

# Overexpression of the S-phase kinase-associated protein 2 in thyroid cancer

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## Abstract

Loss of expression of the cyclin-dependent kinase inhibitor p27 through enhanced protein degradation frequently occurs in human cancer. Degradation of p27 requires ubiquitination by the S-phase kinase-associated protein 2 (Skp2), a member of the F-box family of Skp1–Cullin–F-box protein ubiquitin ligases. In the present study, we have investigated the role of Skp2 in human thyroid tumours. Immunohistochemistry analysis showed that Skp2 was overexpressed significantly in thyroid carcinomas (26 out of 51) compared with goitres (0 out of 12,  $P < 0.001$ ) or adenomas (1 out of 10,  $P < 0.05$ ), and that high Skp2 expression was detected more often in anaplastic thyroid (ATC; 83%,  $n = 12$ ) than follicular thyroid (FTC; 40%,  $n = 20$ ) or papillary thyroid (PTC; 42%,  $n = 19$ ) carcinomas ( $P < 0.05$ ). Thyroid cancer cell lines and tissues with high levels of Skp2 protein presented high p27 degradation activity and there was an inverse correlation between Skp2 and p27 expression in thyroid cancer tissues ( $n = 68$ ;  $P < 0.05$ ). In most cases, the observed overexpression of Skp2 protein was paralleled by an increase in the levels of Skp2 mRNA, and we observed *Skp2* gene amplification at 5p13 in 2 out of 6 cell lines and in 9 out of 23 primary tumours (six out of eight ATCs, two out of nine PTCs and one out of six FTCs) using Q-PCR and/or fluorescence *in situ* hybridization analysis. Finally, *in vitro* experiments demonstrated that suppression of Skp2 expression drastically reduced proliferation of thyroid cancer cells and, conversely, forced expression of Skp2 circumvented serum dependency and contact inhibition in Skp2-negative cells by promoting p27 degradation. These findings indicate that Skp2 plays an important role for the development of thyroid cancer.

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## Introduction

Alteration of cell cycle is a hallmark of cancer (Hanahan & Weinberg 2000). In particular, the importance of the G1/S transition in human tumour cells has been highlighted by the frequent observations of aberrant regulation of molecules involved in this process (Malumbres & Barbacid 2001). Thyroid tumours occur frequently in the general population and although the majority represents benign follicular adenomas (FAs), thyroid cancer constitutes 1% of all

malignancies worldwide (Sherman 2003). Among the differentiated cancers, two main types are recognized, including papillary thyroid carcinoma and follicular thyroid carcinoma forms (PTC and FTC) that originate from the thyrocytes. The undifferentiated form, anaplastic thyroid carcinoma (ATC), is highly aggressive cancer that often originates from pre-existing differentiated cancer (Hedinger *et al.* 1989). As is the case with other common human carcinomas, a series of multiple alterations in cell cycle control-related gene

products are involved in thyroid cancer pathogenesis (Kondo *et al.* 2006). It is therefore important to clarify the cell cycle control mechanisms involved in the development and progression of thyroid cancer.

Skp1–Cullin–F-box protein (SCF) complexes are a multicomponent RING-type evolutionarily conserved class of ubiquitin ligase enzymes (E3) that consists of four subunits: an adaptor protein (Skp1, S-phase kinase-associated protein), a scaffold protein (Cull1/Cullin), a RING-domain protein (Rbx1) and a substrate-binding protein (F-box-containing protein; Patton *et al.* 1998, Deshaies 1999). Cullin-1, Skp1 and ROC1 proteins are constant component of SCF, whereas F-box proteins represent variable subunits that dictate specificity (Patton *et al.* 1998, Deshaies 1999). Each F-box protein recognizes a specific subset of protein substrates and thus promotes their ubiquitination and subsequent degradation (Kipreos & Pagano 2000, Jin *et al.* 2004). The F-box protein Skp2, which was originally identified as a protein that interacts with cyclin A–cyclin-dependent kinase 2 (CDK2; Zhang *et al.* 1995) is one of the substrate recognition units of SCF. Skp2 has been shown to be required for G1/S transition in both diploid fibroblasts and transformed cells (Zhang *et al.* 1995, Sutterluty *et al.* 1999). The levels of Skp2 are low in G0–G1 phases and high in S-phase (Zhang *et al.* 1995), and forced expression of Skp2 induces quiescent fibroblasts to replicate their DNA in low serum (Zhang *et al.* 1995, Sutterluty *et al.* 1999).

Control of cell cycle progression by Skp2 has been linked to its ability to control the levels of the CDK inhibitor p27 (Slingerland & Pagano 2000, Philipp-Staheli *et al.* 2001), a CDK inhibitor that negatively regulates cell cycle (Carrano *et al.* 1999, Tsvetkov *et al.* 1999). Skp2 ubiquitinates and degrades p27, both *in vivo* and *in vitro* (Carrano *et al.* 1999, Tsvetkov *et al.* 1999, Slingerland & Pagano 2000, Philipp-Staheli *et al.* 2001). Degradation of p27 through the ubiquitin–proteasome pathway requires phosphorylation of p27 on Thr187 by cyclin E/CDK2 or cyclin A/CDK2 complexes (Pagano *et al.* 1995, Sheaff *et al.* 1997) and subsequent binding of Skp2 to T187-phosphorylated p27. Accordingly, suppression of Skp2 expression in cultured cells stabilizes p27 protein *in vivo* (Kudo *et al.* 2005), whereas Skp2-deficient mice show high levels of p27 (Nakayama *et al.* 2000).

The loss of p27 expression plays an important role in human cancer pathogenesis (Slingerland & Pagano 2000). The p27 expression is reduced in several tumours including thyroid carcinomas (Erickson *et al.* 1998, Baldassarre *et al.* 1999, Tallini *et al.* 1999); data from our laboratory suggest that

such decreased expression depends on enhanced p27 protein degradation (Motti *et al.* manuscript in preparation). Therefore, although there are different reports of overexpression of Skp2 protein in human haematological and solid cancers (Gstaiger *et al.* 2001, Hershko *et al.* 2001, Kudo *et al.* 2001, Latres *et al.* 2001, Chiarle *et al.* 2002, Masuda *et al.* 2002, Signoretti *et al.* 2002, Li *et al.* 2004), so far there are no studies regarding the clinical significance or the biological behaviour of Skp2 expression in human thyroid carcinomas. Most interestingly, genetic alterations that result in gain of part or of the entire short arm of chromosome 5, where the *Skp2* gene has been assigned at 5p13, frequently occur in PTC, Hurthle carcinomas and ATC (Hemmer *et al.* 1999, Wada *et al.* 2002, Foukakis *et al.* 2005), suggesting that Skp2 may be the target of gene amplification in a fraction of thyroid cancer.

The aim of the present study was to determine the potential role of Skp2 in thyroid cancer development and progression. The findings reported here might provide additional insight into the molecular pathogenesis and identify Skp2 as a novel potential therapeutic target for thyroid ATC.

## Materials and methods

### Clinical samples

Primary thyroid carcinomas and their paired adjacent normal glandular tissues were obtained from patients who underwent surgery from 1993 to 2000, at the Chirurgia B, INT Fondazione G Pascale, Napoli, Italy.

### Cell culture

Cell lines have been established from PTC (NPA (Ohta *et al.* 1997), BHP2.7, BHP10.3 and BHP17.10 (Pang *et al.* 1989), TPC-1 (Tanaka *et al.* 1987), BC-PAP (Fabien *et al.* 1994); FTC (WRO ;Estour *et al.* 1989) and ATC (FB-1 (Fiore *et al.* 1997), FRO (Fagin *et al.* 1993) and ARO (Ohta *et al.* 1997)). Cells were routinely maintained in Dulbecco's modified Eagle's medium (DMEM; Life Technologies Inc.) supplemented with 10% heat-inactivated fetal bovine serum and 100 units/ml penicillin–streptomycin (Life Technologies Inc.), in a CO<sub>2</sub> incubator (5% CO<sub>2</sub> in air) at 37 °C. Control thyroid cells were provided by Dr F Curcio (University of Udine, Italy) and grown as described (Curcio *et al.* 1994).

### RNA and DNA isolation and RT-PCR

Total RNA and high-molecular-weight genomic DNA were prepared as described (Chomczynski & Sacchi

1987, Sambrook *et al.* 1992). Semiquantitative RT-PCR amplification was performed by 24 cycles, using the following primers: Skp2 sense primer, GCTGCTAAA GGTCTCTGGTGT; Skp2 antisense primer AGGCT-TAGATTCTGCAACTTG; actin sense primer GTCAA CCGATTGGTCTGTATT and actin antisense primer AGTCTTCTGGGTGGCAGTGAT.

### Quantitative reverse transcription real-time PCR (Q-RT-PCR) and quantitative real-time PCR (Q-PCR)

Q-RT-PCR and Q-PCR were performed using the Power SYBR Green PCR Master Mix in an ABI Prism 7300 thermocycler (Applied Biosystems, Foster City, CA, USA) in the presence of 0.4  $\mu$ M primers, in a total volume of 25  $\mu$ l. cDNAs were synthesized from 5 mg total RNA by Superscript. In all real-time PCR experiments, we run the final dissociation stage to generate a melting curve for the verification of amplification product specificity. All primers were designed and tested for their specificity using the Primer Express v. 1.5 (Applied Biosystems). For RNA analysis, primers were designed to span two adjacent exons in the *Skp2* gene (exons 4 and 5 respectively) to avoid amplification of contaminating genomic DNA sequences. The target amplicon was 120 bp.

Oligonucleotide sequences used as primers in the Q-RT-PCR experiments were the following: Skp2 forward (on exon 4), 5'-CGCTGCCACGATCATT-TAT-3'; Skp2 reverse (on exon 5), 5'-TGCAACTTG-GAACACTGAGACA-3';  $\beta$ -actin forward, 5'-TGCGT GACATTAAGGAGAAG-3' and  $\beta$ -actin reverse, 5'-GCTCGTAGCTCTTCTCC-3'.

The expression levels of Skp2 mRNA in each sample were normalized on the basis of the respective actin content and recorded as a relative expression level.

For *Skp2* gene dosage analysis, the target amplicon was 98 bp spanning exon 5 and intron 5 of *Skp2* gene. Oligonucleotide sequences used as primers in the Q-PCR genomic DNA quantification experiments were the following: Skp2 forward (exon 5), 5'-CTCCACGGCATACTGTCTCA-3'; Skp2 reverse (intron 5), 5'-ACTCT GCCTGCCACTCACTT-3';  $\beta$ -actin forward, 5'-TGCG TGACATTAAGGA-GAAG-3' and  $\beta$ -actin reverse: 5'-GCTCGTAGCTCT-TCTCC-3'.

Each sample was run thrice and each PCR experiment included two non-template control wells. The relative amounts of mRNA or DNA were calculated by the comparative cycle threshold method

by Livak & Schmittgen (2001) and subsequently normalized by  $\beta$ -actin expression.

### Fluorescence *in situ* hybridization (FISH)

FISH analysis was performed on ten formalin-fixed paraffin-embedded samples (five PTCs, two FTCs and three ATCs). The copy number status of the SKP2 locus was determined using two different probes: 1) three BAC clones covering the gene (RP11-36A10, RP11-262E5 and RP11-624K2) that were labelled with dUTP-SpectrumOrange (Vysis Inc., DownersGrove, IL, USA) and 2) three BAC clones as control probe (RP11-215J12, RP11-397H14 and RP11-620I22) covering the 5q31 region that were labelled with dUTP-SpectrumGreen (Vysis Inc.). The BAC clones used were designed according to the Ensembl database ([www.ensembl.org](http://www.ensembl.org)). The slides were deparaffinized, boiled in a pressure cooker with 1 mM EDTA (pH 8.0) for 10 min and incubated with pepsin at 37 °C for 30 min. The slides were then dehydrated. The probes were denatured at 75 °C for 2 min after overnight hybridization at 37 °C in a humid chamber. Slides were washed with 0.4  $\times$  SSC and 0.3% NP-40. FISH evaluation was performed by two investigators with no previous knowledge of other genetic, clinical or IHC results. Scoring of fluorescence signals was carried out in each sample by counting the number of single copy gene and control probe signals in an average of 130 (60–210) well-defined nuclei. Gain or amplification status was counted as the ratio (in >5% of tumour cells) of red/green signals. The cut-off values for the copy number changes were obtained from the analysis of normal adjacent cells in each experiment.

### Immunohistochemistry

Immunohistochemical studies of Skp2 and p27 in thyroid carcinoma were performed using the avidin–biotin–peroxidase method (LSAB kit; DAKO, Glostrup, Denmark) on formalin-fixed, paraffin-embedded tissues as described previously (Signoretti *et al.* 2002, Li *et al.* 2004). All sections were counterstained with haematoxylin. p27 was scored by observing 500 cancer cells in at least ten high-power fields and were classified as positive (staining in >40% of cells) or negative (staining in <40% of cells) as described previously. The expression of Skp2 was graded as high (>20% of tumour cells showed strong or diffuse immunopositivity) or low (<20% of the tumour cells showed weak focal immunopositivity or absence of staining).

### Western blot and antibodies

Whole cell extracts were prepared from cultured cells by homogenizing cells in NP-40 lysis buffer (10 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% NP-40) containing protease inhibitors. Western blot analysis was carried out by standard methods using whole cell extracts. Mouse monoclonal antibodies to Skp2, p27 and phospho-T187, p27 were purchased from Zymed Laboratory (San Francisco, CA, USA) and Transduction Laboratories (Lexington, KY, USA) respectively.

### Transfection assays

Human Skp2 cDNA was a gift of Dr Giulio Draetta (Milan, Italy). Onto a six-well multiwell plate,  $1 \times 10^5$  cells were plated and allowed to attach for 24 h. Transfection was done with Fugene 6 reagent (Roche Applied Biosciences). After 48 h of transfection, cells were treated with 800  $\mu\text{g/ml}$  G418 (Invitrogen) for 2–3 weeks as a selective marker. Surviving cells were dispersed in a 24-well multiwell plate and several stably transfected cell clones were obtained after 2–3 more weeks. Each clone was screened for Skp2 expression by western blot analysis.

### RNA interference

Antisense experiments targeting Skp2 were performed as described previously (Yokoi *et al.* 2004). Oligonucleotides containing phosphorothioate backbones were synthesized (Invitrogen): AS, 5'-CCTGGGGG ATGTTCTCA-3' (the antisense direction of human Skp2 cDNA nucleotides 180–196) and SC, 5'-GGCTT CCGGGCATTAG-3' (a scrambled control for AS). Oligonucleotides (AS or SC), were delivered into ATC cells at a final concentration of 200 nmol/l each, using oligofectamine reagent (Invitrogen) according to manufacturer's instructions. After 24 or 48 h of transfection, cells were harvested by trypsinization for growth and viability assays.

### Virus generation and infection

The human p27 (NM\_004064) MISSION shRNA set (five individual hairpins individually cloned into pLKO.1-puro; Sigma) was used to generate lentiviral particles in HEK293T packaging cells. Subconfluent HEK293T cells were cotransfected with 13  $\mu\text{g}$  p27 MISSION shRNA set, 18  $\mu\text{g}$  pCMV-deltaR8.91 and 12  $\mu\text{g}$  pCMV-VSVG per 100 mm tissue culture plate by calcium phosphate precipitation (Zufferey *et al.* 1997). Starting 48 h after transfection, supernatants were collected at 8-h intervals, filtered and used for three rounds of transduction of WRO cells in the

presence of 8  $\mu\text{g/ml}$  polybrene (Sigma). Transduced cells were lysed after 72 h from infection, and were analysed by immunoblotting with anti-p27 antibodies. The p27 knockdown in transduced WRO cells was determined by immunoblotting and compared with untransduced WRO cells, to Mission non-target control transduction virus (Sigma SHC002V) and to Mission TurboGFP Control Vector encoding virus (Sigma SHC003V).

### In vitro proliferation assay and flow cytometry assay

Mock- and Skp2-transfected cells were plated at a density of  $1.0 \times 10^5$  cells/well in triplicate and were harvested and counted daily for 6 days. The medium was changed every 48 h. This experiment was repeated thrice.

### Indirect immunofluorescence

Cells were grown to subconfluence on coverslips, fixed in 4% paraformaldehyde and permeabilized with 0.2% Triton X-100. 5'-Bromo-3'-deoxyuridine (BrdU) was added to the culture medium to a final concentration of 10  $\mu\text{M}$  for 2 h. S-phase cells were identified using a BrdU Labeling and Detection Kit (Roche Applied Science). Cell nuclei were identified by Hoechst staining. Fluorescence was viewed with a Zeiss 140 epifluorescent microscope equipped with filters that allowed discrimination between Texas red and FITC.

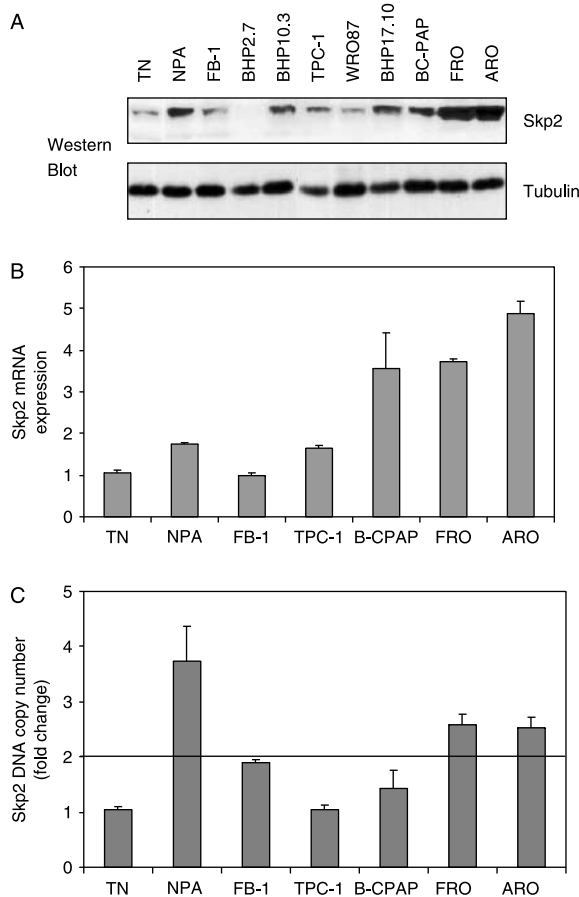
### In vitro p27 protein degradation assay

Human histidine-tagged p27 was generated by RT-PCR and cloned into pET21a plasmid as described previously (Viglietto *et al.* 2001). The 6-histidine tag was at the C-terminus. The p27 degradation assay was performed as described by Pagano *et al.* (1995). Frozen tissue samples and cell pellets were resuspended in lysis buffer containing 20 mM Tris-HCl (pH 8.5) and 1 mM dithiothreitol (DTT), and subsequently frozen and thawed several times. Lysates were then centrifuged at 13 000 *g* and the supernatant was retrieved and stored at  $-80^\circ\text{C}$  for the degradation assay. Purified histidine-tagged p27 was incubated at  $37^\circ\text{C}$  in a degradation mixture containing 100  $\mu\text{g}$  cell extract, 50 mM Tris-HCl (pH 8.5), 5 mM  $\text{MgCl}_2$ , 1 mM DTT, 2 mM ATP, 10 mM creatine phosphokinase (Sigma), 10 mM creatine phosphate (Sigma) and 5  $\mu\text{M}$  ubiquitin (Sigma). The p27 degradation rate was analysed by immunoblotting with an anti-p27 antibody.

## Results

### Expression of Skp2 in thyroid cancer cell lines

To determine the role of Skp2 in thyroid carcinogenesis, we analysed the expression of Skp2 protein in ten cell lines derived from human thyroid tumours: six cell lines were derived from PTC (NPA, BHP2.7, BHP10.3, TPC-1, BHP17.10 and B-CPAP), one from FTC (WRO) and three from ATC (FB-1, ARO and FRO; Fig. 1A). The primary P5 cells were used as control of normal human thyroid cells (TN). Western blot analysis demonstrated that Skp2 expression was moderately increased in four out of nine PTC cell lines (NPA, BHP10.3, BHP17.10 and B-CPAP), and markedly overexpressed in two ATC cell lines (FRO

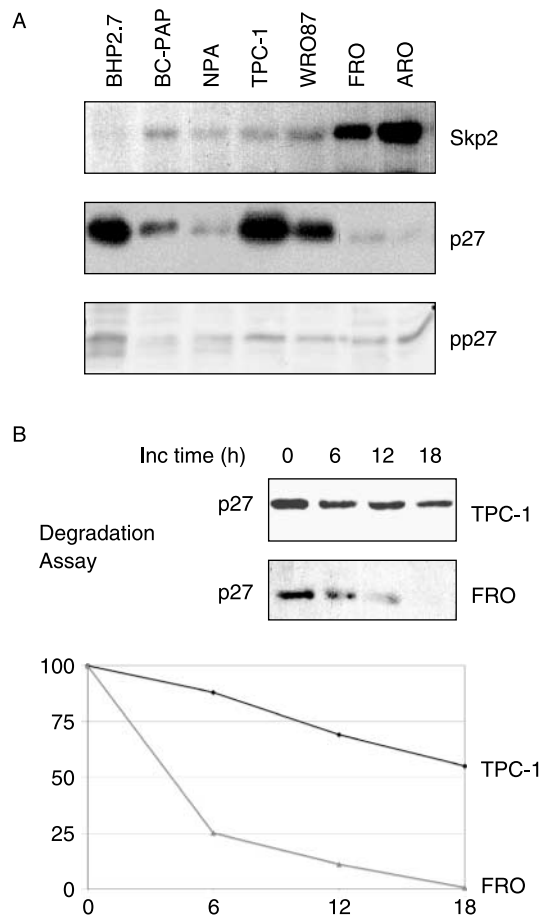


**Figure 1** Skp2 expression in human thyroid cancer cell lines. (A) Immunoblot analysis of Skp2 in human thyroid cancer cell lines. TN, normal thyroid cell line (P5); NPA, BHP2.7, BHP10.3, TPC-1, BHP17.10 and B-CPAP cell lines were derived from papillary carcinomas (PTCs), WRO from follicular carcinoma (FTC), FB-1, ARO and FRO cells were derived from ATC. Antibodies to  $\beta$ -tubulin were used for normalization. (B) Skp2 relative mRNA expression by Q-RT-PCR. Control, RNA from P5 cells. (C) Skp2 relative gene number by Q-PCR. Control, DNA from normal peripheral blood lymphocytes.

and ARO). Subsequently, we examined Skp2 mRNA expression by northern blot (not shown) and quantitative RT-PCR in a selected panel of thyroid cancer cell lines (NPA, B-CPAP, TPC-1, ARO, FRO and FB-1; Fig. 1B). We found a good correlation between Skp2 mRNA and protein expression in most cell lines analysed. In fact, the lines that showed low levels of Skp2 protein (FB-1 and TPC-1) also showed low amount of mRNA, whereas the cell lines with increased Skp2 protein levels showed enhanced amount of Skp2 mRNA (B-CPAP, FRO and ARO).

Previous work has shown that the *Skp2* gene mapping at 5p13 is frequently the target of amplification in lung cancer (Yokoi *et al.* 2002, 2004, Coe *et al.* 2005), in glioblastoma (Saigusa *et al.* 2004) and in carcinoma of the biliary tract (Sanada *et al.* 2004). Interestingly, an increase in the copy number of chromosome 5 involving the locus 5p13, where the *Skp2* gene has been assigned, was reported to occur in FTC, in Hurthle carcinomas (Hemmer *et al.* 1999, Wada *et al.* 2002) and in ATC cell lines including ARO (Foukakis *et al.* 2005). Thus, we examined the amplification status of *Skp2* gene in thyroid carcinoma cell lines (Fig. 1C). Genomic DNA extracted from six thyroid cancer cell lines and from control normal human lymphocytes (Fig. 1C, control) was analysed by Q-PCR as described in Materials and Methods. The DNA copy number of the *Skp2* gene was considered to be increased when the arbitrary values obtained by the quantitative PCR analysis (after normalization with actin levels) were greater than twofold the value obtained for normal diploid human lymphocytes. Using these parameters, our results indicated that at least three cell lines (NPA, FRO and ARO) presented increased copy number of the *Skp2* gene in comparison with genomic DNA from normal lymphocytes or from other cell lines examined. At least, two out of the three cell lines (ARO and FRO) that presented amplification of the *Skp2* gene expressed the highest levels of Skp2 mRNA and protein.

Subsequently, we investigated whether Skp2 was involved in p27 degradation in thyroid cancer cells. First, we found that in thyroid cancer cells the expression of Skp2 and p27 was inversely correlated. p27 was expressed heterogeneously in thyroid cancer cells, with marked accumulation in the TPC-1, BHP2.7 and WRO cells that presented low Skp2 expression. Conversely, the p27 expression was reduced (B-CPAP, NPA, FRO) or lost (ARO) in cells that presented relatively high Skp2 expression (Fig. 2A). The finding that in B-CPAP cells the p27 levels did not correlate with Skp2 levels, suggested that other (unknown) factors, in addition to Skp2, are involved in the regulation of p27 levels in cultured thyroid cancer cells. Since Skp2-dependent degradation of p27 depends on T187 phosphorylation



**Figure 2** Correlation between Skp2 and p27 expression in thyroid cancer cell lines. (A) Immunoblot analysis of Skp2 in human thyroid cancer cell lines. (B) p27 degradation assay. Rate of p27 degradation in extracts from TPC-1 and FRO cells. One microgram of recombinant p27 was incubated at 37 °C with 100 µg proteasome extracts for 0, 6, 12 or 18 h respectively, and the subsequent immunoblot analysis revealed the amount of intact p27 protein in different conditions. Half-life of p27 protein was of ~4–5 h in FRO cells and > 18 h in TPC-1.

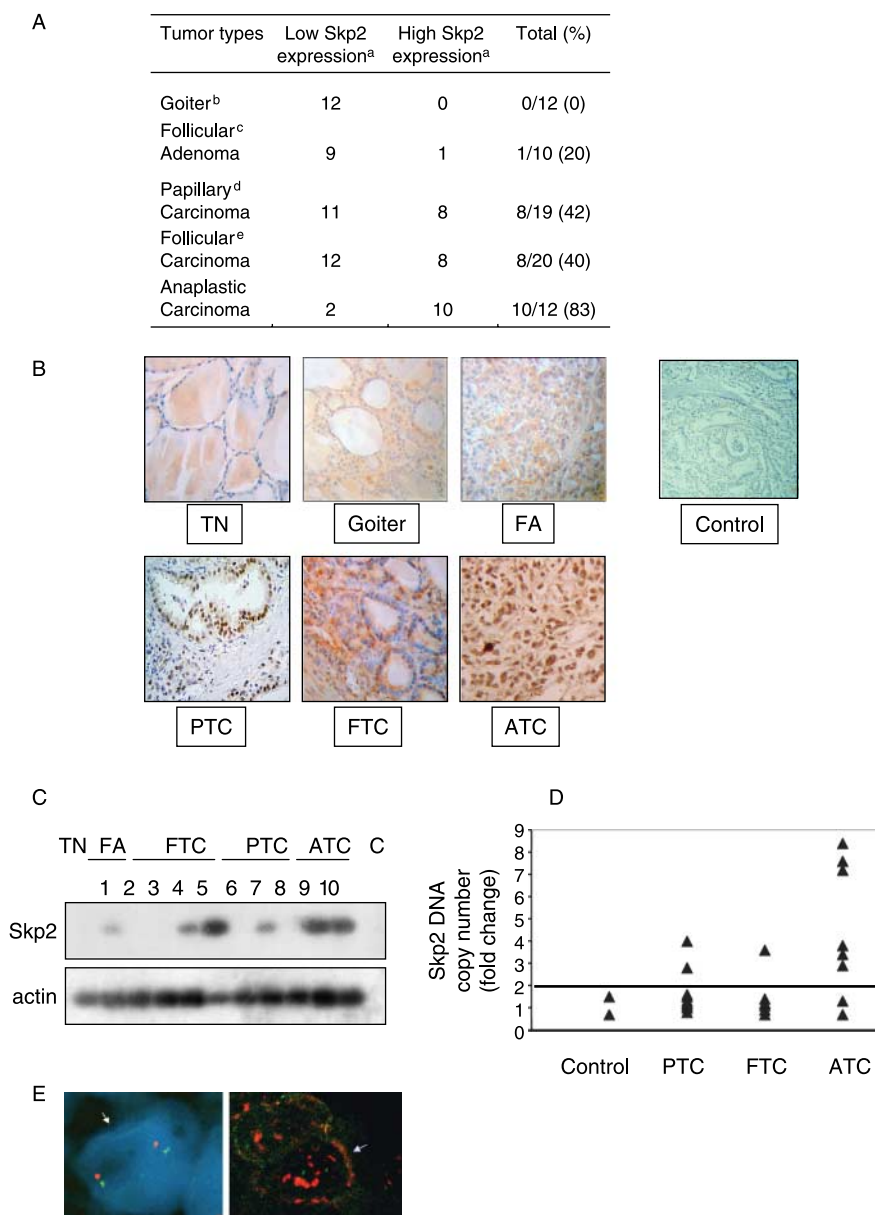
of p27, we also analysed T187 phosphorylation using phospho-specific antibodies. We found that p27 was apparently more abundantly phosphorylated at T187 in those cells which presented reduced or absent p27 expression (see the amount of T187 phosphorylation relative to the levels of p27 in NPA, ARO and FRO cells compared with other cells), suggesting that T187 phosphorylation was implicated in p27 degradation in thyroid cancer cells (Fig. 2A). Subsequently, we evaluated the ability of extracts from cells expressing high levels of Skp2 protein (ATC cells: FRO and ARO) to degrade recombinant p27 *in vitro* in comparison with cell lines that express low levels of Skp2 protein (TPC-1 and WRO). Proteasome extracts prepared from FRO, ARO, TPC-1 and WRO cells were incubated with

recombinant p27 and analysed by western blotting to determine the rate at which the thyroid cancer cells degraded p27. Proteasome extracts derived from TPC-1 (Fig. 2B) and WRO (not shown) cells slowly degraded p27 (half-life: > 18 and ~11.5 h respectively), whereas FRO (Fig. 2B) and ARO (not shown) cells degraded p27 with a shorter half-life (~4.5 and 5 h respectively).

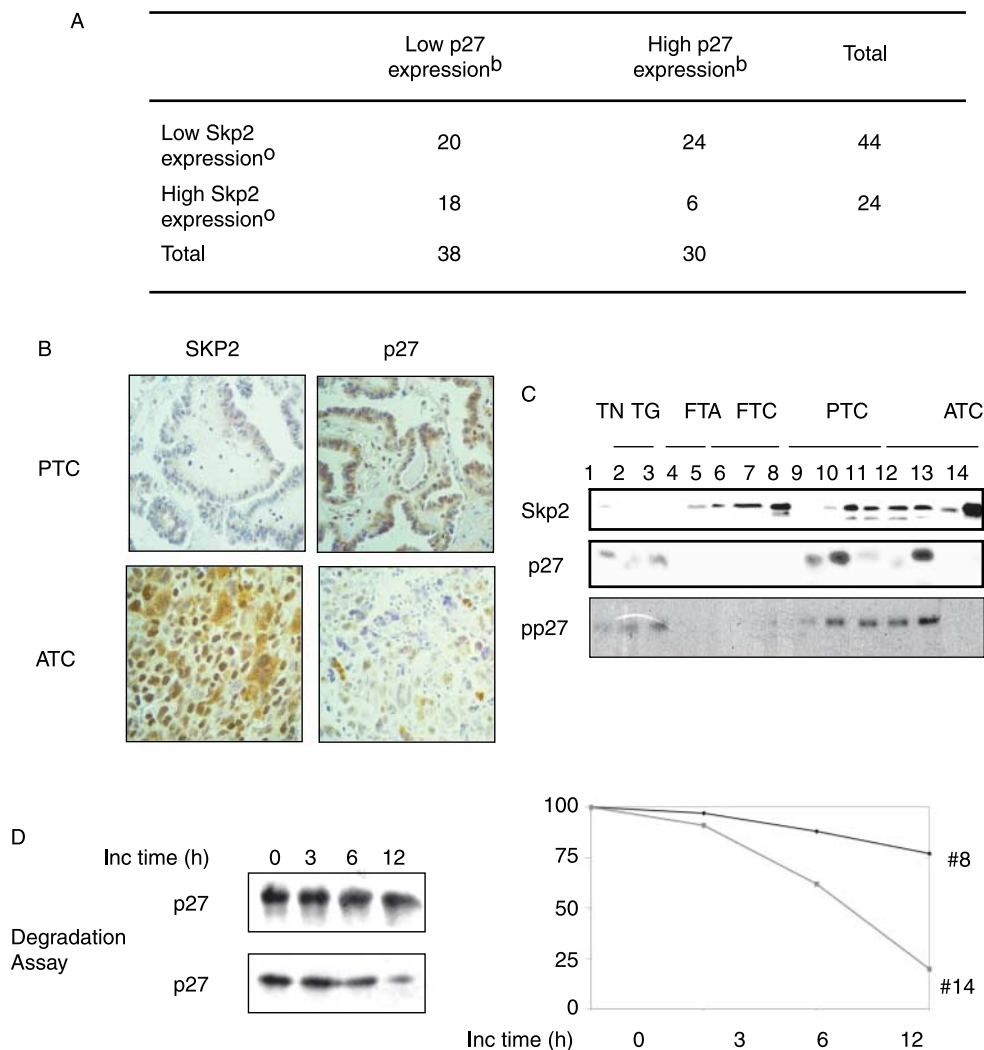
### Expression analysis of Skp2 in thyroid tumours

Although the results described earlier suggest that Skp2 may play a key role in the process of thyroid carcinogenesis, it is necessary to note that the various thyroid cancer cell lines used in this study could not be the most relevant models of primary thyroid tumours. Therefore, we extended our study to the expression of Skp2 in primary carcinomas. To this aim, we performed immunostaining for Skp2 protein in 12 cases of TG, 10 FA and 51 cases of thyroid carcinomas (19 PTC, 20 FTC and 12 ATC). The incidence of Skp2 expression in thyroid disease is summarized in Fig. 3A. We found that Skp2 is not detectable in thyrocytes from normal glands (4 out of 4) and in hyperplastic disease such as goitres (12 out of 12), and is expressed only in one out of ten FA with few (~10%) focally distributed nuclear-positive tumour cells (Fig. 3B). In contrast, Skp2 is overtly overexpressed in 26 out of 51 (~51%) malignant thyroid carcinomas. Thyroid carcinomas were classified as positive for Skp2 expression when showing strong diffuse nuclear staining in several cells/high-power fields (cut-off set at >20% of tumour cells). Positive Skp2 expression was significantly higher in the carcinoma samples than in either goitres or adenomas (carcinoma versus goitre,  $P < 0.001$ ; carcinoma versus adenoma,  $P < 0.05$ ; Fisher's exact test). Among the different types of thyroid carcinomas, Skp2 expression was observed more frequently in ATC (83%, 10 out of 12) than PTC (42%, 8 out of 19) or FTC (40%, 8 out of 20;  $P < 0.05$ ; Fisher's exact test).

The relationship between Skp2 and p27 protein expression was examined in 68 cases of thyroid tumours. The expression of p27 in thyroid tumour tissues was evaluated by immunohistochemistry with anti-p27 monoclonal antibody (Fig. 4A and B). Thyroid cancer cases showed three major patterns of Sp2/p27 expression. Eighteen tumours (26%) were characterized by low p27 and high Skp2 levels. Twenty-four tumours (35%) expressed high p27 and low Skp2 levels. In 20 tumours (29%), both Skp2 and p27 were observed in low levels. Only six tumours expressed both high levels of Skp2 and p27. Fisher's exact test indicated that the percentage of high Skp2 expressors was significantly greater in the low p27



**Figure 3** Skp2 expression in human primary thyroid cancer. (A) Expression of Skp2 in thyroid tumours. <sup>a</sup>The expression of Skp2 was graded as high (>20% of tumour cells with strong or diffuse immunopositivity) or low (<20% of the tumour cells with weak focal immunopositivity or absence of staining). <sup>b</sup>Significant; goitre versus carcinomas:  $P=0.0008$  (Fisher's exact test). <sup>c</sup>Significant; adenoma versus carcinomas:  $P=0.032$  (Fisher's exact test). <sup>d</sup>Significant; papillary versus ATCs:  $P=0.031$  (Fisher's exact test). <sup>e</sup>Significant; follicular versus ATCs:  $P<0.027$  (Fisher's exact test). (B) Representative immunostaining of Skp2 in normal and pathological thyroid tissue. Serial formalin-fixed paraffin-embedded sections from normal thyroid tissue (TN), goitre, FA, PTC, FTC and ATC were analysed for Skp2 expression. Most follicular epithelial cells from normal glandular tissue, goitre and FA are negative for Skp2 expression; several cancer cells show intense nuclear Skp2 expression in PTC, FTC and ATC. Magnification,  $100\times$ . (C) Semiquantitative RT-PCR analysis of Skp2 mRNA expression in thyroid tumours. Twenty-four cycles RT-PCR products were separated onto agarose gel, transferred to nylon membrane and hybridized. Actin expression was used as a loading control. TN, normal thyroid; FTA, follicular adenoma; FTC, follicular thyroid carcinoma; PTC, papillary thyroid carcinoma; ATC, anaplastic thyroid carcinoma and C, blank control without DNA for PCR. (D) Skp2 relative gene number by Q-PCR. Control, DNA from normal peripheral blood lymphocytes. E. FISH analysis of primary thyroid carcinomas. Left, the white arrow points to a cell showing the normal complement of *Skp2* gene (red); right, the white arrow points to a cell showing multiple signals of Skp2 (red).



**Figure 4** Correlation between Skp2 and p27 expression in thyroid primary tumours. (A) <sup>a</sup>Correlation between the expression of Skp2 and p27 in thyroid tumours ( $P=0.023$ ) with two-tailed Fisher's exact test. <sup>b</sup>Cells were classified as p27-positive, if staining was observed in  $>40\%$  of observed cells, or p27-negative, if staining was observed in  $<40\%$  of observed cells. <sup>c</sup>Cells were classified as Skp2-positive, if staining was observed in  $>20\%$  of observed cells. (B) Immunostaining of Skp2 and p27 in a papillary tumour (PTC) that show low Skp2 and high p27 expression; immunostaining of Skp2 and p27 in an anaplastic tumour (ATC) with high Skp2 and low p27 expression. (C) Reciprocal expression of Skp2 and p27 in human primary thyroid carcinoma by western blot. TN, normal thyroid tissue; TG, goitre; FTA, follicular adenoma; FTC, follicular carcinoma; PTC, papillary carcinoma; TC, anaplastic carcinoma. D. Rate of p27 degradation in extracts from sample 8 or 14. One microgram of recombinant p27 was incubated at  $37^\circ\text{C}$  with  $100\ \mu\text{g}$  proteasome extracts from tumours 8 and 14 for 0, 3, 6 or 12 h respectively, and the subsequent immunoblot analysis revealed the amount of intact p27 protein in different conditions. Half-life of p27 protein was of  $\sim 7$  h in sample 14 and  $> 12$  h in sample 8.

expression group than in the high p27 expression group ( $P<0.05$ ). Benign tumours that presented low expression of Skp2 (12 TG and 6 out of 8 FA) showed high nuclear expression of p27, whereas 18 out of 24 carcinomas with high Skp2 expression showed reduced levels of p27 protein. Figure 4B shows a representative immunostaining. Results were also confirmed by immunoblotting (Fig. 4C). To determine the contribution of T187 phosphorylation to p27 degradation, we

also determined p27 phosphorylation at T187 in the same samples of primary thyroid carcinomas (Fig. 4C). We found that most samples expressed high levels of Skp2 protein and thus lacked p27 and T187 phosphorylation, whereas a few samples (no. 10 and 13) showed low levels of Skp2 and high levels of p27. In these samples, the relative amount of T187-phosphorylated p27 was rather low. Conversely, in samples 11 and 12, which presented low levels of p27 protein,

the signal detected by the phospho-T187 antibody indicated that a great amount of p27 protein in those samples was phosphorylated. These studies indicated that Skp2 expression was inversely correlated with p27 levels and that both Skp2 levels and the different extent of T187 phosphorylation of p27 may contribute to determine p27 expression in thyroid cancer cells.

Since the reduction in p27 protein is brought about by ubiquitin–proteasome-mediated degradation, we also examined p27 degradation activity in these tissues. We compared the ability of proteasome extracts deriving from three different ATCs (that showed high levels of Skp2 protein) to degrade recombinant p27 in an *in vitro* degradation assay with that of two different PTCs with low Skp2 expression. Extracts from ATC showed higher p27 degradation activity in agreement with the very low level of p27 protein expression; conversely, extracts from PTC showed much lower p27 degrading activity (compare kinetics of p27 degradation rate in nos. 8 and 14 in Fig. 4D).

Subsequently, we investigated the molecular mechanisms whereby the expression of the Skp2 protein occurred in thyroid carcinomas. To this aim, we performed semiquantitative PCR analysis on RNA extracted from three normal thyroid tissues and 42 thyroid tumours (3 GT, 6 FA, 8 FTC, 16 PTC and 9 ATC). In a preliminary study, we determined the linearity of the PCR amplification and set the ideal conditions for Skp2 semiquantitative detection at 24 cycles. RT-PCR products were separated onto 1.2% agarose gels, transferred to nylon membranes and hybridized with a Skp2-specific probe. At this number of cycles, we found very low Skp2 mRNA expression in 3 out of 3 normal thyroid tissues and in 3 out of 3 GTs, in 4 out of 6 FAs; in contrast, 12 out of 16 PTCs, 5 out of 8 FTCs and 8 out of 9 ATCs showed consistent levels of Skp2 mRNA expression, confirming expression of Skp2 at the transcriptional level. Figure 3C shows an example of the semiquantitative PCR results, using actin primers as internal control of Skp2 expression. Similar results were obtained by Q-RT-PCR (not shown). As in cell lines, we found a significant relationship between Skp2 mRNA and protein levels also in primary tumours.

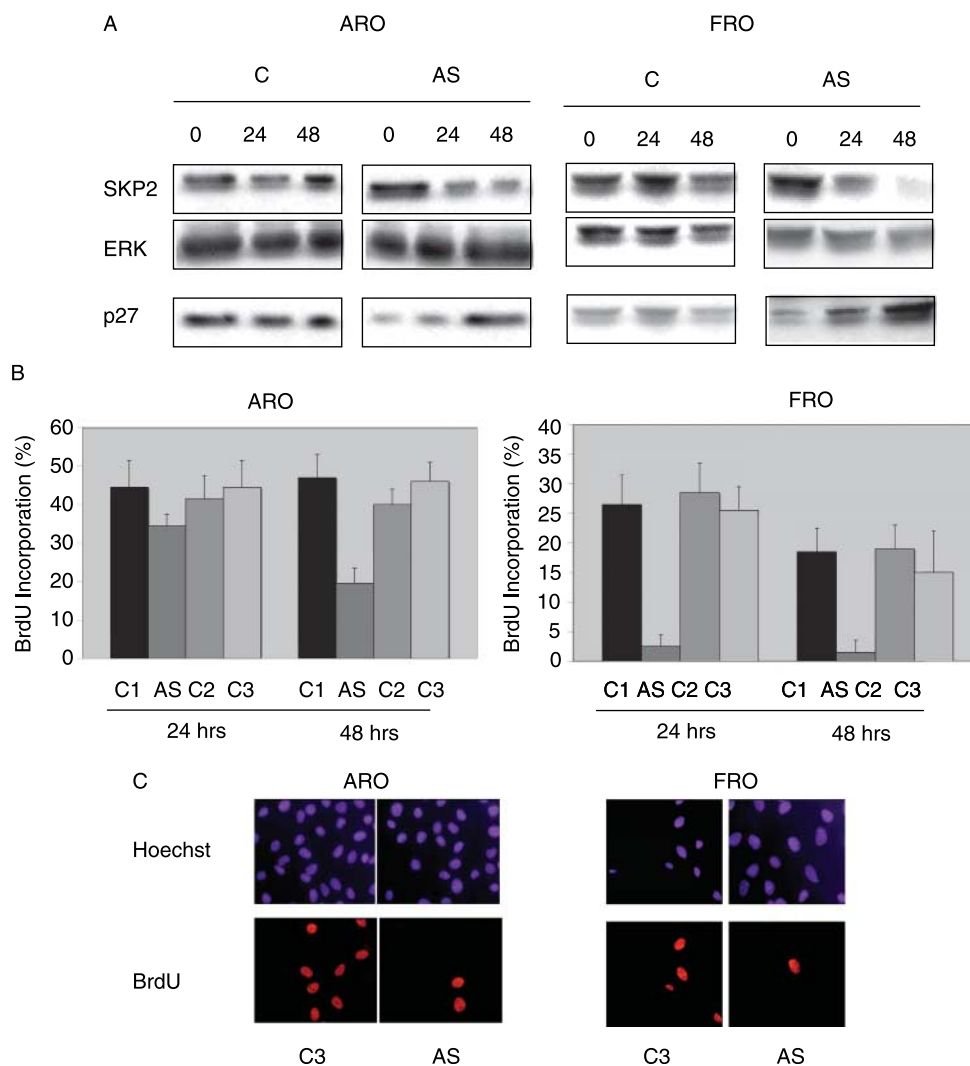
Subsequently, to determine whether the observed increased expression of Skp2 could be ascribed to gene amplification at 5p13, we examined the amplification status of the *Skp2* gene in the same samples analysed for protein and mRNA expression. Therefore, we performed Q-PCR on genomic DNA extracted from 23 thyroid tumours (nine PTCs, six FTCs and eight ATCs) using normal human lymphocytes as control. As with cell lines, the DNA copy number of the *Skp2* gene was

considered to be increased when the values obtained by Q-PCR analysis with the Skp2 primers, were greater than twofold the value obtained for normal diploid human lymphocytes. We found that, compared with normal lymphocytes, six out of eight ATCs, one out of nine PTCs and one out of six FTCs showed *Skp2* gene increased copy number (Fig. 3D). DNA amplification at 5p13 was also evaluated by performing FISH analysis on ten cases as described in Materials and Methods. FISH analysis demonstrated that the copy number of the *Skp2* gene was increased in all three ATCs analysed. Conversely, gain of chromosome 5p13 was observed only in one out of five PTCs and in one out of two FTCs (see Fig. 3E for a representative case). Therefore, our data suggest that gene amplification of the Skp2 locus at 5p13 represents a common event in ATC and may account for the observed increased expression of Skp2 mRNA and protein frequently observed in these tumours.

### Skp2 is necessary for p27 degradation and cell proliferation in ATC cells

To determine the effects exerted by the block of Skp2 expression on p27 expression and cell proliferation in ATC cells, we made use of antisense technology. FRO and ARO cells were transfected with Skp2 antisense or control oligonucleotides (200 nM). After 48 h, cells were incubated with 10  $\mu$ M BrdU for 2 h and processed for western blot and indirect immunofluorescence. As shown in Fig. 5A, we were able to reduce Skp2 expression by >70% in ARO cells and almost 90% in FRO cells, whereas the same dose of control sequence-scrambled oligonucleotides (Fig. 5A and C) had no effect on the cellular levels of Skp2. In parallel with the decrease in Skp2 expression, the treatment of ATC cells with anti-Skp2 antisense oligonucleotides induced a marked induction of p27 protein levels. Such increase in p27 levels in AS-treated WRO cells results in increased binding of p27 to CDK2 and decreased activity of the kinase using the histone H1 as substrate (not shown).

Finally, we found that the capacity to incorporate BrdU of ARO and FRO cells was markedly reduced in cells that were administered antisense oligonucleotides (Fig. 5B, AS) compared with untransfected (Fig. 5B, C1), mock-transfected ATC cells (Fig. 5B, C2) or cells transfected with control oligonucleotides (Fig. 5B, C3). Similar results were observed when cells were analysed after 24 and 48 h of treatment. Figure 6C shows a representative BrdU incorporation experiment. The results derived from antisense experiments pointed to a prominent role of Skp2 in the proliferative potential of ATC cells.



**Figure 5** Suppression of Skp2 protein expression inhibits proliferation of human ATC cells. To suppress the expression of SKP2 in ATC cells, ARO and FRO cells were plated onto glass coverslips in DMEM containing 10% FBS and then transfected with SKP2 antisense or scrambled control oligonucleotides with oligofectamine. After 24 and 48 h, cells were incubated with 10  $\mu$ M BrdU and processed for western blot (A) or indirect immunofluorescence (B and C). A. Western blot analysis of cyclin SKP2 expression in the presence of control or anti-SKP2 antisense oligonucleotides. C1, untransfected cells; AS, anti-Skp2 oligonucleotide-transfected cells; C2, mock-transfected cells; C3, cells transfected with an oligonucleotide of scrambled sequence. B. Graphs indicate the percentage of BrdU-positive cells. BrdU-positive cells were evidenced using FITC-conjugated secondary antibodies. C. A representative BrdU incorporation experiment. Magnification, 63 $\times$ .

### Skp2 promotes cell in proliferating thyroid carcinoma cells

To further investigate the role played by Skp2 in the control of thyroid cell cycle progression, we transfected WRO cells with a plasmid that carry the cDNA encoding human Skp2 cDNA. Transfected cells were selected in G418-containing medium and several WRO-neo and WRO-Skp2 clones were isolated and expanded. The presence of the exogenous Skp2 protein in transfected cells was detected by western blot analysis (Fig. 6A). Two different WRO-neo (c1.2 and c1.9) and three

different WRO-Skp2 (c1.3, c1.5 and c1.17) clones were selected for biological studies. Since the same data were obtained with the three clones in all our experiments, we will refer to them as 'WRO or WRO-Skp2 cells'. As expected, forced expression of Skp2 protein in WRO cells promoted increased binding to T187-phosphorylated p27 (not shown) and reduced p27 levels (Fig. 6A).

We investigated whether the constitutive expression of Skp2 modified specific proliferative parameters of thyroid cells, including proliferation rate, serum requirement and contact inhibition of WRO cells.

To this aim, WRO or WRO-Skp2 cells were plated onto glass coverslips and, after allowing cells to attach for 24 h in complete medium, the medium was changed and the cells were grown in DMEM containing 10, 5, 1 and 0.1% FBS for further 24 h, incubated with 10  $\mu$ M BrdU and processed for indirect immunofluorescence. As shown in the graphs of Fig. 6B that indicate the percentage of BrdU-positive cells, BrdU incorporation of WRO-Skp2 cells was higher than that of parental WRO and WRO-neo cells. This effect was more evident for cells grown in serum as low as 1 and 0.1%. Figure 6B reports a representative experiment.

Although BrdU incorporation is a good measure of the rate of DNA synthesis, it does not measure cell division. To further characterize the effect of Skp2 overexpression on the proliferative potential of thyroid cancer cells, we measured the growth rates of parental and Skp2-transfected WRO cells in low or high serum content. WRO and WRO-Skp2 cells were plated in DMEM supplemented with 1% (low serum) or 10% (high serum) serum and counted daily for 6 days. A representative experiment of three is reported in Fig. 6C. As shown in the figure, the growth rate of WRO-Skp2 cells was higher compared with parental WRO cells, especially when grown in low serum-containing medium. Moreover, when grown at confluence, WRO-Skp2 cells incorporated more BrdU compared with WRO cells, which suggested that Skp2 relieved the growth arrest induced by high-density culturing in WRO cells (Fig. 6D).

Skp2 interacts with several cell cycle regulatory proteins (Zhang *et al.* 1995, Patton *et al.* 1998, Sutterluty *et al.* 1999, Philipp-Staheli *et al.* 2001). To determine whether the stimulation of proliferation induced by overexpression of Skp2 in WRO cells was entirely mediated by the reduction of p27 levels, we silenced p27 expression using lentiviral-transduced shRNA to p27 both in confluent and proliferating WRO cells. Although interference with p27 expression was complete in both confluent and proliferating WRO cells (not shown), we observed a partial but reproducible increase in the BrdU uptake of transduced confluent WRO cells compared with untransduced WRO cells or transduced with Mission non-target control transduction virus (Sigma SHC002V; Fig. 6D). This proliferative effects induced by interfering with p27 expression were observed only in confluent but not in proliferating WRO cells (Fig. 6D). These findings suggest that the effect on the proliferation of WRO cells exerted by Skp2 was mediated only in part by the control of p27 expression and that the regulation of other cell cycle regulatory proteins by Skp2 must

contribute to thyroid cancer cell proliferation, especially in proliferating cells.

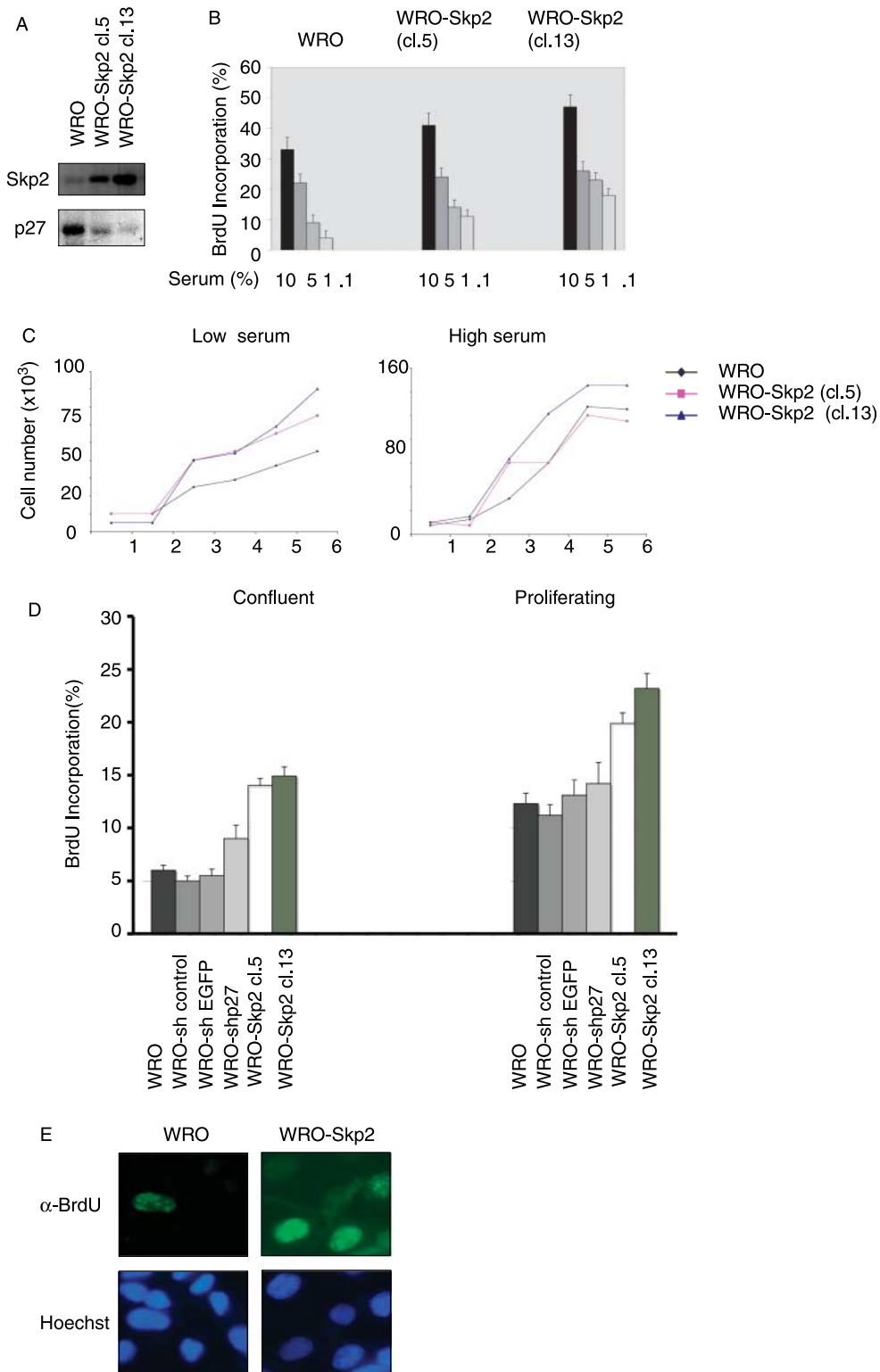
In summary, these data indicate that Skp2 can act as a rate-limiting factor for G1/S transition in cultured thyroid cancer cells and its unrestrained expression removes the serum dependency constraint and impairs contact inhibition-induced G1 arrest.

## Discussion

Here, we demonstrate that Skp2 protein is overexpressed in  $\sim$ 37% of surgically resected specimens from thyroid cancer patients. Interestingly, we found that Skp2 is significantly overexpressed in carcinomas but not in goitres or adenomas. Among malignant cancers, poorly differentiated carcinomas exhibit significantly higher levels of Skp2 protein compared with well-differentiated carcinomas of both follicular and papillary histotypes. These results are in agreement with previous studies that have shown Skp2 overexpression in different cancerous tissues (23–30), with a striking correlation between Skp2 overexpression and grade of malignancy, lymph node metastasis and poor prognoses (Gstaiger *et al.* 2001, Hershko *et al.* 2001, Kudo *et al.* 2001, Latres *et al.* 2001, Chiarle *et al.* 2002, Masuda *et al.* 2002, Signoretti *et al.* 2002, Li *et al.* 2004), and shed light on the molecular alterations that occur during thyroid carcinogenesis.

Skp2 is the substrate-recognizing subunit of an ubiquitin ligase named SCF complex (Deshaies 1999), and p27 is the target for ubiquitination and subsequent proteolysis by SCFSkp2 (Sutterluty *et al.* 1999, Slingerland & Pagano 2000, Philipp-Staheli *et al.* 2001). It is well known that reduced expression of p27 is frequently found in various cancers and correlated with poor survival of cancer patients (Slingerland & Pagano 2000, Philipp-Staheli *et al.* 2001). Cancer cells express low levels of p27 because of its decreased stability (Slingerland & Pagano 2000, Philipp-Staheli *et al.* 2001). Although some studies have proposed that the reduced stability of p27 protein observed in human cancer could be due to increased levels of Skp2, the detailed mechanism of abnormal degradation of p27 protein in cancer cells is still unclear.

Different groups including ours have previously reported that a substantial fraction of thyroid cancer presented reduced p27 expression (Erickson *et al.* 1998, Baldassarre *et al.* 1999, Tallini *et al.* 1999, Motti *et al.* 2005) and, most importantly, this was correlated with lymph node involvement and the presence of distant metastasis (Erickson *et al.* 1998, Kondo *et al.* 2006). In a recent study, we found that in more than two-third of the thyroid cancer samples analysed, the



**Figure 6** Skp2 promotes proliferation of thyroid cancer cells. (A) Effects of adoptive Skp2 expression on the growth rate of thyroid cancer cells. A. Skp2 and p27 expression in WRO and WRO-Skp2 (Cl.5 and Cl.13) cells. (B) WRO and WRO-Skp2 (Cl.5 and Cl.13) cells were plated onto glass coverslips in complete DMEM and, after 24 h, the medium was replaced with DMEM containing 10, 5, 1 and 0.1% FBS for further 24 h. Subsequently, cells were incubated with 10  $\mu$ M BrdU and processed for indirect immunofluorescence.

reduction in the levels of p27 protein was not accompanied by a parallel reduction in the levels of the corresponding mRNA; conversely, tumours with low levels of p27 protein presented high rate of p27 degradative activity (Motti *et al.* manuscript in preparation). In the present study, we further extend these data and report that the enhanced degradation of p27 protein observed in thyroid cancer can be attributed to Skp2 overexpression. In fact, immunohistochemistry and immunoblot analysis demonstrated an inverse correlation between Skp2 and p27 expression ( $n=68$ ;  $P<0.05$ ). Thyroid tissue specimens presented three major patterns of Sp2/p27 expression: low p27 and high Skp2 levels in 26% of tumours, high p27 and low Skp2 levels in 35% of tumours and low Skp2 and p27 levels in 20% of tumours. Of note, only few cases expressed both high levels of Skp2 and p27. Accordingly, thyroid cancer cell lines and tissues that showed high levels of Skp2 protein presented high p27 degradation activity. The role of Skp2 in the regulation of p27 in thyroid cancer cells was further demonstrated by *in vitro* experiments with cultured model systems. Forced expression of Skp2 in WRO cells drastically reduced p27 expression and, in contrast, antisense oligonucleotide mediated suppression of Skp2 expression in two ATC cell lines (ARO and FRO) resulted in enhanced levels of p27 protein. These results suggest that the reduction in p27 protein at least in some thyroid cancer cases may be brought about by high Skp2-mediated degradation.

Moreover, the existence of some thyroid cancer cases that showed low expression of Skp2 and p27 implied that other systems might also regulate p27 protein expression. Recent studies showed that CDK subunit 1 (Cks1) was required for the ubiquitination of p27 by bridging Skp2 and its substrate, p27, *in vivo* and *in vitro* (Ganoth *et al.* 2001, Spruck *et al.* 2001). On the other hand, p27 degradation may be accomplished through Skp2-independent mechanisms (Hara *et al.* 2001, Kamura *et al.* 2004).

The mechanism for the overexpression of Skp2 in most cancer is still unknown, though amplification of the Skp2 locus was found in some tumours (lung, bladder and glioblastoma; Yokoi *et al.* 2002, 2004, Sanada *et al.* 2004, Saigusa *et al.* 2004, Coe *et al.* 2005,

Ohno *et al.*, 2005). In the work reported here, we showed that Skp2 was amplified in at least three out of six thyroid cancer-derived cell lines and in a significant fraction (35%) of primary thyroid carcinomas analysed, in particular ATC, providing evidence that amplification of the Skp2 locus at 5p13 represents one of the mechanisms capable of enhancing Skp2 expression in thyroid cancer. It is of note that our results confirm cytogenetic data indicating that gain of part or of the entire short arm of chromosome 5, where the *Skp2* gene maps, frequently occur in PTC, Hurthle carcinomas and ATC (Hemmer *et al.* 1999, Wada *et al.* 2002, Foukakis *et al.* 2005). However, other mechanisms must be involved in the dysregulation of Skp2 expression in thyroid cancer, since our results indicate that a fraction of thyroid carcinomas over-expresses Skp2 mRNA and protein in the absence of gene amplification of the Skp2 locus. Accordingly, it has been suggested that the expression of Skp2 protein may be regulated at post-transcriptional level (Bashir & Pagano 2004, Wei *et al.* 2004).

To investigate the effect of Skp2 in the proliferative activity of ATC cells, we made use of an antisense strategy. Transfection of an antisense oligonucleotide (AS) into cultured FRO and ARO cells decreased Skp2 protein levels and suppressed the growth of ATC cells *in vitro* by inhibiting the degradation of p27 via the ubiquitin–proteasome pathway. Conversely, AS treatment had no significant effect on cell viability during 24–70 h after transfection (not shown). In contrast, Skp2 transfection resulted in the promotion of proliferation in thyroid cancer cells that expressed low levels of Skp2. Forced expression of Skp2 in WRO cells circumvented serum dependency and contact inhibition, allowing WRO cells to cycle also in the presence of 1% of serum and in conditions of high density. Taking into account, our results of the inverse correlation between Skp2 and p27 in primary thyroid cancer tissues and cell lines and the observation that Skp2-overexpressors presented increased p27 degrading activity, the promotion of proliferation activity by Skp2 transfection into thyroid cancer cells may be attributable to p27 degradation following Skp2 overexpression. However, it is likely that different cell cycle regulatory proteins which are targeted by Skp2

Graphs indicate the percentage of BrdU-positive cells. (C) Growth rates of parental and Skp2-transfected WRO cells in low or high serum content. WRO and WRO-Skp2 (Cl.5 and Cl.13) cells were plated in DMEM supplemented with 1% (low serum) or 10% (high serum) FBS and counted daily for 6 days. A representative experiment of three is reported. (D) WRO and WRO-Skp2 (Cl.5 and Cl.13) cells were plated onto glass coverslips in complete DMEM, at <25% (low density) or >80% (high density) and allowed to grow as described in the text. Cells were fixed in 4% paraformaldehyde and permeabilized with 0.2% Triton X-100. BrdU-positive cells were evidenced using FITC-conjugated secondary antibodies. Cell nuclei were identified by Hoechst staining. Fluorescence was visualized with a Zeiss 140 epifluorescent microscope. (E) A representative BrdU incorporation experiment. Magnification, 100×.

may contribute to thyroid cancer cell proliferation, since depletion of p27 in WRO cells only partially mimick the effects exerted by Skp2 cell proliferation.

Taken together, our findings are consistent with the current opinion indicating that Skp2 is an oncogene (Nakayama *et al.* 2000), regulating proliferation rate, resistance to apoptosis and higher potential for invasiveness, and motility in different carcinoma cells. In conclusion, these findings indicate that Skp2 may play an important role for the development of thyroid cancer, and that Skp2 overexpression could modulate the malignant phenotype of thyroid carcinoma, possibly by regulating the p27 protein level. However, it must be noted that in a certain fraction of thyroid carcinomas, p27 expression is not reduced (Erickson *et al.* 1998, Baldassarre *et al.* 1999, Tallini *et al.* 1999, Motti *et al.* 2005). Therefore, in these case mechanisms other than Skp2-dependent degradation of p27, such as sequestration into cyclin D/CDK complexes (Baldassarre *et al.* 1999) or phosphorylation-dependent cytoplasmic relocalization of p27 (Baldassarre *et al.* 1999, Vasko *et al.* 2004, Motti *et al.* 2005) have been proposed to contribute to inactivate p27 function.

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