Biochemical and Biophysical Research Communications 418 (2012) 186–190

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Stat3 induces oncogenic Skp2 expression in human cervical carcinoma cells

Hanhui Huang [a], Wenrong Zhao [b], Dan Yang [c,*]

[a] Shanghai Medical College of Fudan University, Shanghai 200032, China
[b] Department of Gynecology, Obstetrics and Gynecology Hospital of Fudan University, Shanghai 200011, China
[c] Department of Gynecology, Shanghai First Maternity and Infant Hospital, Tongji University, Shanghai 200040, China

ARTICLE INFO

Article history:
Received 25 December 2011
Available online 9 January 2012

Keywords:
Cervical carcinoma
Stat3
Skp2
Protein degradation

ABSTRACT

Dysregulated Skp2 function promotes cell proliferation, which is consistent with observations of Skp2 over-expression in many types of human cancers, including cervical carcinoma (CC). However, the molecular mechanisms underlying elevated Skp2 expression have not been fully explored. Interleukin-6 (IL-6) induced Stat3 activation is viewed as crucial for multiple tumor growth and metastasis. Here, we demonstrate that Skp2 is a direct transcriptional target of Stat3 in the human cervical carcinoma cells. Our data show that IL-6 administration or transfection of a constitutively activated Stat3 in HeLa cells activates Skp2 mRNA transcription. Using luciferase reporter and ChIP assays, we show that Stat3 binds to the promoter region of Skp2 and promotes its activity through recruiting P300. As a result of the increase of Skp2 expression, endogenous p27 protein levels are markedly decreased. Thus, our results suggest a previously unknown Stat3–Skp2 molecular network controlling cervical carcinoma development.

1. Introduction

Skp2 (S-phase kinase associated protein-2) is a critical component of the Skp2–SCF (Skp1/Cul1/F-box) complex, which is responsible for substrate recognition and subsequent proteosome-mediated degradation [1–3]. It has been suggested that Skp2 promotes cell-cycle progression through ubiquitylation and degradation of p27 [4,5]. Besides, numerous cell-cycle regulators including FoxO1 and p130 were identified as substrates for Skp2 [6,7]. Skp2 deficiency shows elevated p27 protein levels and a significant impairment in proliferation accompanied by nuclear enlargement, polypoidy and centrosome overduplication [8,9]. In addition, accumulation of Skp2 is frequently observed in many tumors, including breast, prostate and cervical carcinomas, whereas a reduced level of p27 is characterized as an adverse prognostic marker in most human cancers [10,11]. Although substantial advances have been made in understanding the mechanisms that control its levels of expression, the molecular mechanisms by which Skp2 is accumulated in cervical carcinomas remain largely unexplored.

Recent evidence shows a crucial role for Stat3 in selectively inducing and maintaining a procarcinogenic inflammatory microenvironment, both at the initiation of malignant transformation and during cancer development [12,13]. In addition, it is suggested that Stat3 is highly interconnected with NF-κB signaling, which has been recognized as a major pathway responsible for both inflammation-induced carcinogenesis and anti-tumor immunity [14,15]. Hence, Stat3 is a promising target to redirect inflammation for cancer therapy.

Here, we show that mRNA and protein levels of Skp2 are markedly increased in human cervical carcinoma cells treated with Interlukin-6 (IL-6). We also suggest that Stat3 activation up-regulates Skp2 expression, leading to the degradation of p27. Our findings thus link Stat3 signaling directly with the Skp2–p27 pathway, which have profound biological and therapeutic implications for cervical carcinoma.

2. Material and methods

2.1. Cell culture

HEK293T and HeLa cell lines were cultured in Dulbecco modified Eagle's medium supplemented with 10% fetal calf serum, 100 IU/ml penicillin and 100 mg/ml streptomycin.

2.2. Reagents

IL-6 was purchased from Merck, China. The following antibodies were purchased: anti-Stat3 (Cell Signaling), anti-Skp2 (Abcam), anti-p27 (Santa Cruz), anti-p53 (Santa Cruz) and anti-P300 (Santa Cruz).

2.3. Transient transfections and luciferase reporter assays

Human Skp2 promoter was cloned into PGL3-basic plasmid (Promega). All the transient transfections were performed by

* Corresponding author. Address: Department of Gynecology, Shanghai First Maternity and Infant Health Hospital, Tongji University, School of Medicine, No. 536 Changle Road, Jing'an District, Shanghai 200040, China. Fax: +86 21 54045772.
E-mail address: yangdandr@gmail.com (D. Yang).

0006-291X/$ - see front matter © 2012 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2012.01.004

Lipofectamin 2000 (Invitrogen), according to the manufacturer's instructions. For luciferase reporter assay, cells were seeded in 12-well plates and transfected with the indicated plasmids. Cells were harvested 48 h after transfection. Luciferase activity was measured using the Dual Luciferase Reporter Assay System (Promega, USA).

2.4. RNA extraction and Real-Time PCR analysis

Total RNAs were extracted from cells by TRIzol reagent, and reverse transcriptions were performed by Takara RNA PCR kit (Takara, China) following the manufacturer's instructions. In order to quantify the transcripts of the interest genes, Real-Time PCR was performed using a SYBR Green Premix Ex Taq (Takara, Japan) on ABI 7300 (ABI, USA). The primer sequences used are available upon request.

2.5. Western Blot

Cells were lysed in radioimmunoprecipitation (RIPA) buffer containing 50 mM Tris–HCl, 150 mM NaCl, 5 mM Mgcl2, 2 mM EDTA, 1 mM NaF, 1% NP40 and 0.1% SDS.

2.6. ChIP assays

A chromatin immunoprecipitation (ChIP) assay kit was used (Upstate, USA). In brief, HeLa cells were fixed with formaldehyde. DNA was sheared to fragments at 200–1000 bp using sonications. The chromatin were incubated and precipitated with antibodies against the indicated antibodies or IgG (Santa Cruz, USA).

2.7. Statistical analysis

Values were shown as mean ± SEM. Statistical differences were determined by a Student's *t* test. Statistical significance is displayed as $^{*}(P < 0.05)$, $^{**}(P < 0.01)$ or $^{***}(P < 0.001)$.

3. Results

3.1. Up-regulation of Skp2 by IL-6 treatment or Stat3 activation

As suggested by the frequent up-regulation of Skp2 and persistent activation of Stat3 in cervical cancers, we then used Real-Time PCR and Western Blot to confirm the correlation between Stat3 activity and Skp2 expression in HeLa cells. As shown in Fig. 1A and B, IL-6 treatment significantly increased Skp2 levels in HeLa cells. Besides, overexpression of a constitutively activated Stat3 (Stat3C) also increased the expression of Skp2 (Fig. 1C and D). Conversely, knockdown of Stat3 by small interfering RNA (siRNA) decreased Skp2 mRNA and protein levels in HeLa cells (Fig. 1E and F). Together, these data suggest that Stat3 could be an important regulator in the control of Skp2 expression in cervical carcinoma cells.

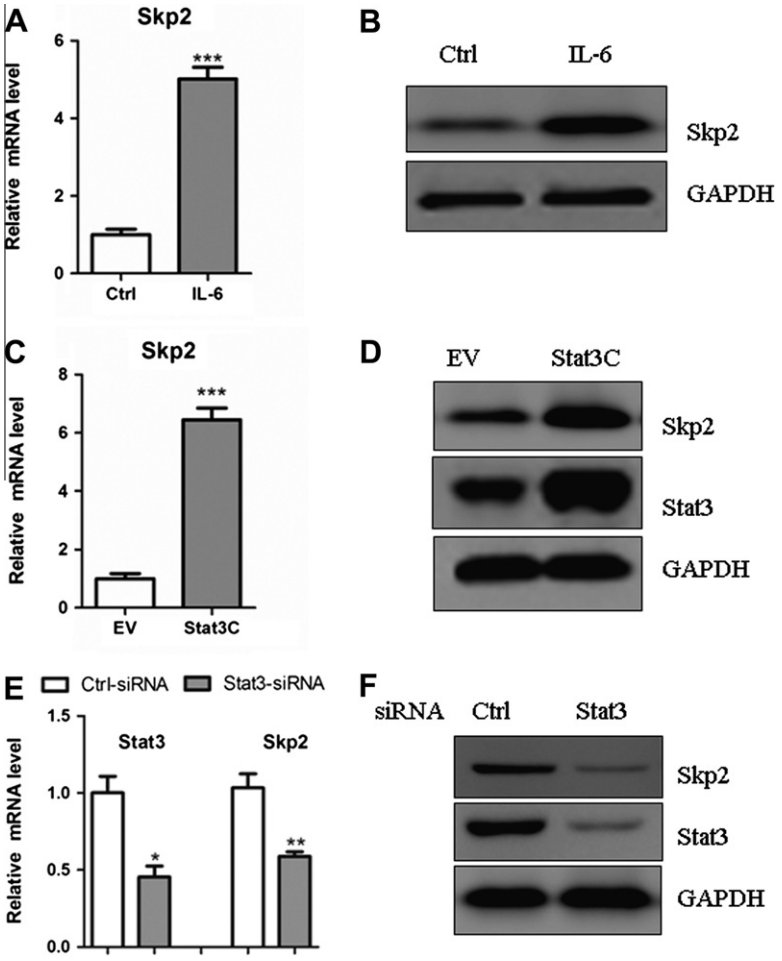

Fig. 1. Upregulation of Skp2 by IL-6 or Stat3 activation. (A and B) mRNA and protein levels of skp2 were analyzed by Real-Time PCR and Western Blot in HeLa cells treated with or without IL-6 (20 ng/ml). (C and D) mRNA and protein levels of skp2 were analyzed by Real-Time PCR and Western Blot in HeLa cells transfected with empty vector (EV) or Stat3C. (E and F) HeLa cells were transfected for nonspecific control or Stat3 siRNA. Endogenous mRNA and protein levels of Stat3 and Skp2 were determined.

3.2. Stat3 activates Skp2 expression through bound to its promoter region

We next determined whether Stat3 could be a transcriptional regulator of the Skp2 gene. We transfected HeLa cells with a reporter vector encoding luciferase under control of the Skp2 promoter (WT-Luc). Concurrent expression of Stat3C with the Skp2 reporter construct increased Skp2 promoter activity (Fig. 2A), which was abrogated by mutation of the Stat3 DNA-binding site in the Skp2 promoter (Mut-Luc). The stimulatory effect of Stat3 on Skp2 promoter activity was also shown in HEK293T cells (Fig. 2B). We next carried out chromatin immunoprecipitation (ChIP) assays to assess whether Stat3 directly binds the Skp2 promoter. Indeed, Stat3 protein bound the Skp2 promoter was significantly increased in HeLa cells treated with IL-6 (Fig. 2C).

3.3. Stat3 activates Skp2 expression through recruitment of P300

Positive regulation of gene transcription by Stat3 has been partly attributed to the recruitment of co-activators such as P300 [16]. Indeed, we observed that P300 was highly recruited to the promoter region of Skp2 in HeLa cells treated with IL-6 (Fig. 3A).

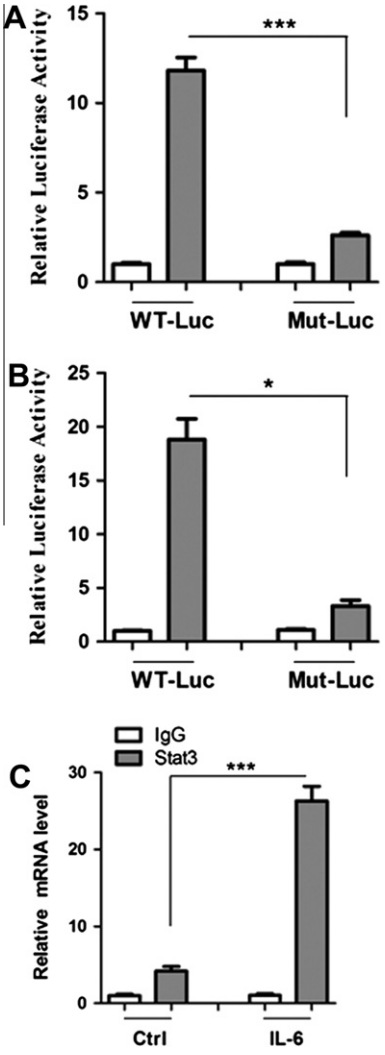

Fig. 2. Stat3 activates Skp2 expression through bound to its promoter region. (A and B) HeLa cells were co-transfected with the indicated plasmids for 48 h, and the luciferase activity was measured. (C) Two antibodies (anti-IgG and -Stat3) were used in the ChIP assays using HeLa cells treated with or without IL-6.

Besides, overexpression of P300 enhanced endogenous Skp2 mRNA and protein levels (Fig. 3B and C). Consistently, knockdown of P300 using small interfering RNA also reduced Skp2 mRNA and protein levels (Fig. 3D and E), suggesting that Stat3 recruits nuclear P300 to confer its transcriptional repression roles.

3.4. Stat3 activation decreases the p27 stability

Since the proteolytic role of Skp2 in the p27 pathway, we determined whether Stat3 activation could affect the p27 stability. As shown in Fig. 4A and B, p27 protein levels were markedly decreased in HeLa cells treated with IL-6 or transfected with Stat3C, whereas protein levels of P53, a classical upregulator of P27 were not changed (Fig. 4C). Consistently, siRNA knockdown of Stat3 induced p27 protein levels and its activation (Fig. 4D). To characterize the role of Skp2 mediated p27 degradation, Skp2 was deleted in HeLa cells. As shown in Fig. 4E and F, p27 protein levels were accumulated and not affected by IL-6 treatment or transfection of Stat3C.

4. Discussion

In the present study, we show that activation of Stat3 is required for the Skp2 expression in human cervical carcinoma cells. Aberrant expression of Skp2 provides a molecular switch that orchestrates the formation of SCF complex, which then promotes cell proliferation through protein degradation of p27. Indeed, we also provide data that persistent activation of Stat3 reduces the protein levels of p27, which leads to the cell-cycle progression in HeLa cells. Taken together, these findings identify Stat3 signaling as a major regulatory pathway of Skp2 expression and function.

Previous studies have suggested that the proto-oncogenic PI3K/Akt pathway can up-regulate Skp2 levels through the enhancement of its transcription [17,18]. It is shown that the tumor suppressor Pten-deficiency in mouse embryonic stem cells causes a decrease of p27 levels with concomitant increase of Skp2 expression [19]. Besides, Myc is reported to bind to the promoter region of Skp2 gene and induce its mRNA level in many cell types, including human myeloid leukemia, lymphoid, fibroblastic and epithelia cell lines [20]. As a result of this up-regulation, Myc overexpression leads to a decrease in p27 protein. Since hyperactivation of the Akt pathway and Myc overexpression are considered a hallmark of many cancers, it will be interesting to determine that whether up-regulation of Skp2 by Stat3 could cross talk with Akt or Myc signaling.

Stat3 is linked to inflammation-associated tumorigenesis that is initiated by genetic alterations in malignant cells, as well as by many environmental factors, including chemical carcinogens, infection, cigarette smoking and stress [21]. Interestingly, significant level of Stat3 expression is predominantly found in cervical cancer cases as compared to normal controls [22]. Besides, Stat3 gene polymorphism was found to be associated with the susceptibility as well as poor differentiation and parametrial invasion of cervical cancer in Chinese woman [23]. In addition, there is a significant correlation of Stat3 expression in cases infected with HPV [22], which represents the main etiological factor in the development of cervical cancer [24]. Although an unanswered question in the field is how Stat3 is initially activated and remains persistently activated in cancer, our data suggest that Stat3 activation leads to tumor cell cycle progression, at least through protein degradation of p27 by induction of Skp2 expression.

In summary, our results provide new insight into how Stat3 activation could influence the Skp2/p27 pathway, which is a known hotspot in human cervical cancer. On the other hand, our findings provide a mechanism by which Stat3 influences cell-cycle

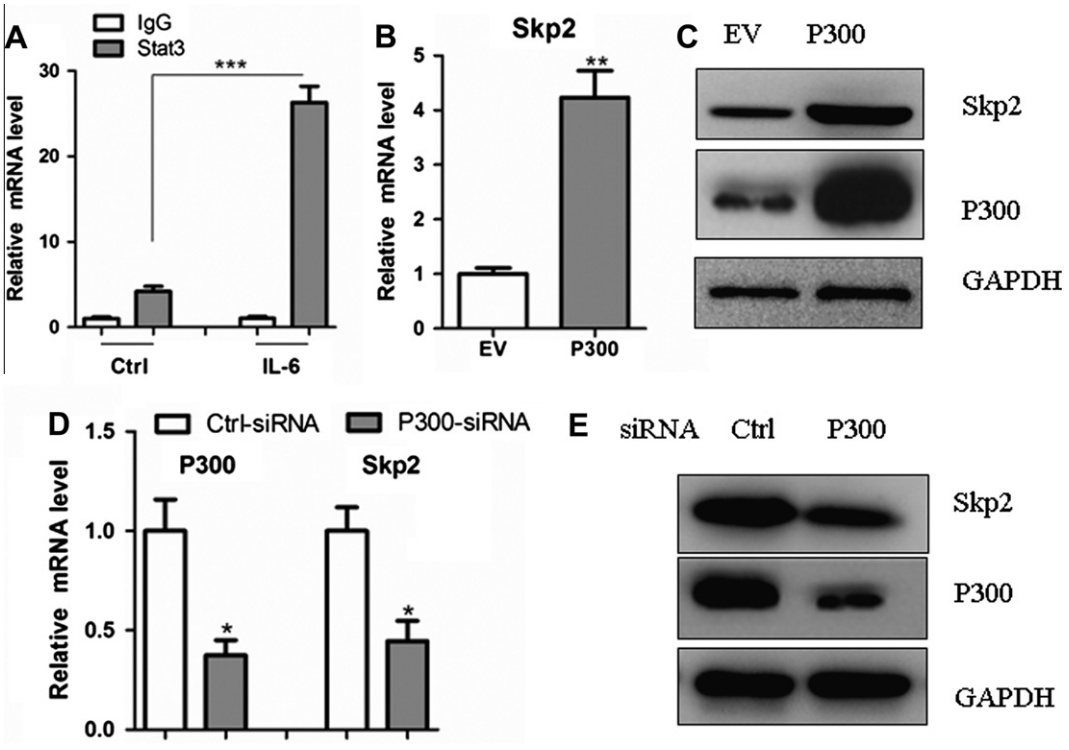

Fig. 3. Stat3 activates Skp2 expression through recruitment of P300. (A) Two antibodies (anti-IgG and -p300) were used in the ChIP assays in HeLa cells treated with or without IL-6. (B and C) mRNA and protein levels of Skp2 were analyzed by Real-Time PCR and Western Blot in HeLa cells transfected with empty vector (EV) or P300. (D and E) HeLa cells were transfected for nonspecific control or P300 siRNA. Endogenous mRNA and protein levels of P300 and Skp2 were determined.

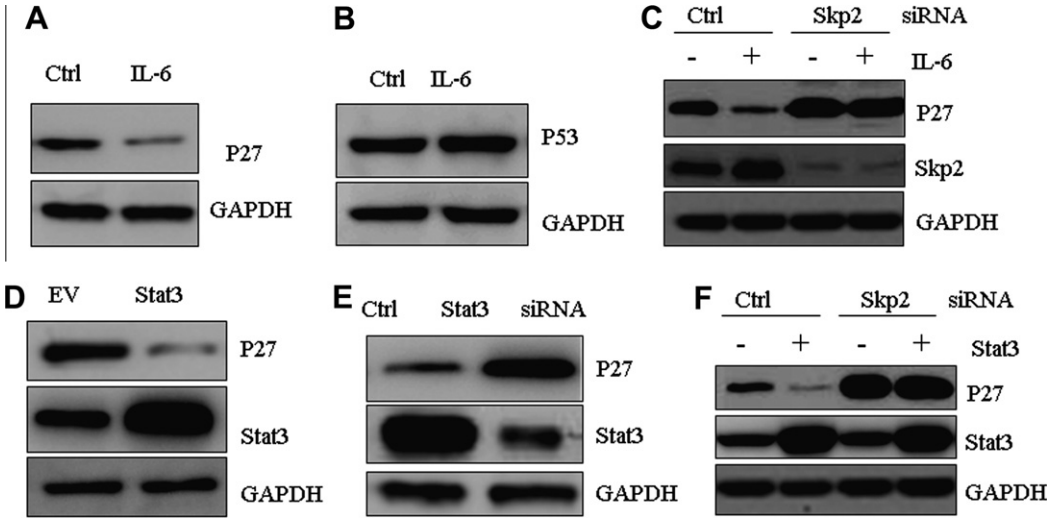

Fig. 4. Stat3 activation decreases the P27 stability. (A) HeLa cells were treated with or without IL-6, the protein level of p27 and stat3 were analyzed by Western Blot. (B) HeLa cells were transfected with the indicated plasmids, the protein level of p27 and stat3 were analyzed by Western Blot. (C) HeLa cells were treated with or without IL-6, the protein level of p53 was analyzed by Western Blot. (D) Protein level of Stat3 and p27 were analyzed by Western Blot in HeLa cells transfected with nonspecific control or Stat3 siRNA. (E) HeLa cells were transfected with the indicated siRNAs and treated with or without IL-6, the protein level of p27 and Skp2 were analyzed by Western Blot. (F) HeLa cells were transfected with indicated siRNAs and then transfected with indicated plasmids, the protein level of p27, Skp2 and Stat3 were analyzed by Western Blot.

progression. Ultimately, these data may provide the rationale for the development of specific Stat3 inhibitors as efficient anti-cancer drugs.

References

[1] B. Amati, J. Vlach, Kip1 meets SKP2: new links in cell-cycle control, Nat. Cell Biol. 1 (1999) E91–E93.
[2] T. Cardozo, M. Pagano, The SCF ubiquitin ligase: insights into a molecular machine, Nat. Rev. Mol. Cell Biol. 5 (2004) 739–751.
[3] K.I. Nakayama, K. Nakayama, Regulation of the cell cycle by SCF-type ubiquitin ligases, Semin. Cell Dev. Biol. 16 (2005) 323–333.
[4] A.C. Carrano, E. Eytan, A. Hershko, M. Pagano, SKP2 is required for ubiqui-tin-mediated degradation of the CDK inhibitor p27, Nat. Cell Biol. 1 (1999) 193–199.
[5] L.M. Tsvetkov, K.H. Yeh, S.J. Lee, H. Sun, H. Zhang, p27 Kip1 ubiquitination and degradation is regulated by the SCF Skp2 complex through phosphorylated Thr187 in p27, Curr. Biol. 9 (1999) 661–664.
[6] T. Huang, D.J. Tindall, Regulation of FOXO protein stability via ubiquitination and proteasome degradation, Biochim. Biophys. Acta. 11 (2011) 1961–1964.
[7] K. Kitagawa, Y. Kotake, M. Kitagawa, Ubiquitin-mediated control of oncogene and tumor suppressor gene products, Cancer Sci. 8 (2009) 1374–1381.

[8] E.J. Andreu et al., BCR-ABL induces the expression of Skp2 through the PI3K pathway to promote p27 Kip1 degradation and proliferation of chronic myelogenous leukemia cells, Cancer Res. 65 (2005) 3264–3272.
[9] P.W. van Duijn, J. Trapman, PI3K/Akt signaling regulates p27 kip1 expression via Skp2 in PC3 and DU145 prostate cancer cells, but is not a major factor in p27 kip1 regulation in LNCaP and PC346 cells, Prostate 66 (2006) 749–760.
[10] S. Signoretti et al., Oncogenic role of the ubiquitin ligase subunit Skp2 in human breast cancer, J. Clin. Invest. 110 (2002) 633–641.
[11] J. Bloom, M. Pagano, Deregulated degradation of the cdk inhibitor p27 and malignant transformation, Semin. Cancer Biol. 13 (2003) 41–47.
[12] H. Yu, R. Jove, The STATs of cancer-new molecular targets come of age, Nat. Rev. Cancer 4 (2004) 97–105.
[13] H. Yu, D. Pardoll, R. Jove, STATs in cancer inflammation and immunity: a leading role for STAT3, Nat. Rev. Cancer 9 (2009) 798–809.
[14] H. Yu, M. Kortylewski, D. Pardoll, Crosstalk between cancer and immune cells: role of STAT3 in the tumour microenvironment, Nat. Rev. Immunol. 7 (2007) 41–51.
[15] H. Lee et al., Persistently activated Stat3 maintains constitutive NF-κB activity in tumors, Cancer Cell 15 (2009) 283–293.
[16] J.L. Lee, M.J. Wang, J.Y. Chen, Acetylation and activation of Stat3 by nuclear translocation of CD44, J. Cell Biol. 6 (2009) 949–957.
[17] M. Reichert, D. Saur, R. Hamacher, R.M. Schmid, G. Schneider, Phosphoinositide-3-kinase signaling controls S-phase kinase-associated protein 2 transcription via E2F1 in pancreatic ductal adenocarcinoma cells, Cancer Res. 67 (2007) 4149–4156.
[18] B. Barre, N.D. Perkins, A cell cycle regulatory network controlling NF-κB subunit activity and function, EMBO J. 26 (2007) 4841–4855.
[19] R. Mamillapalli, N. Gavrilova, V.T. Mihaylova, et al., PTEN regulates the ubiquitin-dependent degradation of the CDK inhibitor p27(KIP1) through the ubiquitin E3 ligase SCF(SKP2), Curr Biol. 11 (2001) 263–267.
[20] G. Bretones, J.C. Acosta, J.M. Caraballo, N. Ferrándiz, et al., SKP2 oncogene is a direct MYC target gene and MYC down-regulates p27(KIP1) through SKP2 in human leukemia cells, J. Biol. Chem. 11 (2001) 9815–9825.
[21] R. Catlett-Falcone et al., Constitutive activation of Stat3 signaling confers resistance to apoptosis in human U266 myeloma cells, Immunity 10 (1999) 105–115.
[22] S. Shukla, G. Shishodia, S. Mahata, et al., Aberrant expression and constitutive activation of STAT3 in cervical carcinogenesis: implications in high-risk human papillomavirus infection, Mol. Cancer 27 (2010) 282.
[23] K. Wang, B. Zhou, J. Zhang, Y. Xin, et al., Association of signal transducer and activator of transcription 3 gene polymorphisms with cervical cancer in Chinese women, DNA Cell Biol. 11 (2011) 931–936.
[24] R. Faridi, A. Zahra, K. Khan, M. Idrees, Oncogenic potential of Human Papillomavirus (HPV) and its relation with cervical cancer, Virol J. 3 (2011) 269.