

## Thrombospondin-1 Receptor Mediates Autophagy of RAS-expressing Cancer Cells and Triggers Tumour Growth Inhibition

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**Abstract.** *Background: The anti-angiogenic activity of thrombospondin-1 (TSP-1) is suppressed in cancer cells, fact which has generated considerable interest in generating the respective therapeutic mimetics. These efforts were almost exclusively centered on peptides targeting CD36, the known TSP-1 receptor. Since the effects of these agents were less dramatic than those of full-length TSP-1 questions could be raised about the cancer-related roles of additional TSP-1 domains and receptors, such as CD47, which bind the C-terminal sequences of this protein. Materials and Methods: The MDFB6 and B6ras cell lines were treated with the anti-CD47 antibody or C-terminal TSP-1 peptide 4N1K and cell viability was monitored using Aqueous Non-Radioactive Cell Proliferation Assay, DNA fragmentation, caspase-3 activation, and cell membrane depolarization assays. The cells were also stained with acridine orange and the anti-LC3 antibody to detect autophagy. B6ras tumours were generated in SCID-NOD mice and tumour responses to injections of the 4N1K peptide were recorded over time. Results: We demonstrated a selective loss of viability of RAS-transformed cells upon ligation of the CD47 receptor. Affected cells did not exhibit hallmarks of apoptosis, but instead were stained with acridine orange and exhibited a punctuate pattern of immunoreactivity for LC3, both features of autophagy. The 4N1K peptide administration also caused a modest but specific and significant tumour growth inhibition in vivo. Conclusion: Our study offers an additional mechanism whereby TSP-1 affects tumour cells directly, in an angiogenesis-independent and*

*CD47-mediated manner. This is consistent with near complete loss of TSP-1 expression in RAS-transformed cells and may open new avenues for therapies involving TSP-1 peptides, in a subset of tumours.*

Several large extracellular matrix proteins such as thrombospondin-1 (TSP-1), endostatin, tumstatin and their proteolytic fragments have recently attracted considerable attention due to their endogenous anticancer effects, mainly attributed to inhibition of tumor angiogenesis (1, 2). TSP-1 is a regulatory target of several oncogenic pathways, including the loss of *p53* tumor suppressor gene, the oncogenic activation of *RAS* and *myc*, and other common transforming events (3-6). These events relieve the negative paracrine effect of TSP-1 on the survival, migration, and proliferation of tumor-associated endothelial cells. At the molecular level, TSP-1 activities were largely attributed to the interaction between the endothelial receptor CD36 and the region of TSP-1 containing the type-1 repeats (TSR) and related signaling (7-10). Findings such as these sparked considerable interest in developing anticancer agents with TSP-1-related antiangiogenic activity, mainly by exploiting the CD36-binding of peptides derived from the second type-1 repeat of TSP-1. Some of these agents (ABT510 and ABT526) have shown antitumor efficacy in pre-clinical studies and have also recently entered clinical trials (11-13). Although the antitumor activity of CD36-directed peptides has been encouraging, their effects are ostensibly less dramatic than might be expected from the impact exerted by the corresponding full-length TSP-1 molecule, for example, when it is exogenously re-introduced into various types of cancer cell lines (8, 9). This raises the possibility that other TSP-1 domains and functions could possess hitherto unappreciated anticancer properties, either related or unrelated to antiangiogenesis (11, 14). In this regard, the C-terminal domain of TSP-1 is known to interact with the CD47 receptor expressed on the surface of various cells, and

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in a manner that has no known antiangiogenic consequences. This binding is dependent on the decapeptide sequence (KRFYVVMWKK) of TSP-1, from which functional peptide analogues (4N1K) have been recently derived and extensively characterized (15).

CD47, also known as integrin-associated receptor, is a 50-kDa membrane glycoprotein and a member of the immunoglobulin superfamily, known to be ubiquitously expressed in various types of cells (16). Of note, CD47 has been implicated in induction of programmed cell death of lymphocytes (17), macrophages (14) and fibroblasts (18, 19) and these effects have been linked to the concomitant expression of BCL2/adenovirus E1B 19-kDa protein-interacting protein-3 (BNIP3). BNIP3 is a BH3-only member of the BCL-2 family of cell death-regulating mitochondrial proteins (17, 19, 20), which in our previous studies was found to be up-regulated by *H-RAS* oncogene (19). This raises the possibility that RAS may render cancer cells prone to BNIP3-dependent cell death induction, possibly *via* the CD47-dependent pathways activated by TSP-1.

Here we demonstrate that the TSP-1/CD47, but not TSP-1/CD36, pathway may contribute to direct tumor cell growth suppression *via* a mechanism involving autophagy-like changes. This effect is induced by the CD47-binding peptide (4N1K) only in cells harboring mutant RAS (B6ras), but not in their non-transformed counterparts, and this translates into significant, albeit not dramatic, tumour growth inhibition *in vivo*. We suggest that targeting CD47 in cancer may represent a viable therapeutic avenue, but the respective endogenous (TSP-1) and exogenous (4N1K) agents would likely require a synergistic combination with other modalities.

## Materials and Methods

**Cells and reagents.** The immortalized, non-tumorigenic, polyclonal mouse dermal fibroblastic cell line MDFB6 was derived from dermal explants of C57Bl/6 mice. These cells and their polyclonal variant B6ras harboring the mutant *H-RAS* oncogene have been described elsewhere (4). The cells were cultured in Dulbecco's Modified Eagle's medium (HyClone, Thermo Fisher Scientific Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS; Gibco, Invitrogen Corp., Carlsbad, CA, USA). All cultures were supplemented with antibiotic and antimycotic solution (Sigma-Aldrich, St. Louis, MO, USA). Culture plates and flasks were purchased from Nunc (Thermo Fisher Scientific Inc.). The CD47 directed peptide 4N1K (KRFYVVMWKK) and control peptide 4NGG (KRFYGGMWKK) and CD36 directed peptide were purchased from Lipopharm (Gdansk, Poland). Phosphate Buffered Saline (PBS), trypsin, paraformaldehyde, hematoxylin, HEPES, Triton X-100, etoposide, Tris-HCl, MgCl<sub>2</sub>, propidium iodide were purchased from Sigma Aldrich. Ethanol, NaOH, NaCl, KCl, CaCl<sub>2</sub> were obtained from POCh (Gliwice, Poland).

**Determination of CD47 and CD36 expression.** These antigens were determined by flow cytometry using 0.5 µg/ml rat anti-CD47; MIAP301 or mouse anti-CD36 (both Santa Cruz Biotechnology,

Inc., Dallas, TX, USA) primary antibodies and FITC-conjugated anti-rat or anti-mouse secondary antibodies (both Dako Denmark A/S, Glostrup, Denmark). Briefly, MDFB6 or B6ras cells were detached using Non-enzymatic Cell Dissociation Solution (Sigma Aldrich), then washed with PBS and incubated with the primary antibody (30 min, 4°C). After three-time wash cells were stained with the appropriate FITC-conjugated antibody (30 min, 4°C). Flow cytometric data were acquired using a FACSCalibur flow cytometer (Becton-Dickinson, Franklin Lakes, NJ, USA) and WinMDI 2.8 software. The filled histogram represents background control staining with FITC-conjugated secondary antibody only. Isotypic control antibody resulted in identical staining (data not shown). Representative results of three independent repeats are shown.

**MTS assay.** Cell viability was determined using the CellTiter 96® Aqueous Non-Radioactive Cell Proliferation Assay (MTS, Promega Corp., Madison, WI, USA), as recommended by the supplier. Briefly, 5,000 cells/well (96-well plate) were cultured (37°C, 5% CO<sub>2</sub>) for 72 h in the presence of 4 µg/ml of the anti-CD47 antibody, 50 µM of the 4N1K TSP-1 peptide, 50 µM of the peptide directed against CD36. Then MTS reagent was added and after 2 h incubation (37°C, 5% CO<sub>2</sub>) 490-nm absorbance was read on a Vallac Victor2 plate reader (PerkinElmer, Waltham, MA, USA). The data were presented as percentages of the untreated samples (control). Results of three independent experiments are shown on graphs.

**Annexin V staining.** After 48 h of treatment with 4 µg/ml of the anti-CD47 antibody or 50 µM of the 4N1K TSP-1 peptide B6ras cells were collected, washed and subsequently stained with 1 mg/ml FITC-Annexin V (Sigma-Aldrich) and 1 mg/ml propidium iodide diluted in binding buffer (10 mM HEPES/NaOH, pH 7.4, 150 mM NaCl, 5 mM KCl, 1 mM MgCl<sub>2</sub>, 1.8 mM CaCl<sub>2</sub>) for 10 min at room temperature. After washing flow cytometric data were acquired and analyzed using FACSCalibur flow cytometer and CellQuest software (Becton-Dickinson). Representative dot-plots of three independent repeats are shown.

**Apoptotic DNA profile.** The detection of apoptosis was based on evaluating DNA content with the use of propidium iodide and flow cytometry. Briefly, after 48 h of treatment with 4 µg/ml of the anti-CD47 antibody or 50 µM etoposide (Sigma Aldrich) B6ras cells were washed twice with PBS containing 2.5% FBS, fixed with ice cold 70% ethanol for 30 min at 4°C, washed again with PBS, and then stained with 10 µg/ml solution of propidium iodide in PBS overnight at 4°C. The cell suspensions were analyzed with a FACSCalibur flow cytometer. The DNA content was evaluated on the basis of FL-2 histograms using WinMDI 2.8 software. The M1 mark indicates cells with sub-G<sub>1</sub> DNA content characteristic of apoptosis, which was prominent in etoposide-treated cells and absent in the untreated control or anti-CD47-treated groups. Histograms representative of three independent repeats are shown.

**Cell count.** B6ras cells incubated for 72 h in the presence of 50 µM 4N1K peptide, were trypsinized and counted. Cell number was evaluated using hemocytometer and bright field microscope (Nikon Instruments Inc., Melville, NY, USA). Averages±SD of two independent experiments are shown; asterisks indicate statistically significant results (*p*<0.02; *n*=24). Non-transformed MDFB6 cells were insensitive to this treatment (data not shown).

**Caspase-3 activity assay.** B6ras cells plated in 24-well plate were treated for 18h with 50  $\mu$ M etoposide (the proapoptotic anticancer drug) or with 50  $\mu$ M 4N1K peptide for 72h. After treatment cells were lysed and processed using the Fluorometric Caspase-3 Cellular Assay Kit (Calbiochem, San Diego, CA, USA) according to manufacturer's instructions. Aliquots (10  $\mu$ l) of the lysate were transferred to a 96-well plate and caspase substrate was added, the fluorescence was analyzed on a Wallac Victor2 plate reader. The data were converted to caspase-3 activity units (U in 100  $\mu$ g of protein) according to the standard curve, which was made using the supplied human recombinant caspase-3. Average values ( $\pm$ SD) obtained in two independent experiments are shown. Asterisks indicate statistically significant results ( $p < 0.02$ ;  $n = 6$ ).

**Immunodetection of LC3.** B6ras cells were placed in chamber slides and incubated with 4  $\mu$ g/ml of the anti-CD47 antibody or control medium for 24 h. After the treatment cells were fixed in 4% paraformaldehyde and permeabilized with 0.15% Triton X-100 in PBS containing 10% horse serum (Sigma Aldrich). The slides were then incubated overnight with 1:3000 rabbit polyclonal anti-LC3B antibody (Abcam, Cambridge, UK) followed by the secondary antibody conjugated with AlexaFluor488 (1:200; Molecular Probes, Invitrogen Corp.). Nuclei were counter-stained with 10  $\mu$ l/ml propidium iodide. The results were viewed under an LSM510 Meta confocal microscope (Carl Zeiss MicroImaging GmbH, Jena, Germany). Control samples were processed without the primary antibody. Representative images are shown.

**Acridine orange staining.** Acridine orange staining of acidic bodies present in B6ras cells after 72 h treatment with 50  $\mu$ M of 4N1K peptide, 4  $\mu$ g/ml of anti-CD47 antibody (MIAP301) or 50  $\mu$ M of control NGG peptide. The volume of the cellular acidic compartment can be quantified by acridine orange staining (21, 22). Cell cultures were stained with 1  $\mu$ g/ml acridine orange (Invitrogen Corp.) for 15 min, trypsinized, and single-cell suspensions were prepared using phenol red-free growth medium (Invitrogen Corp.). The green (510-530 nm) and red (650 nm) fluorescence emissions were measured with a FACSCalibur flow cytometer at an excitation wavelength of 488 nm and the data were analyzed using WinMDI 2.8 software. Representative dot-plots from three independent experiments are presented.

**Analysis of tumor growth.** The animal experiments were performed according to the Interdisciplinary *Principles and Guidelines for the Use of Animals in Research* and approved by the institutional Committee for Experiments with the Use of Laboratory Animals, Wrocław, Poland. SCID-NOD female mice (University Children's Hospital, Cracow, Poland) were inoculated subcutaneously with  $5 \times 10^4$  B6ras tumor cells (10 mice per group). 20 mg/kg 4N1K or control (4NGG) peptide was administered daily *s.c.* in the region of the flank located outside of the tumor. Tumors were measured every other day and their volumes (TVs) were calculated using the formula:  $TV = (a^2 \times b) / 2$  [mm<sup>3</sup>], where "a" is the smaller and "b" the larger of the two perpendicular tumor diameters [mm]. The mice were sacrificed in accordance with ethical guidelines, examined for metastasis (none found), and the tumors were removed at autopsy. The average tumor volume  $\pm$ SD was plotted as a function of time post injection. The statistical significance of the recorded differences is designated by asterisks: \* $p > 0.05$ , \*\* $p > 0.005$ , \*\*\* $p > 0.0005$  ( $n = 10$ ), as estimated by the Mann-Whitney U test. Body weights of tumor-bearing mice receiving 4N1K peptide were also recorded.

**Immunohistochemistry.** The indirect peroxidase-antiperoxidase test was performed on 4- $\mu$ m-thick fixed tumor cryosections. After inhibition of the endogenous peroxidase and 30-min incubation with 1% normal rabbit serum (Sigma Aldrich), the tissue preparations were treated with the primary antibody against Ki-67 antigen (Abcam) at a 1:200 dilution. After washing, the slides were treated with peroxidase-conjugated swine anti-rabbit IgG antibody (Dako) and visualization was carried out using 3,3'-diaminobenzidine chromogen (Sigma Aldrich). For microscopic evaluation, sections were counter stained with hematoxylin and mounted. Control slides were processed in a similar manner except for replacement of the primary antibodies with 0.1 M Tris-buffer, pH 7.4. Ki67-positive cells were counted in 5-7 fields per tumor sample, 5 tumors per group; an asterisk indicates statistically significant results,  $p > 0.001$ .

**Statistical analysis.** All experiments were repeated at least 2-3 times with similar results. For *in vivo* experiments One-way analysis of variance (ANOVA) followed by a Tukey-Kramer multiple comparison test or a Mann-Whitney U-test was applied.  $p$ -Values  $< 0.05$  were considered significant. For other experiments unpaired Student's  $t$ -test was used.

## Results

**Selective loss of viability of RAS-transformed cells upon ligation of CD47 receptor.** We observed that both non-transformed, immortalized, murine dermal fibroblasts (MDFB6) and their tumorigenic counterparts harboring mutant *H-RAS* oncogene (B6ras) expressed TSP-1 receptors, CD36 and CD47, the latter at considerably greater but comparable levels in both cell lines (Figure 1A). This suggests that cellular transformation by oncogenic *H-RAS* does not impact CD36 or CD47 even though this event plays a major role in the down-regulation of TSP-1 (4).

To explore the function of these receptors we exposed them to peptides that bind, and selectively activate either CD36 or CD47. While the ligation of CD36 was relatively inconsequential, the 4N1K peptide derived from the CD47-binding region of TSP-1 produced a pronounced and selective diminution of the viability of B6ras cells, but not of their non-transformed MDFB6 counterparts, as measured by the MTS assay (Figure 1B). The control peptide NGG produced no such effect (Figure 1C), but the similar loss of viability was also recapitulated in the presence of the validated agonistic anti-CD47 antibody (Figure 1D). The activity of both of these CD47-directed agents was robust and dose-dependent across a wide spectrum of previously described (17) effective concentrations (Figure 1CD) and it indicates that expression of mutant RAS sensitizes cancer cells to anti-survival effects associated with ligation of the TSP-1 receptor CD47.

**Anticancer activity of anti-CD47 peptide *in vivo*.** Based on our aforementioned results we reasoned that loss of TSP-1 in cells harboring oncogenic *H-RAS* (4) may have both angiogenesis-related and angiogenesis-unrelated consequences, the latter

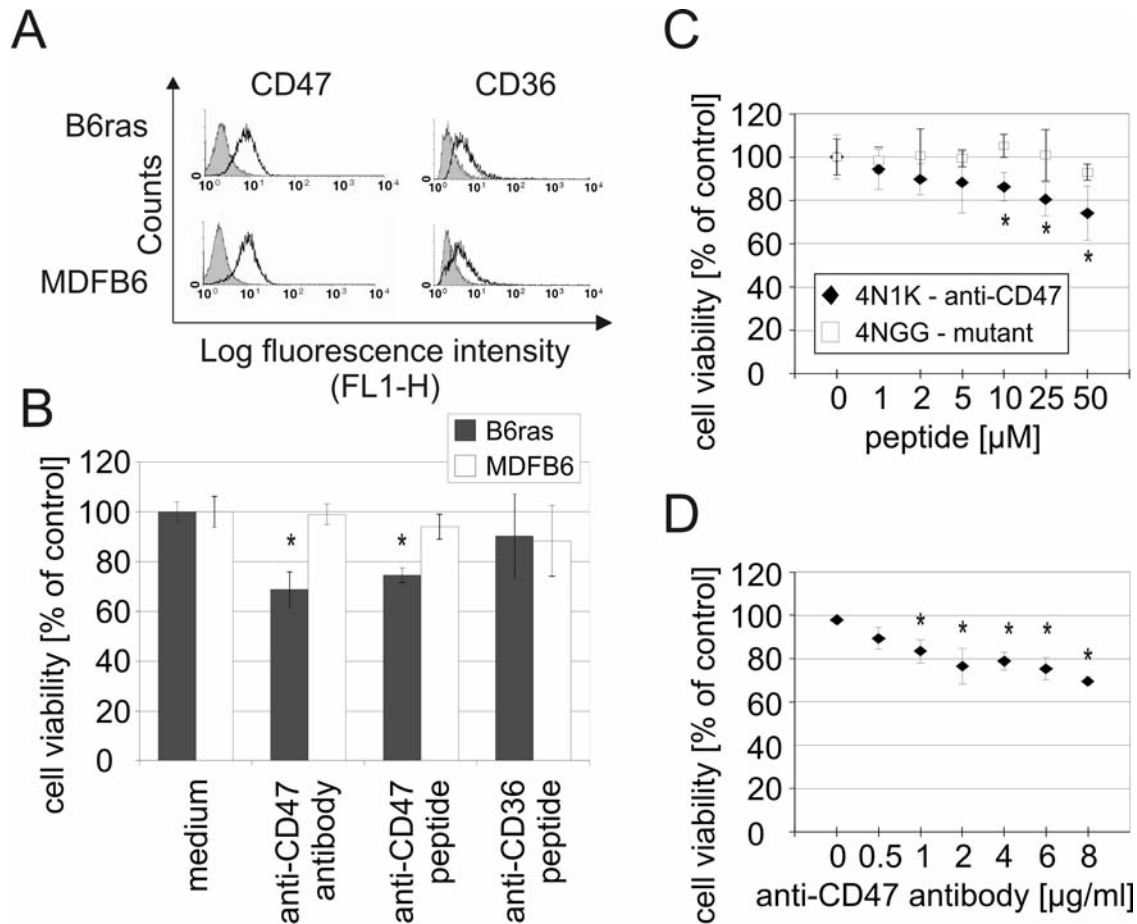


Figure 1. Differential sensitivity of RAS-transformed and non-transformed cells to ligation of the CD47 receptor. (A) CD47 and CD36 expression on the surfaces of non-transformed mouse dermal fibroblasts (MDFB6) and their H-RAS-transformed counterparts (B6ras). Introduction of H-RAS oncogene had no impact on the expressions of these receptors, as detected by flow cytometry. (B) Impact of CD47 ligation on survival of MDFB6 (empty bars) and B6ras (dark bars) cells, measured by the MTS Assay. The presence of the H-RAS oncogene sensitizes cells to CD47-mediated cell death. The values correspond to the average $\pm$ SD, where asterisks indicate statistically significant results ( $p < 0.02$ ;  $n = 9$ ). (C, D) Dose-dependent cytotoxicity of CD47-directed 4N1K and 4NGG peptides (C) and anti-CD47 antibody (D). Treatment of B6ras fibroblasts with increasing concentrations of 4N1K peptide or anti-CD47 antibody resulted in a gradual loss of cell viability as measured by the MTS assay. Data are presented as the percentage of the untreated control (average $\pm$ SD). The results are of three independent experiments and asterisks indicate statistically significant results ( $p < 0.01$ ;  $n > 9$ ).

linked to direct viability loss resulting from CD47 ligation. To assess whether the CD47-dependent mechanism may be operative and targetable *in vivo*, we generated B6ras tumors in NOD/SCID immunodeficient mice and subjected them to daily injection of the CD47-specific 4N1K peptide at 20 mg/kg/day beginning on day 1 after tumor inoculation. Notably, this treatment resulted in a 30% inhibition of tumor growth in mice receiving 4N1K (Figure 2A) compared with those injected with the vehicle or the equivalent dose of the control peptide 4NGG (Figure 2D). This effect was associated with a marked reduction in the number of Ki67-positive cells in tumors exposed to 4N1K (Figure 2A, inset). We observed no apparent clinical toxicity or weight loss in mice that

received 4N1K injections. Some reduction in weight of both treated and control mice did occur over time and can be attributed to the impact of the developing tumor (Figure 2B). These results are consistent with the notion that TSP-1-related peptides interacting with the CD47 receptor are generally non-toxic and may possess an independent and direct anticancer activity *in vivo* against tumor cells harboring activated RAS.

**Ligation of CD47 induces autophagy.** To understand the nature of anti-growth effects associated with ligation of CD47 in B6ras cells, we performed a series of viability, growth and cell-cycle assays (Figure 3). We found that ligation of CD47 using either 4N1K peptide or anti-CD47



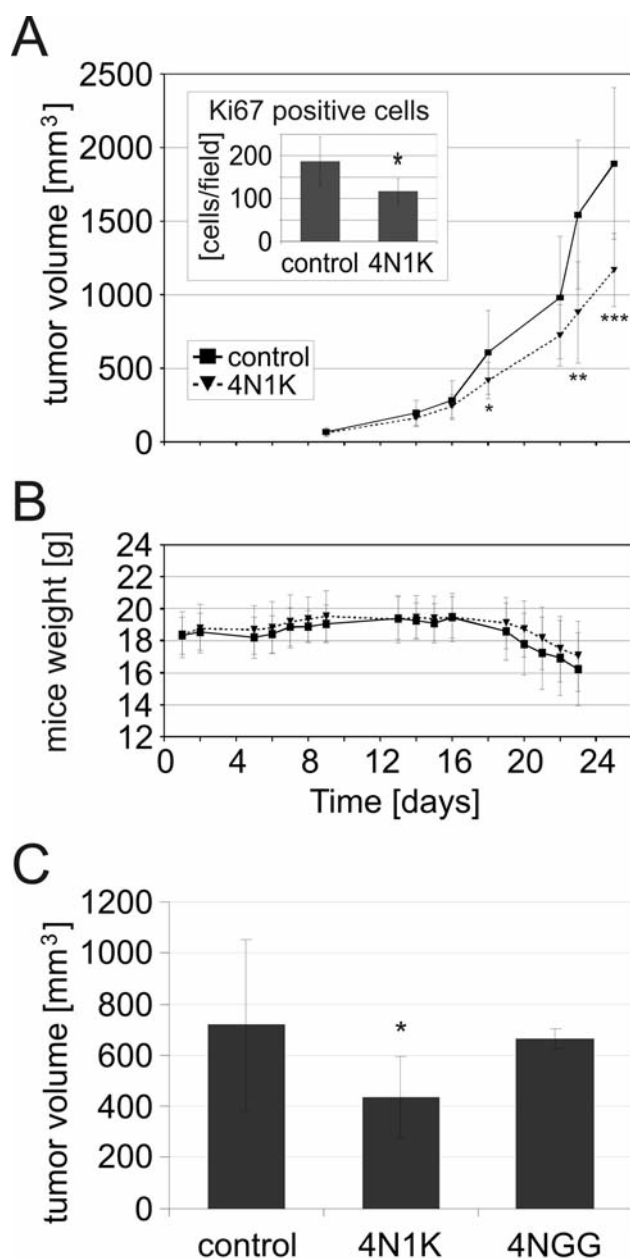


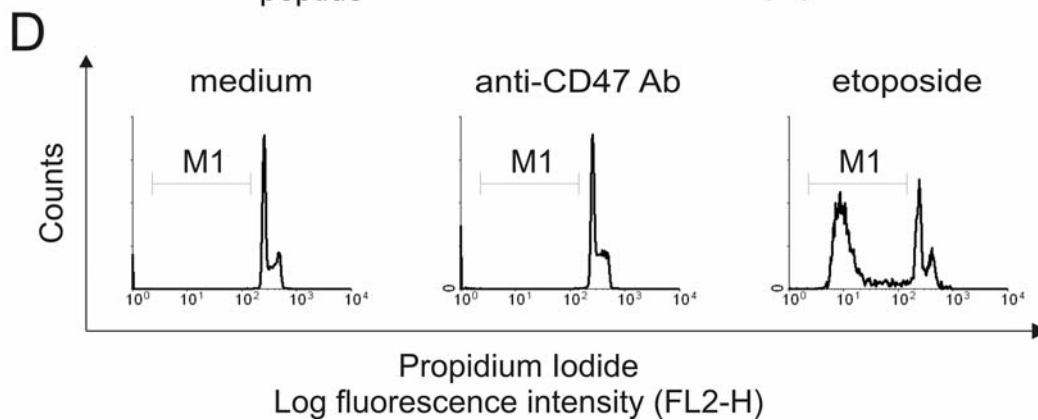
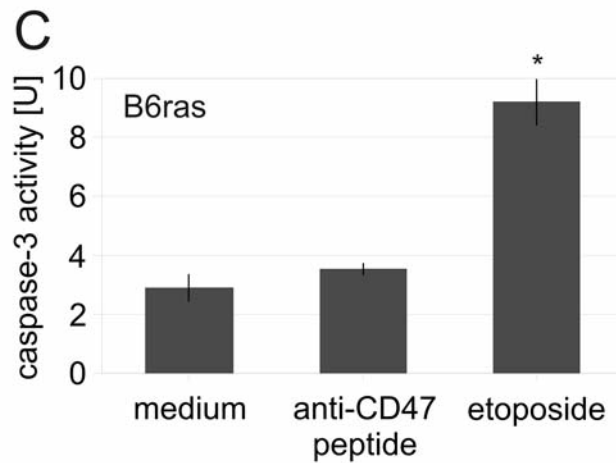
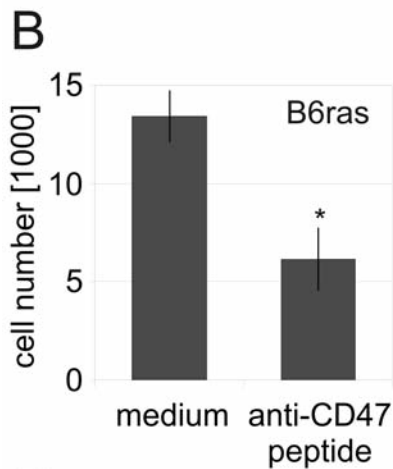
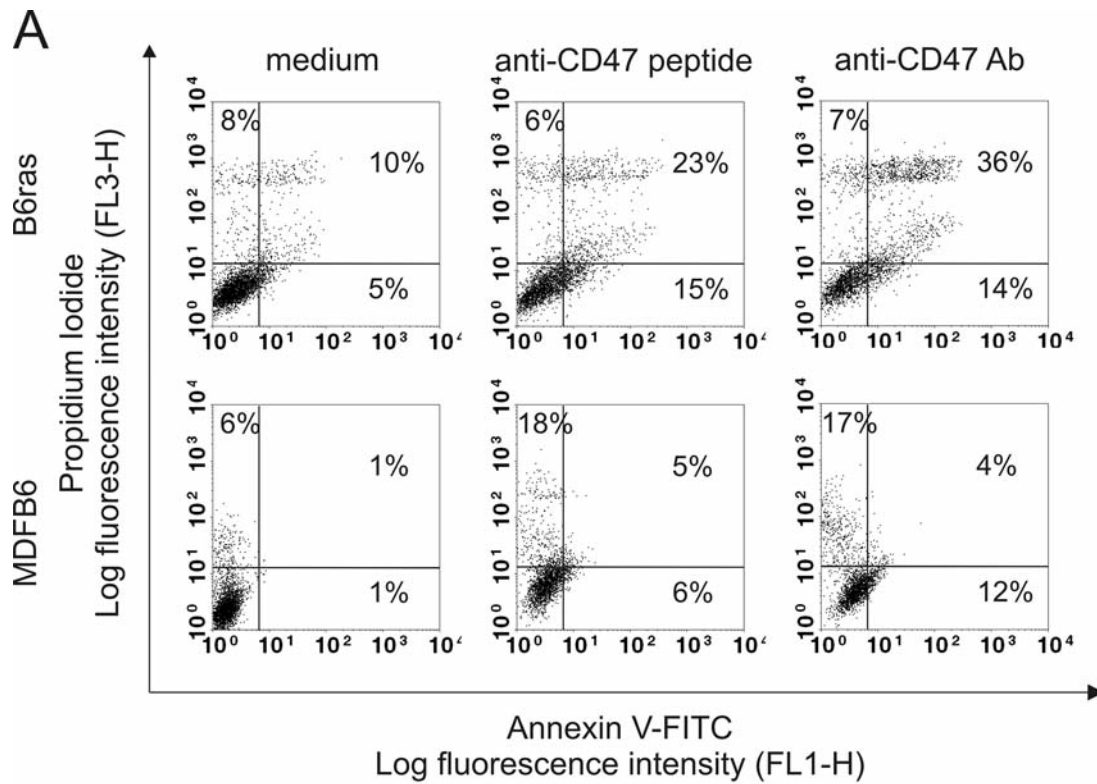
Figure 2. The impact of CD47-directed peptides on RAS-driven tumorigenesis in vivo. (A) Growth of B6ras tumor in vivo with and without treatment with 4N1K peptide. The average tumor volume $\pm$ SD was plotted. The statistical significance of the recorded differences is designated by asterisks: \* $p>0.05$ , \*\* $p>0.005$ , \*\*\* $p>0.0005$  ( $n=10$ ), (inset). The number of Ki67-positive cells in 4N1K-treated and control tumors. An asterisk indicates statistically significant results,  $p>0.001$ . (B) Body weights of tumor-bearing mice receiving 4N1K peptide. The average weight of the tumor-bearing mice showed no indication of treatment-related toxicity, although the body mass did decline with disease progression (average's $\pm$ SD are shown;  $n=10$ ). (C) Volumes of B6ras tumors in vivo without treatment (control), with treatment with 4N1K peptide or peptide 4NGG as for 14 days post-injection. The average tumor volume $\pm$ SD from two independent experiments were plotted (control  $n=25$ , 4N1K  $n=20$ , 4NGG,  $n=5$ ). Asterisks indicate statistically significant difference from the control ( $p<0.002$ ).

antibody selectively increased the reactivity of B6ras cells with Annexin V and markedly reduced their overall numbers (Figure 3AB), both changes consistent with the activation of a programmed cell death pathway. However, the death of B6ras cells exposed to anti-CD47 agents (peptide or antibody) did not exhibit hallmarks of classical apoptosis, as no apparent activation of caspase-3 was detected in the treated cells (Figure 3C) and no sub-G<sub>1</sub> peak was observed in their cell-cycle profile (Figure 3D). Notably, we have previously observed a link between the expression of oncogenic *H-RAS* and up-regulation of BNIP3, which is a regulator of autophagy, a process that may sometimes lead to a programmed cell death. Therefore, we asked whether this process could be triggered by the CD47 ligation. Interestingly, the 4N1K treatment of Bras cells but not of their non-transformed MDFB6 counterparts led to an increase in staining with acridine orange which is a marker of intracellular acidification that accompanies autophagy (Figure 4A). Moreover, punctuate staining for LC3, characteristic for autophagosome formation (Figure 4B) was also detected in *RAS*-transformed cells exposed to 4N1K (23). Thus, our observations suggest that *RAS*-transformed cells become sensitive to induction of autophagy by ligation of the TSP-1 receptor CD47. This is a selective and *RAS*-related process, as such death does not occur in CD47-expressing but non-transformed isogenic MDFB6 cells.

## Discussion

Expression of the *H-RAS* oncogene was linked with profound re-arrangement of virtually every aspect of cell physiology, including gain of angiogenic potential by induction of Vascular endothelial growth factor (VEGF) and inhibition of TSP-1 expression (24-26). Indeed, the increasing appreciation of the multifaceted involvement of oncogenic pathways in the progression of human malignancies has recently fueled considerable interest in developing targeted anticancer therapies (27, 28). In this regard it is recognized that while activated oncogenes, including mutant *RAS*, may impart a selective growth advantage on the affected cancer cells, notably through cell proliferation, survival, angiogenesis, inflammation (26), and other features, this is achieved at the cost of certain inevitable vulnerabilities, commonly described as the process of "oncogene addiction" (29). This term refers to the mechanism by which a withdrawal of the oncogenic signalling, or some of its essential molecular consequences, could lead to a precipitous and selective demise of cancer cells unable to function in a given context.

One aspect of this change that has attracted notable interest is the observation that *RAS*-driven cancer cells rely on the continuous presence of this oncogene to form overt tumors *in vivo* (30, 31). This is believed to be, at least in part,



a consequence of the RAS-dependent stimulation of angiogenesis, as cultured cells can readily withstand withdrawal of RAS signaling, unless exposed to restrictive growth conditions (4, 25, 32). Down-regulation of TSP-1 is believed to be among the key events related to the angiogenic switch in the context of RAS-mediated cellular transformation (33, 34). The anti-angiogenic effects of TSP-1, which are mainly attributed to the activation of the endothelial CD36 receptor, followed by their growth inhibition and apoptotic death (35). These observations have provided a strong rationale for the development of TSP-1-derived CD36-specific peptides as anti-angiogenic agents. It has been reasoned that such agents would restore the anti-angiogenic TSP-1 activity within the tumor mass and thereby achieve anticancer effects in an indirect manner. Surprisingly, the anticancer efficacy of these agents in clinical trials has been relatively modest (36, 37).

In this study we sought to understand the circumstances surrounding the changes in the expression of TSP-1 and its receptors as they occur in cancer cells harboring mutant or activated RAS oncoproteins. First, it is of note that cancer cells may express the two major TSP-1 receptors CD36 and CD47 and may, thereby, be potentially sensitive to the direct influences of their ligands, (beyond angiogenesis) including native TSP-1 and its fragments, peptides, and agonistic antibodies. In our previous studies we have shown that 4N1K peptide selectively impairs the growth of *H-RAS*-expressing cells *in vitro* (19), but the nature and *in vivo* implications of this effect have remained unstudied. Here we describe induction of autophagy and antitumour effect of 4N1K peptide. Autophagy is recognized as a tumor protective process which may off-set the effects of various therapeutics (38, 39). However, under certain circumstances induction of autophagy may lead to cellular demise *via* mechanisms

involving downstream elements of the apoptotic circuitry (40, 41). The latter scenario is consistent with our findings which suggest that CD47 binding results in autophagy, especially in RAS-transformed cells in which there is a concomitant and oncogene-driven up-regulation of BNIP3, one of the key autophagy regulators (19). It is of interest that this mechanism also produced some tumour growth inhibition, which may suggest that in combination with other agents CD47-directed peptides may possess an antitumour activity worthy of clinical exploration. It is tempting to speculate that the positivity for mutant RAS or BNIP3 might serve as an indication that targeting this receptor could lead to selective antitumour cytotoxicity. It is also of interest whether co-expression of endogenous TSP-1 (*e.g.* released from platelets or stromal cells) may influence tumour growth through a dual anti-angiogenic (CD36-mediated) and cytotoxic (CD47-mediated) effects. At the same time we observed that, RAS-transformed cells could be selected against a high expression of TSP-1, which impairs their viability *in vitro* (data not shown). However, the involvement of autophagy in responses of RAS-transformed cells to CD47 stimulation may also be a source of certain therapeutic ambiguity. Autophagy may provide a rescue mechanism to cancer cells under severe metabolic stress conditions (42, 43), a property that could place TSP-1 in a hitherto unexplored role of a modulator of therapeutic resistance. This may be among the reasons for the relatively modest anticancer effect of the 4N1K peptide in our experiments, as larger tumours would likely have been hypoxic and metabolically-deprived. Indeed, the role of TSP-1 and its receptors in direct control of cancer cell behavior require further explorations.

## Conclusion

Overall, our study offers new element in the interactions between cancer cells and their surrounding matrix, including TSP-1. We propose that the loss of TSP-1 expression in RAS-transformed cells could bear a non-appreciated significance (and selective advantage), as a way to remove the growth-restraining effects of TSP-1/CD47 interactions. These findings speak to the role of peri-cellular and paracrine networks that may control oncogene function, oncogene addition, therapeutic responses and tumour-stromal interactions of transformed cells.

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Figure 3. Atypical pattern of cellular death markers of RAS-expressing tumor cells upon CD47 ligation. (A) Differential staining of B6ras and MDFB6 cells with Annexin V and propidium iodide upon treatment with CD47 agonists. Triggering the CD47 receptor resulted in an increase in the percentage of Annexin V-positive (single and double positive populations) cells only in the case of B6ras, but not in their non-transformed MDFB6 counterparts. (B) Reduced cell number in cultures of B6ras cells incubated in the presence of 4N1K peptide. Averages  $\pm$  SD of two independent experiments are shown; asterisks indicate statistically significant results ( $p < 0.02$ ;  $n = 24$ ). Non-transformed MDFB6 cells were insensitive to this treatment (data not shown). (C) Absence of caspase-3 activation in dying B6ras cells exposed to 4N1K peptide. Etoposide was used as a positive control. Average values ( $\pm$  SD) obtained in two independent experiments are shown. Asterisks indicate statistically significant results ( $p < 0.02$ ;  $n = 6$ ). (D) Absence of the apoptotic sub-G<sub>1</sub> peak (M1 mark) in the DNA profile of dying B6ras cells exposed to anti-CD47 antibody. Etoposide induced prominent DNA fragmentation. Representative histograms are shown.

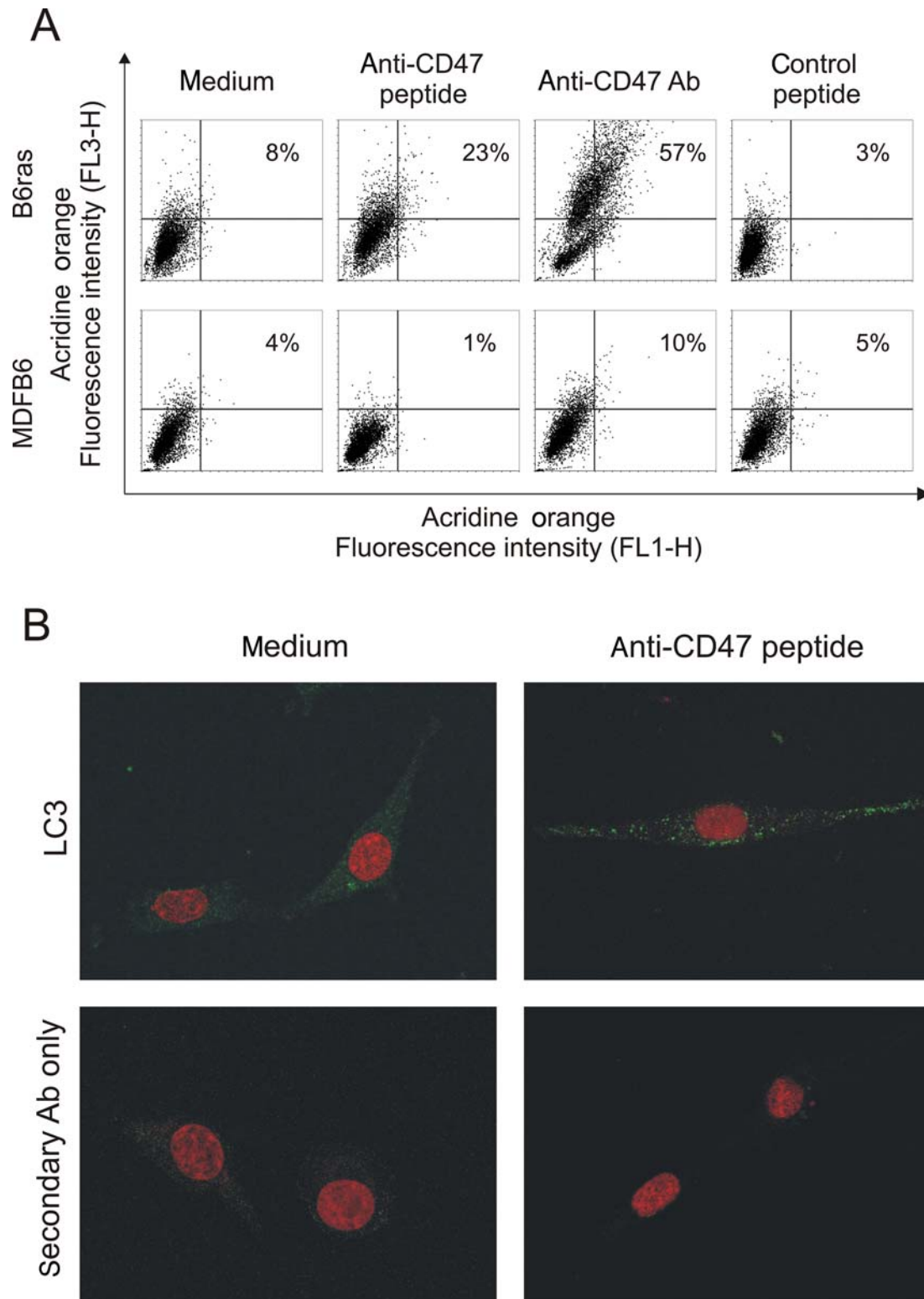


Figure 4. Characteristics of the atypical cell death program induced in RAS-expressing cells by CD47 ligation. A: Increase in acridine orange staining of acidic bodies present in B6ras cells after CD47 triggering. Representative dot-plots are shown B: Punctuate pattern of LC3 protein staining in B6ras cells treated with 4N1K peptide resembles the pattern associated with the formation of autophagosomes. Nuclei were counter-stained with propidium iodide. Representative confocal microscope micrographs are shown.



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