SKP2 confers resistance of pancreatic cancer cells towards TRAIL-induced apoptosis

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Abstract. Pancreatic ductal adenocarcinoma (PDAC) is characterized by a dismal prognosis and no effective conservative therapy exists. Although the F-box protein S-phase kinase associated protein 2 (SKP2) is highly expressed and regulates cell cycle progression in PDAC, alternative SKP2 functions in PDAC are unknown. Using RNA interference we now demonstrate that SKP2 confers resistance of a subset of PDAC cell lines towards the tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), but not the topoisomerase II inhibitor etoposide. We observed accelerated cleavage of the BH3-only protein Bid and augmented downregulation of cFLIPL, XIAP and MCL1 upon treatment of SKP2-depleted MiaPaCa2 cells with TRAIL. Our data disclose a novel SKP2 function in PDAC cells and therefore define SKP2 as a molecular target.

Introduction

Despite increasing knowledge concerning the molecular and genetic alterations of pancreatic ductal adenocarcinoma (PDAC) (1-7), the 5-year survival rate remains <5% and no significant improvement in patient overall survival was documented over the last decades (8). In addition, most clinical phase III studies have failed to establish novel and efficient therapies for this disease (7,9). Therefore, there is a need to increase our understanding of therapeutic resistance at the molecular level in PDAC.

Therapeutic resistance in PDACs can be divided in cell intrinsic and extrinsic mechanisms (10). Recent work in genetically engineered murine PDAC models, clearly demonstrated that cell extrinsic resistance contributes to therapeutic failure in PDAC. Here, it was demonstrated that the very low blood vessel density combined with the characteristic desmoplasia of PDAC contributes to impaired drug delivery and therapeutic failure (11). In addition to extrinsic therapeutic resistance, cell intrinsic therapeutic resistance accounts for the treatment failure of PDAC. Since many therapeutics induce apoptosis, evasion of apoptosis is a main mechanism contributing to cell intrinsic therapeutic resistance and the insufficiency of current therapies (12). Therefore, restoring the sensitivity of PDAC cells towards cell death inducers is a therapeutic goal (13,14).

The oncogene S-phase kinase associated protein 2 (SKP2) is a F-box protein that functions as a receptor component of the SCF (Skp1-Cullin-F-box protein) ubiquitin ligase complex (15,16). SKP2 is overexpressed in most human cancers and mediates oncogenic function by ubiquitination and degradation of proteins including the cyclin-dependent kinase inhibitors $p27^{Kip1}$, $p21^{Cip1}$, $p57^{Kip2}$ or the retinoblastoma-like 2 (p130) protein (15,17). In PDAC cells, the SKP2 gene is activated by the phosphatidylinositol 3-kinase (PI3K)- and NF-κB-signaling pathways (18,19). Furthermore, high levels of SKP2 expression were observed in about one third of pancreatic cancer specimens and was shown to be an independent predictor of worse patient outcome (20). In this study we show that SKP2, in addition to the regulation of cell cycle progression, mediates resistance of PDAC cells towards tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)-induced apoptosis.

Materials and methods

Cell culture, siRNA transfection and reagents. The pancreatic cancer cell lines MiaPaCa2, Panc1, DanG and PatuII were cultivated as recently described (18,19,21,22). TRAIL and etoposide were purchased from EMD (EMD Biosciences, San Diego, CA, USA). Untreated controls received vehicle alone. Double-stranded siRNAs were transfected at a final concentration of 50 nM as described (21,22). siRNAs were purchased from Eurofins, Ebersberg, Germany. Target sequences of the used siRNAs were: control siRNA 5'-CAG TCGCGTTTGCGACTGGdtdt-3', SKP2-1 siRNA 5'-GGGA GTGACAAAGACTTTGdtdt-3 ', SKP2-3 siRNA 5'-GCATG TACAGGTGGCTGTTdtdt-3'.

Statistical methods. All data were obtained from at least three independent experiments performed in triplicate, and the results are presented as mean and standard error of the mean (SEM). To demonstrate statistical significance a two-tailed Student's t-test was used. p-values are indicated, and an asterisk in the figures denotes $p<0.05$.

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Quantitative reverse-transcriptase PCR. Total RNA was isolated from pancreatic carcinoma cell lines using the RNeasy kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. Quantitative mRNA analyses were performed as previously described using real-time PCR analysis (TaqMan, PE Applied Biosystems, Norwalk, CT) (21,22). Expression levels were normalized using cyclophilin. Primer sequences are available upon request.

Total cell lysates and Western blot analyses. Whole cell lysates were prepared and Western blot analyses were done as recently described (18,19,21,22). The following antibodies were used: XIAP (R&D Systems, Minneapolis, MN, USA); Bid, bcl_{XL} (Cell Signaling Technology Inc., Danvers, MA, USA); $p27^{Kip1}$ (Santa Cruz Biotechnology, Santa Cruz, CA, USA); MCL1 (Alexis Biochemicals, San Diego, CA, USA); SKP2 (Invitrogen, Frankfurt, Germany); cFLIP, cleaved PARP (BD Biosciences, Heidelberg, Germany); ß-actin (Sigma-Aldrich, Munich, Germany). One representative Western blotting out of at least three independent experiments is shown. Western blot analyses were quantified using Odyssey Infrared Imaging System (LI-COR Biosciences, Bad Homburg, Germany).

Hoechst-stain, MTT- and Caspase-3/7-assay. Viability of the cells was measured using MTT-assays performed according to the manufacturer's protocol (Roche Applied Science, Mannheim, Germany). Caspase-3/7 activity was determined using Promega's Caspase-Glo-3/7 assay according to the manufacturer's instructions (Promega, Madison, WI, USA). Chromatin was stained with Hoechst 33342 (4 μM) and typical apoptotic morphological changes were visualized using fluorescence microscopy as described (23). The apoptotic fractions of 300 cells were counted per individual experiment.

Cell cycle analysis. BrdU incorporation was measured using the colorimetric BrdU-Assay according to the manufacturer's instructions (Roche Applied Science). For cell cycle analysis, cells were washed twice in PBS and redissolved in propidium iodide (PI) staining buffer containing 0.1% sodium citrate, 0.1% Triton X-100, and 50 μg/ml PI. After 1 h of incubation at 4˚C, flow cytometry was performed using a BD Biosciences FACScan. The distribution of cells in different cell cycle stages (G1/S/G2+M) was determined according to their DNA content as described (18,19).

Results

SKP2 controls G1-phase progression and resistance of MiaPaCa2 cells towards TRAIL. Recently we have demonstrated that signaling pathways activating SKP2 transcription are linked to an accelerated G1- to S-phase progression (18,19,24). To prove the contribution of SKP2 towards the cell cycle control in the model investigated, we used RNA interference (RNAi). Forty-eight hours after the transfection of a SKP2-specific siRNA, SKP2 protein (Fig. 1A) and mRNA levels (Fig. 1B) were distinctly decreased compared to control siRNA transfected MiaPaCa2 cells. Consistent with an important function of SKP2 for cell cycle regulation, we observed increased expression levels of the SKP2 target

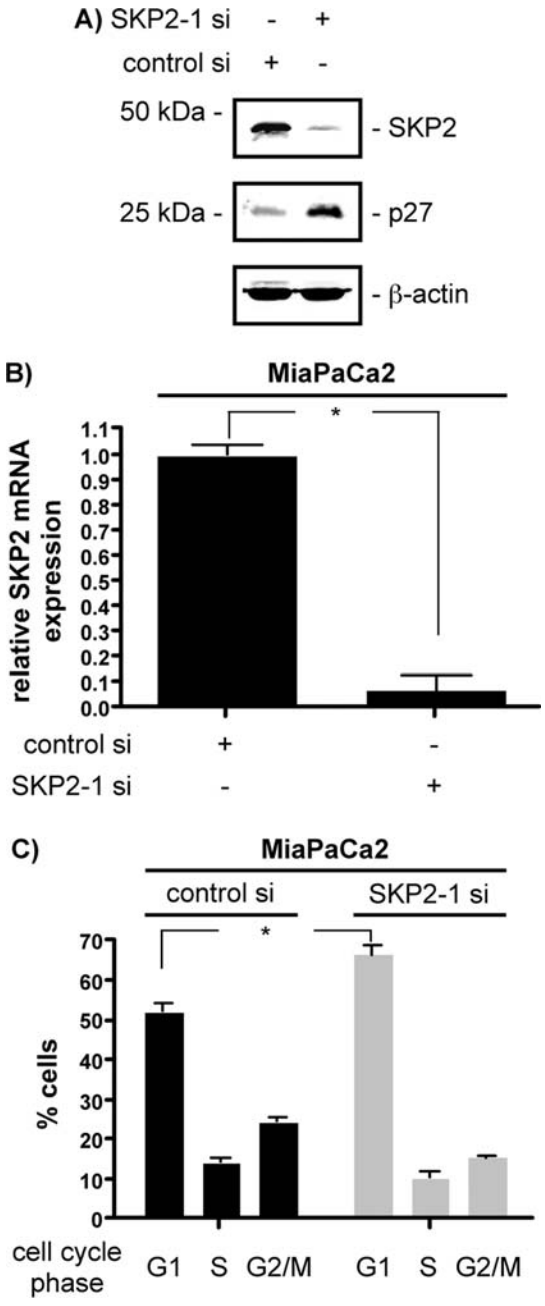

Figure 1. SKP2 knockdown induces a G1-phase arrest of the cell cycle in MiaPaCa2 cells. (A) Western blot analysis of SKP2 and $p27^{Kip1}$ 48 h after the transfection of MiaPaCa2 cells with a control siRNA or a SKP2-specific (SKP2-1) siRNA. ß-actin controls equal protein loading. (B) Quantitative SKP2 mRNA expression analysis in MiaPaCa2 cells after the transfection of a control or a SKP2-specific (SKP2-1) siRNA. Total RNA was prepared 48 h post-transfection. SKP2 mRNA levels were quantified using real-time PCR analysis and normalized to cyclophilin expression levels (Student's t-test: $^{*}p<0.05$ versus controls). (C) MiaPaCa2 cells were transfected with the indicated siRNAs. Forty-eight hours post-transfection, cells were stained with PI and cell cycle distribution was analyzed by FACS. The fraction of the cells in G1, S and G2/M phases is indicated (Student's t-test: $^{*}p<0.05$ versus controls).

$p27^{Kip1}$ (Fig. 1A) and a consecutive accumulation of the cells in the G1-phase of the cell cycle (Fig. 1C). Since the G1-phase of the cell cycle of PDAC cells is especially sensitive for TRAIL-induced apoptosis (25), we treated SKP2-depleted MiaPaCa2 cells with TRAIL and investigated viability and

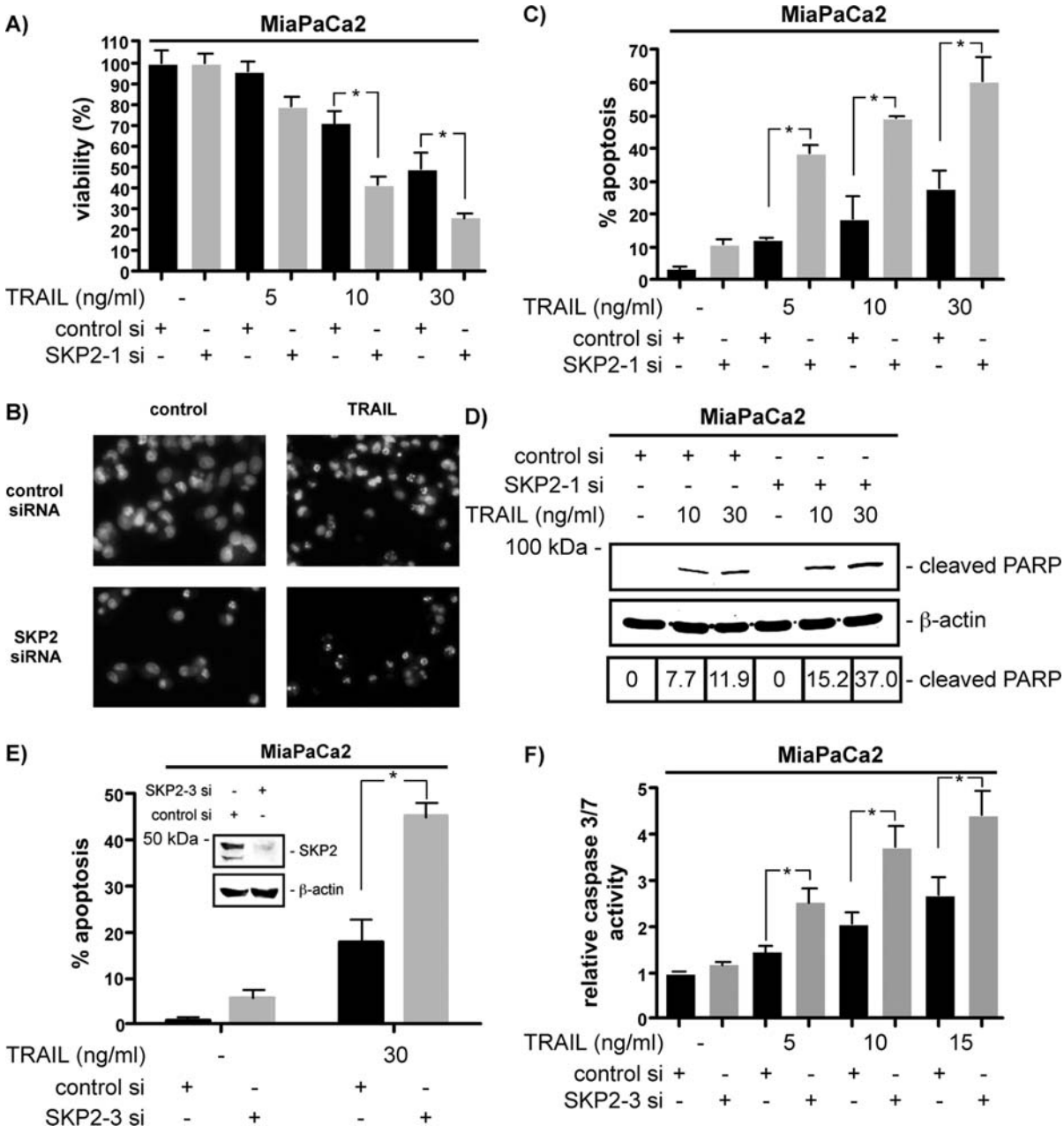

Figure 2. SKP2 controls TRAIL sensitivity of MiaPaCa2 cells. (A) MiaPaCa2 cells were transfected with a control siRNA or an SKP2-specific (SKP2-1) siRNA. Forty-eight hours after the transfection the cells were treated with increasing doses of TRAIL as indicated for additional 24 h or left as an untreated control. Viability was determined using MTT assays (Student's t-test: $^{*}p<0.05$ versus controls). (B) MiaPaCa2 cells were transfected with a control siRNA or an SKP2-specific (SKP2-1) siRNA. Forty-eight hours after the transfection the cells were treated with 30 ng/ml TRAIL for additional 24 h or left as an untreated control. Photomicrographs (original magnification x40) of MiaPaCa2 cells after the staining of the nuclei with Hoechst reveal apoptotic changes. (C) MiaPaCa2 cells were transfected with a control siRNA or an SKP2-specific (SKP2-1) siRNA. Forty-eight hours after transfection the cells were treated with increasing doses of TRAIL as indicated for additional 24 h or left as an untreated control. Apoptotic cells were quantified by fluorescence microscopy after Hoechst staining (Student's t-test: $^{*}p<0.05$ versus controls). (D) MiaPaCa2 cells were transfected with a control siRNA or an SKP2-specific (SKP2-1) siRNA. Forty-eight hours after transfection the cells were treated with increasing doses of TRAIL as indicated for additional 24 h or left as an untreated control. Western blot analysis of PARP cleavage. ß-actin controls equal protein loading. Cleaved PARP was quantified using the Odyssey near-infrared fluorescence scanner ensuring measurements in the linear range. Relative expression of cleaved PARP is indicated. (E) Western blot analysis of SKP2 48 h after the transfection of MiaPaCa2 cells with a control siRNA or an SKP2-specific (SKP2-3) siRNA. ß-actin controls equal protein loading. MiaPaCa2 cells were transfected with a control siRNA or an SKP2-specific (SKP2-3) siRNA. Forty-eight hours after transfection the cells were treated with TRAIL as indicated for additional 24 h or left as an untreated control. Apoptotic cells were quantified by fluorescence microscopy after Hoechst staining (Student's t-test: $^{*}p<0.05$ versus controls). (F) MiaPaCa2 cells were transfected with a control siRNA or an SKP2-specific (SKP2-3) siRNA. Forty-eight hours after transfection the cells were treated with TRAIL as indicated for additional 2 h or left as an untreated control. Caspase activity was measured using caspase-3/7 activity assays (Student's t-test: $^{*}p<0.05$ versus controls).

cell death. As shown in Fig. 2A, SKP2-depleted cells revealed an increased TRAIL-induced loss of viability, which was due to a distinctly increased apoptotic fraction (Fig. 2B and C). SKP2-depleted MiaPaCa2 cells show an augmented caspase activation as measured indirectly by the cleavage of the caspase-substrate poly-(ADP-ribose) polymerase 1 (PARP)

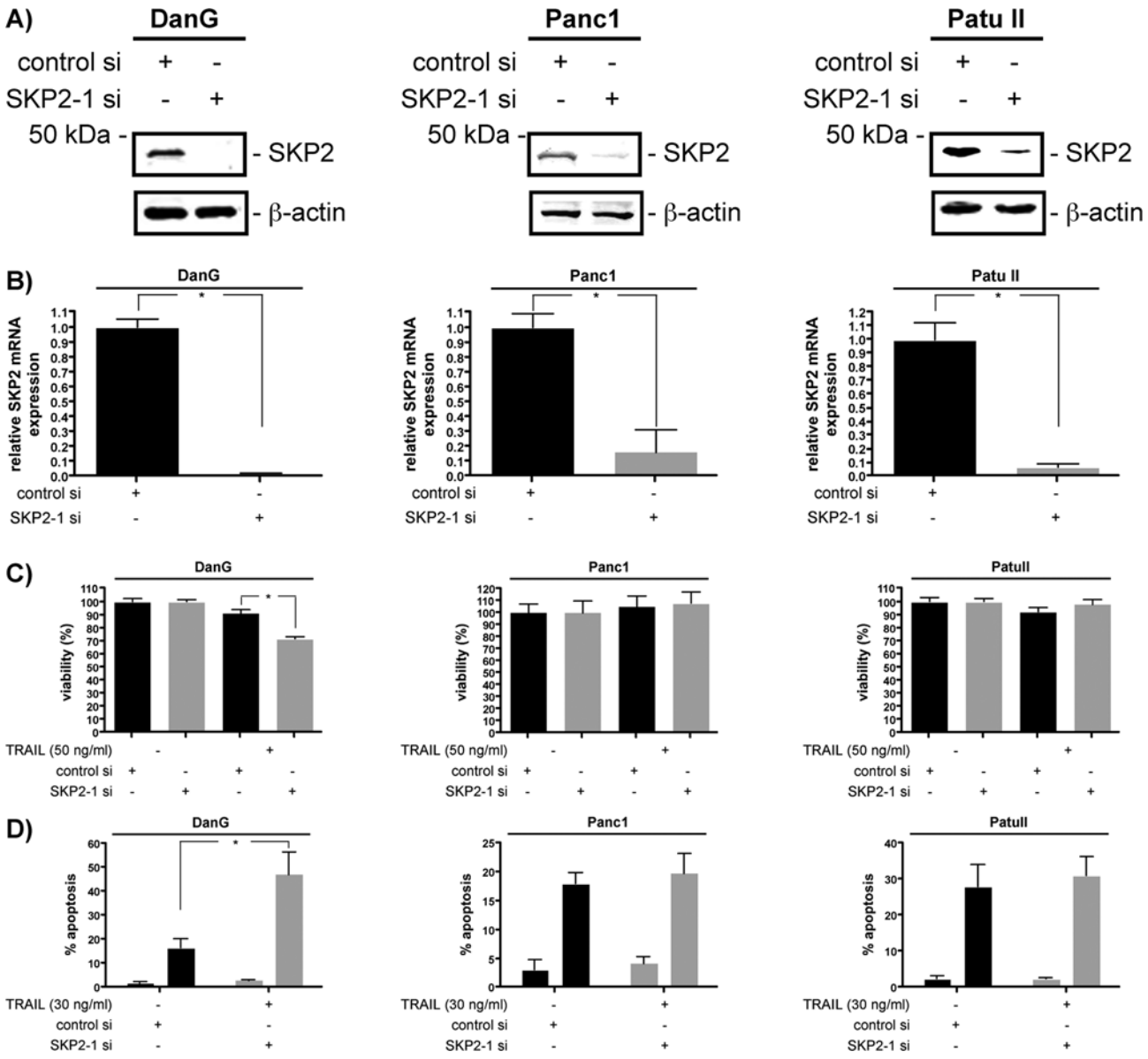

Figure 3. SKP2 and TRAIL sensitivity of DanG, Panc1 and PatuII cells. (A) Western blot analysis of SKP2 48 h after the transfection of DanG, Panc1 and PatuII cells with a control siRNA or an SKP2-specific (SKP2-1) siRNA. ß-actin controls equal protein loading. (B) Quantitative SKP2 mRNA expression analysis in DanG, Panc1 and PatuII cells after the transfection of a control or an SKP2-specific (SKP2-1) siRNAs. Total RNA was prepared 48 h post-transfection. SKP2 mRNA levels were quantified using real-time PCR analysis and normalized to cyclophilin expression levels (Student's t-test: $^{*}p<0.05$ versus controls). (C) DanG, Panc1 and PatuII cells were transfected with a control siRNA or an SKP2-specific (SKP2-1) siRNA. 48 h after the transfection the cells were treated with 50 ng/ml TRAIL as indicated for additional 24 h or left as an untreated control. Viability was determined using MTT assays (Student's t-test: $^{*}p<0.05$ versus controls). (D) DanG, Panc1 and PatuII cells were transfected with a control siRNA or an SKP2-specific (SKP2-1) siRNA. Forty-eight hours after transfection the cells were treated with TRAIL as indicated for additional 24 h or left as an untreated control. Apoptotic cells were quantified by fluorescence microscopy after Hoechst staining (Student's t-test: $^{*}p<0.05$ versus controls).

(Fig. 2D). To validate these results we used a second SKP2-specific siRNA. Again, the SKP2 knockdown (Fig. 2E) lead to a prominent increase of the TRAIL-induced apoptotic fraction (Fig. 2E) and TRAIL-induced caspase activation (Fig. 2F). Together, these data suggest that SKP2 increases the sensitivity of MiaPaCa2 cells for TRAIL-induced apoptosis.

Control of TRAIL-sensitivity by SKP2 is cell-type specific. To determine whether SKP2 is generally involved in the regulation of sensitivity for TRAIL-induced apoptosis, we transfected the PDAC cell lines DanG, Panc1, and PatuII with the SKP2-specific siRNA. The knockdown was verified 48 h after the transfection of the SKP2-specific siRNA at the level of SKP2 protein (Fig. 3A) and mRNA (Fig. 3B). Although depletion of SKP2 significantly increased TRAIL-induced loss of viability (Fig. 3C) and distinctly increased TRAIL-induced apoptotic fraction in DanG cells (Fig. 3D), SKP2 depletion did not change TRAIL sensitivity of Panc1 and PatuII cells (Fig. 3C and D). These data argue that SKP2-dependent regulation of TRAIL sensitivity is cell-type specific, reflecting tumor heterogeneity.

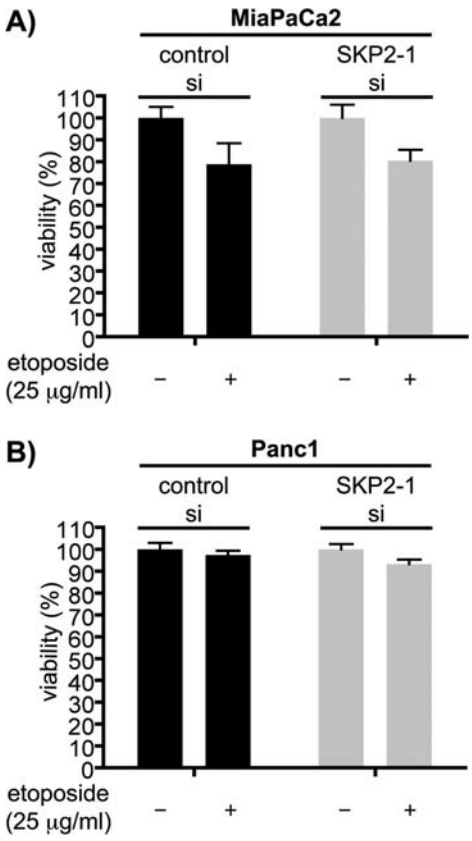

Figure 4. SKP2 and etoposide sensitivity of MiaPaCa2 and Panc1 cells. (A) MiaPaCa2 and (B) Panc1 cells were transfected with a control siRNA or an SKP2-specific (SKP2-1) siRNA. Forty-eight hours after the transfection the cells were treated with etoposide as indicated for additional 24 h or left as an untreated control. Viability was determined using MTT assays.

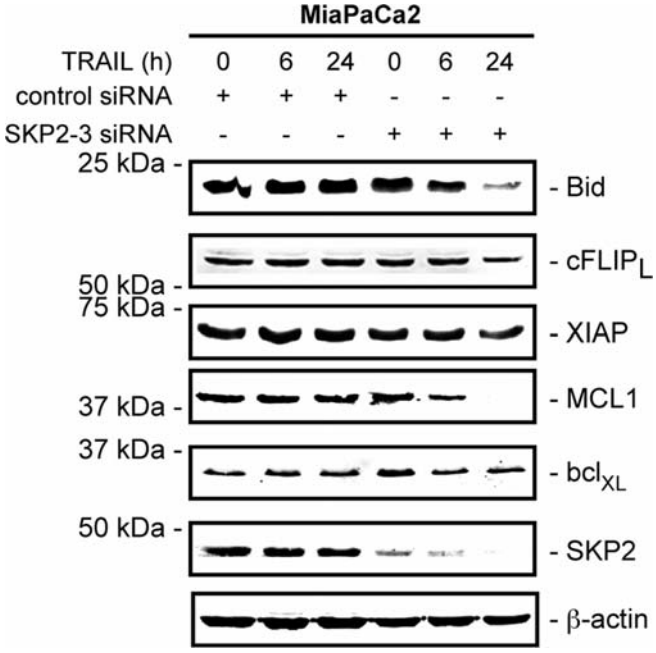

Figure 5. SKP2 and regulation of TRAIL resistance proteins in MiaPaCa2 cells. Forty-eight hours after the transfection of MiaPaCa2 cells with a control siRNA or an SKP2-specific siRNA cells were treated with TRAIL over time as indicated. Western blot analysis of Bid, $cFLIP_L$, XIAP, MCL1, bcl_{XL} and SKP2. ß-actin controls equal protein loading.

SKP2 is not involved in regulation of topoisomerase II inhibitor sensitivity of PDAC cells. The use of DNA-damaging agents is a current strategy to treat solid tumors. Since recent work demonstrated that SKP2 confers resistance towards DNA-damage induced apoptosis (26,27), we tested the sensitivity of SKP2-depleted PDAC cells towards the topoisomerase II inhibitor etoposide. As shown in Fig. 4, sensitivity of SKP2 depleted MiaPaCa2 (Fig. 4A) and Panc1 cells (Fig. 4B) was not changed compared to control siRNA transfected cells, suggesting that SKP2 is not linked to the regulation of topoisomerase II inhibitor sensitivity in PDAC cells.

TRAIL treatment leads to an accelerated cleavage of Bid and depletion of cFLIPL, XIAP and MCL1 in SKP2 depleted MiaPaCa2 cells. Sensitivity of PDAC cells towards TRAIL is controlled at the level of the death inducing signaling complex (DISC), at the level of the inhibitors of apoptosis proteins (IAPs), and, since PDAC cells are type II cells depending on the activation and cleavage of the BH3-only protein Bid, at the level of the mitochondrium (13,14). Consistent with increased sensitivity towards TRAIL-induced apoptosis, we observed accelerated cleavage of Bid in SKP2-depleted MiaPaCa2 cells over time (Fig. 5). Twenty-four hours after TRAIL treatment we detected a slightly pronounced depletion of XIAP and $cFLIP_L$ in SKP2 siRNA transfected MiaPaCa2 cells. Furthermore, we observed already 6 h after the TRAIL treatment a downregulation of the anti-apoptotic BCL2 family member MCL1 in SKP2 siRNA transfected MiaPaCa2 cells (Fig. 5). Whereas the basal expression of bcl_{XL} was slightly increased in SKP2-depleted MiaPaCa2 cells (Fig. 5), basal expression of cIAP1, cIAP2 and survivin was not changed in SKP2-depleted MiaPaCa2 cells (data not shown).

Discussion

TRAIL agonistic therapeutic approaches are currently investigated in various clinical trials (28-31). In PDAC, high TRAIL expression was correlated with an increased apoptotic index in the human pancreas arguing that a TRAIL-based therapy might be a feasible strategy for the treatment of PDAC (32,33). Nevertheless, primary human tumor cells often resist TRAIL-induced apoptosis (34) and PDAC cells reveal a high half-maximal inhibitor concentration (IC_{50}) for TRAIL despite they express the TRAIL receptors DR4 (TRAIL-R1) and DR5 (TRAIL-R2) as well as relevant mediators of the TRAIL receptor signaling pathway (13,14,35). Therefore, defining molecular targets whose inhibition synergizes with TRAIL agonists is important for defining new therapies. Several proteins, conferring TRAIL resistance to PDAC cells, including histone deacetylase 2 (HDAC2) (21), STAT3 (36), CUX1 (37), cFLIP (38-40), XIAP (41-45), MCL1 (36,46), bcl_{XL} (36,47,48) or survivin (25) were described. In addition our current work now demonstrates that SKP2 can confer TRAIL resistance to PDAC cells.

The most prominent function of SKP2 is to drive cell cycle progression, mainly by inducing ubiquitination and degradation

of $p27^{Kip1}$ (15-17). However, recent evidence revealed that SKP2 can also regulate cancer-relevant processes like senescence (49), anoikis (50) or apoptosis (26,27,51-56). To our knowledge, we provide for the first time evidence that SKP2, highly expressed in pancreatic cancer (20), confers resistance towards extrinsic induction of apoptosis. Although we detected accelerated Bid cleavage and slightly pronounced depletion of $cFLIP_L$ and XIAP, the molecular mechanism by which SKP2 controls TRAIL sensitivity remains unclear at the moment and the described changes can thus occur secondary to increased TRAIL-induced apoptosis in SKP2-depleted cells. In contrast to this late occurring events, the expression of MCL1, a pro-survival BCL2 family member, was already decreased 6 h after treatment with TRAIL in SKP2 depleted cells. Although MCL1 can protect PDAC cells from TRAIL- (36), TNFα- and chemotherapy-induced apoptosis (unpublished data), the regulation of this short-lived protein in PDAC cells is complex and regulation of MCL1 occurs at the level of transcription, translation, and degradation (57). How, directly or indirectly, SKP2 is involved in the regulation of MCL1 in the model system investigated is unclear and awaits further investigation.

SKP2 regulates the response of cancer cells towards DNA-damaging agents (26,27,53). At the molecular level, SKP2 was shown to suppress p53-dependent apoptosis by forming a complex with the histone acetyltransferase p300. Thereby, SKP2 is antagonizing the p53/p300 interaction, needed to activate pro-apoptotic programs (26). We did not observe changes of PDAC cells sensitivity towards etoposide-induced apoptosis in SKP2-depleted cells. The fact that MiaPaCa2 ($p53^{R248W}$) as well as Panc1 ($p53^{R273H}$) cells harbor p53 mutations might explain this discrepancy.

Collectively, additional studies are needed to decipher the regulation of TRAIL sensitivity by SKP2 in molecular detail. Nevertheless, our findings provide clear evidence that SKP2 can contribute to the resistance of cancer cells towards extrinsic induction of apoptosis.

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