2 in triple-negative and basal-like breast cancers that express high levels of both SKP2 and pSer10p27 regardless of p27 levels may be an appropriate therapeutic approach. Agents that can specifically decrease SKP2 activity and/or phosphorylation of p27, specifically at Serine 10, will provide a significant therapeutic impact and new, targeted therapeutic options for patients who otherwise solely depend on the radiation and chemotherapy available to them. Several studies have already identified agents, which can abrogate SKP2's activity (rapamycin, compound A, and compound #25) (Shapira et al., 2006; Chen et al., 2008; Totary-Jain et al., 2012; Chan et al., 2013) or decrease the expression of SKP2 (SMIP0004 as well as several phytochemicals) (Dow et al., 2001; Huang et al., 2008, 2011; Rico-Bautista et al., 2010). However, further preclinical and clinical studies are needed to verify the efficacy of these compounds.

The results of the present study provide additional and important information about patients with triple-negative and basal-like breast cancers, and provide insight into the significance of alterations in the cell cycle regulatory gene SKP2 on tumor progression. Our findings suggest that SKP2 overexpression could modulate the malignant phenotype of triple-negative and basal-like breast cancers, through regulation of p27 phosphorylation. The precise molecular characterization of p27, direct and indirect phosphorylation by SKP2 will be relevant to delineate the properties of these breast cancer subtypes and identify patients with triple-negative and/or basal-like tumors driven by SKP2 pathway dysregulation and will be the focus of future studies. It also would be valuable to examine the role cytoplasmic SKP2 may play in the Serine 10 phosphorylation of p27, as the study examined nuclear expression. A recent study by Liu et al. (2012) found that cytoplasmic SKP2 expression is associated with larger tumor size and advanced histological grade in breast cancer, and that the combination of cytoplasmic SKP2 and pAkt1 expression may be prognostic in invasive breast cancer. Patients with breast carcinomas that express high levels of SKP2 could possibly improve their prognostic outcomes if treated with a targeted molecular therapy aiming to reduce increased SKP2 levels by either small molecules and/or natural inhibitors. The combination of high levels of SKP2 and pSer10p27 may be a good indicator of whether this type of targeted molecular therapy will result in slowing cell cycle and proliferation, and overall tumor growth in patients.

Acknowledgments

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