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Rac1 inhibition promotes antitumor activity of statins in bone-seeking breast cancer cells

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ABSTRACT

Background: Bone metastases are the most common site of cancer spread in breast cancer (BC) and are associated with a poor prognosis. Statins elicit antitumor activity *in vitro*, but supraphysiological doses are often required to achieve these effects. Inhibition of the mevalonate pathway by statins prevents the prenylation of small Rho-GTPases including Rac1. Therapeutic approaches to increase statin sensitivity of tumor cells remain scarce. We investigated whether the combined inhibition of the mevalonate pathway and Rac1 potentiates antitumor effects in osteotropic triple-negative BC (TNBC) cells.

Methods: MDA-MET, MDA-BONE, and MDA-MB-231 TNBC cells were exposed to statins and either pharmacological Rac1 inhibitors or Rac1 knockdown via siRNAs. Antitumor effects were quantified by viability and apoptosis analysis, Western blot analyses, and gene expression of tumor-related mediators.

Results: The combined inhibition of the mevalonate pathway by statins and Rac1 via siRNAs induced significant additive antitumor effects in TNBC cell lines. Required concentrations of statins to induce apoptosis and to reduce vitality could be reduced when cells were genetically depleted of Rac1. Triple knockdown of Rac1, RhoA, and CDC42 partially prevented the potentiated antitumor effects by the statin and Rac1-specific siRNA combination.

Conclusion: Genetic inhibition of Rac1 sensitizes osteotropic TNBC cell lines to statin treatment. Our results support a pro-tumorigenic function of Rac1 and indicate a potential benefit of combining Rac1 inhibition with statin treatment in osteotropic TNBC.

1. Introduction

Breast cancer (BC) affects one in eight women during their lifetime resulting in a high clinical and socioeconomic burden [1]. Although advances in diagnostics and therapy have reduced the overall mortality in recent years, BC remains a leading cause of cancer-related death in women and metastases to the bone represent a prognostically relevant complication [2]. Triple-negative BC (TNBC) remains particularly difficult to treat due to the comparatively aggressive nature, higher rates of recurrence and often younger age at primary diagnosis [3].

Bisphosphonates are well-established in clinical practice to treat metastatic bone disease and to prevent bone loss. Modern amino-bisphosphonates exert their action mainly by inhibiting the mevalonate pathway [4]. Another class of mevalonate pathway inhibitors are statins, which are the gold-standard to treat patients with hypercholesterolemia [5]. The mevalonate pathway is responsible for providing isoprenoid precursors for the synthesis of a plethora of products. An aberrant activation of the mevalonate pathway can drive BC initiation and progression [6]. However, studies have provided controversial results regarding the antitumor potential of statins in preclinical and clinical studies in BC [5–9]. Several reasons may account for such discrepancies including supraphysiological concentrations used in most preclinical settings [10], resistance mechanisms [11], and molecular features that differ between the different subtypes [6,8].

Antitumor effects of statins result, in part, from inhibition of isoprenoid synthesis, which is responsible for the prenylation of Rho-

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GTPases such as RhoA, Rac,1, and CDC42 and, thus, their subcellular localization [12,13]. Through a cycle between an inactive guanosine diphosphate (GDP)-bound form and active guanosine diphosphate (GTP)-bound form, Rho-GTPases modulate more than 70 downstream effectors [14]. Overactivation of Rho-GTPases is associated with dysregulation of transcription, cell cycle activation, adhesion, and angiogenesis, thereby fueling tumor progression and metastasis [14]. BC cells show an increased Rac1 activity, especially in recurrent tumors [15]. Rac1-GTP is an independent prognostic marker with both increased risk of recurrence and mortality [16]. In this study, we tested a new approach to sensitize TNBC cells for statins by targeting the Rho-GTPase Rac1.

2. Materials and methods

2.1. Reagents

Statins and Rac1 inhibitors were purchased from Sigma-Aldrich, Hamburg, Germany or SelleckChem, Munich, Germany. Primary antibodies for Western Blots were Rac1 (16118X) and CDC42 (16119X) from Thermo Fisher Scientific, Schwerte, Germany; RhoA (2127) and cleaved PARP (9541) from Cell Signaling, Danvers, USA; and GAPDH (sc-25778) from Santa Cruz, Heidelberg, Germany.

2.2. Cell culture

MDA-MB-231, MDA-BONE, and MDA-MET BC cells were purchased from ATCC (Manassas, VA, USA), acquired from the University of Texas (San Antonio, TX, USA) and kindly provided by Prof. L. Suva (Center for Orthopedic Research, University of Arkansas, AR, USA), respectively [17,18]. All cell lines were maintained in DMEM/Ham's F12 (Gibco Life Technologies, Darmstadt, Germany) supplemented with 10 % fetal bovine serum (FBS; Biochrome, Berlin, Germany) and 1 % penicillin/streptomycin (Gibco Life Technologies) in a humidified atmosphere of 95 % air and 5 % CO_2 at 37 °C.

2.3. Transfection

Transfections using gene-specific small interfering (si)RNAs for Rac1 (HSS184182, HSS184181, HSS184180), RhoA (HSS100654) and CDC42 (HSS190760), all obtained from Thermo Fisher Scientific, were performed as previously described [19].

2.4. Metabolic and apoptosis analyses

Cell viability and caspase 3/7 activity were performed using the CellTiterBlue® and Caspase 3/7 Glo® assays (Promega, Mannheim, Germany). Crystal violet staining was used for cell counts as previously described [11].

2.5. Clonogenic assay

Transfected cells were scraped after 24 h (h) and seeded at a cell number of 250 cells per well in 6-well plates and treated with statins immediately afterwards for 48 h. After 13 days, the colonies were quantified and quantified using crystal violet staining [20].

2.6. RNA isolation and quantitative real-time-PCR

Gene expression was analyzed using a protocol described previously [20]. The primer sequences (Sigma-Aldrich) for human genes were as following: GAPDH (glyceraldehyde 3-phosphate-dehydrogenase): AGCCACATCGCTCAGACAC, GCCCAATACGACCAAATCC; BCL-2 (B cell lymphoma 2): TGTGTGTGGAGAGCGTCAAC, GACAGCCAGGAGAAATCAAAC, SVV (Survivin): GAACTGGCCCTTCTTGGAG, AAGTCTGGCTCGTTCTCAGTG, RAC1 (Ras-related C3 botulinum toxin substrate 1): AATCTGGGCTTATGGGATACAG, ATGGGAGTGTTGGGACAGTG; RHOA (Ras homolog gene family, member A): GGAGTGTTCAGCAAAGACCA, CAAGACAAGGCACCCAGATT; and CDC42 (cell division cycle 42): ACGACCGCTGAGTTATCCAC, CCCAACAAGCAAGAAAGGAG.

2.7. Subcellular protein fragmentation

Cells were treated as described above. For the subcellular protein fragmentation, a commercially available fractionation kit was used (Pierce, Thermo Fisher Scientific) according to the manufacturer's instructions. Eluates were subsequently analyzed by Western blot.

2.8. Pulldown assay of activated (GTP-bound) CDC42, Rac1 and RhoA

For the analysis of the GTP-bound forms of CDC42, Rac1 and RhoA, a commercially available pulldown assay was used (Pierce, Thermo Fisher Scientific) according to the manufacturer's instructions and as previously described [21]. GTP-bound forms of RhoA, CDC42, and Rac1 were analyzed by Western Blot.

2.9. Western Blot

Cells were washed with phosphate-buffered saline (PBS) and total protein was isolated using sodium dodecyl sulfate (SDS)-based lysis buffer (20 mM Tris/HCl pH 7.4; 1 % SDS; protease inhibitor cocktail (Roche)). After quantifying protein contents by a commercially available BCA protein assay kit (Thermo Fisher Scientific, Schwerte, Deutschland), 20 µg of protein were separated using a protocol as described earlier [20].

2.10. Software and statistical analysis

Figures are presented as mean ± standard deviation (SD) of at least three independent experiments. Outliers were determined via Grubb's test. A student's t-test was used to perform single group comparisons and group analysis was performed using one-way analysis of variance (ANOVA) by GraphPad Prism 6.0 (GraphPad, La Jolla, CA, USA). p-values <0.05 were considered statistically significant.

3. Results

3.1. Individual and combined antitumor effects of statins and Rac1 inhibitors in TNBC cells

We first analyzed individual effects of statins and Rac1 inhibitors on triple-negative MDA-MB-231 cells (Fig. 1.) Here, simvastatin (SIM), and rosuvastatin (ROSU) significantly reduced viability (up to - 50 %; $p \leq 0.001$; Fig. 1A). Comparable results were achieved in MDA-MET and MDA-BONE cells (Fig. 1B). These results show that high concentrations of statins induce dose-dependent antitumor effects in TNBC cell lines with high osteotropic properties. For the pharmacological Rac1 inhibition, EHop-016 (EHop), NSC23766 (NSC), and EHT1864 (EHT), showed a significant reduction in viability by up 70 % (Fig. 1C–E; $p \leq 0.01$). As statins inhibit the mevalonate pathway and Rac1 activation depends on prenylation [22], we asked whether a dual inhibition of Rac1 and the mevalonate pathway would lead to potentiated effects. However, none of the combinations using statins and Rac1 inhibitors were able to potentiate the individual antitumor effects of statins in MDA-MB-231 cells (Fig. 1F).

3.2. Knockdown of Rac1 potentiates statin-induced antitumor effects in TNBC cells

Interestingly, the combination of statins with siRNA-mediated suppression of Rac1 led to a significant additive reduction in viability (up to

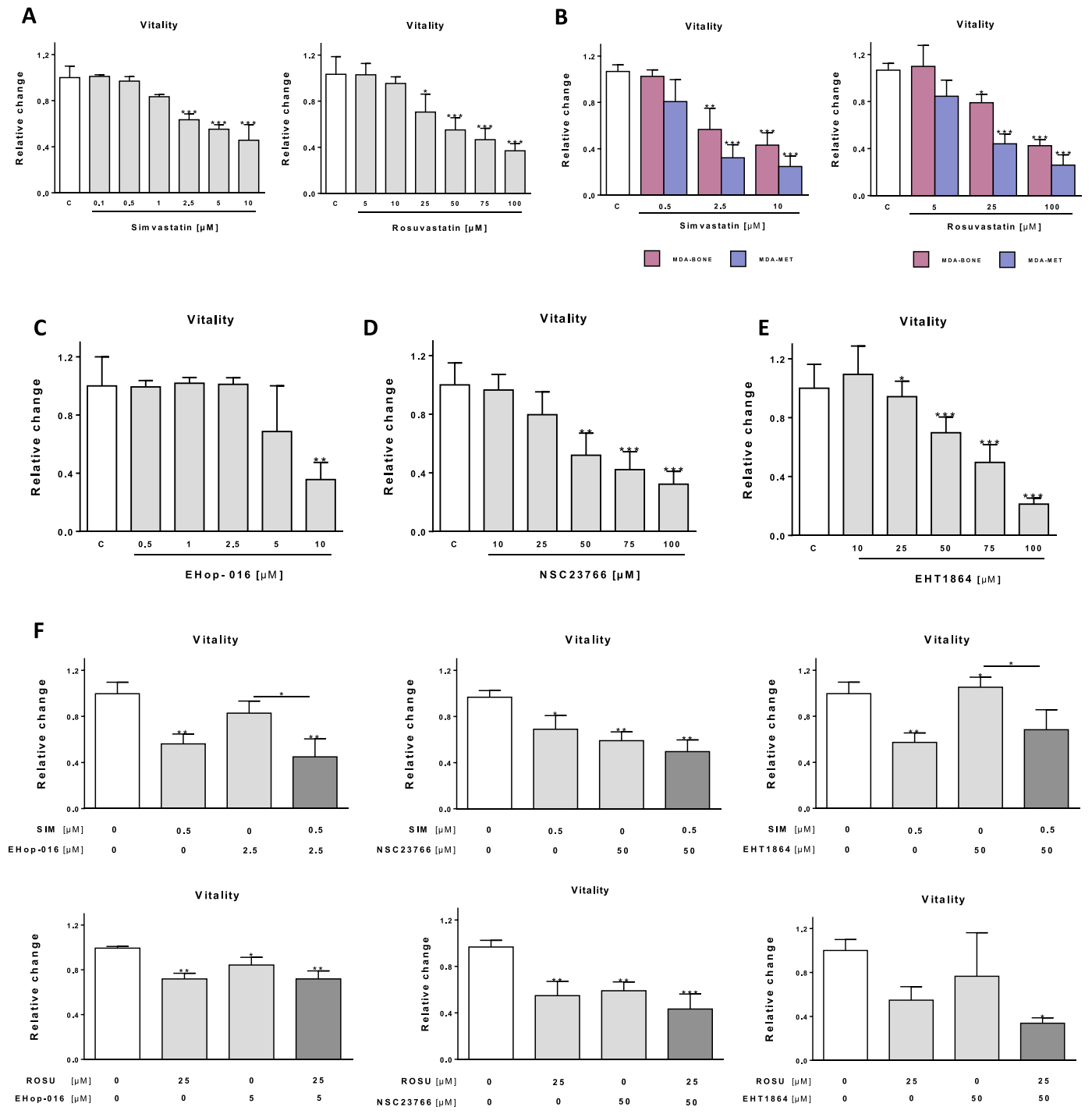

Fig. 1. Pharmacological inhibition of Rac1 does not potentiate the antitumor effects of statins in triple-negative breast cancer cells. MDA-MB-231(**A**), MDA-MET (**B**), and MDA-BONE (**B**) cells were treated with increasing concentrations of simvastatin (SIM) or rosuvastatin (ROSU) **(A)** for 48 h (h). MDA-MB-231 cells were treated with increasing concentrations of EHop-016 (EHop) **(C)**, NSC23766 (NSC) **(D)** and EHT1864 (EHT) **(E)** for 48 h. Vitality in all experiments was quantified using Cell Titer Blue assay. Individual combinations of statins and Rac1 inhibitors were assessed (**F**). Dimethyl sulfoxide (DMSO) was used as a control (C). Data are shown as mean ± standard deviation of at least three independent experiments (*$p \leq 0.05$; **$p \leq 0.01$; ***$p \leq 0.001$). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

– 50 %; $p \leq 0.001$), as well as in the clonogenic potential of MDA-MB-231 cells (Fig. 2A–E). To analyze whether siRNA and statin treatment would modulate the subcellular localization of Rac1, subcellular fragmentation was performed. Here, SIM treatment reduced total, membrane-bound and nuclear expression of Rac1, whereas cytosolic fractions were increased (Fig. 2F). Combinations of SIM with Rac1-specific siRNA further reduced Rac1 protein in all compartments. In addition, pulldown analyses revealed that activated, GTP-bound Rac1 is only absent upon the combination of SIM and Rac-specific siRNA (Fig. 2G). The combinatory approach of statins and Rac1 knockdown significantly induced apoptosis measured by increase of caspase 3/7 activity ($p \leq 0.001$; Fig. 3A), accumulation of cleaved PARP (cPARP) (Fig. 3B), and significant suppression of the anti-apoptotic genes B cell lymphoma 2 (*BCL-2*) and survivin (*SVV*), compared to individual

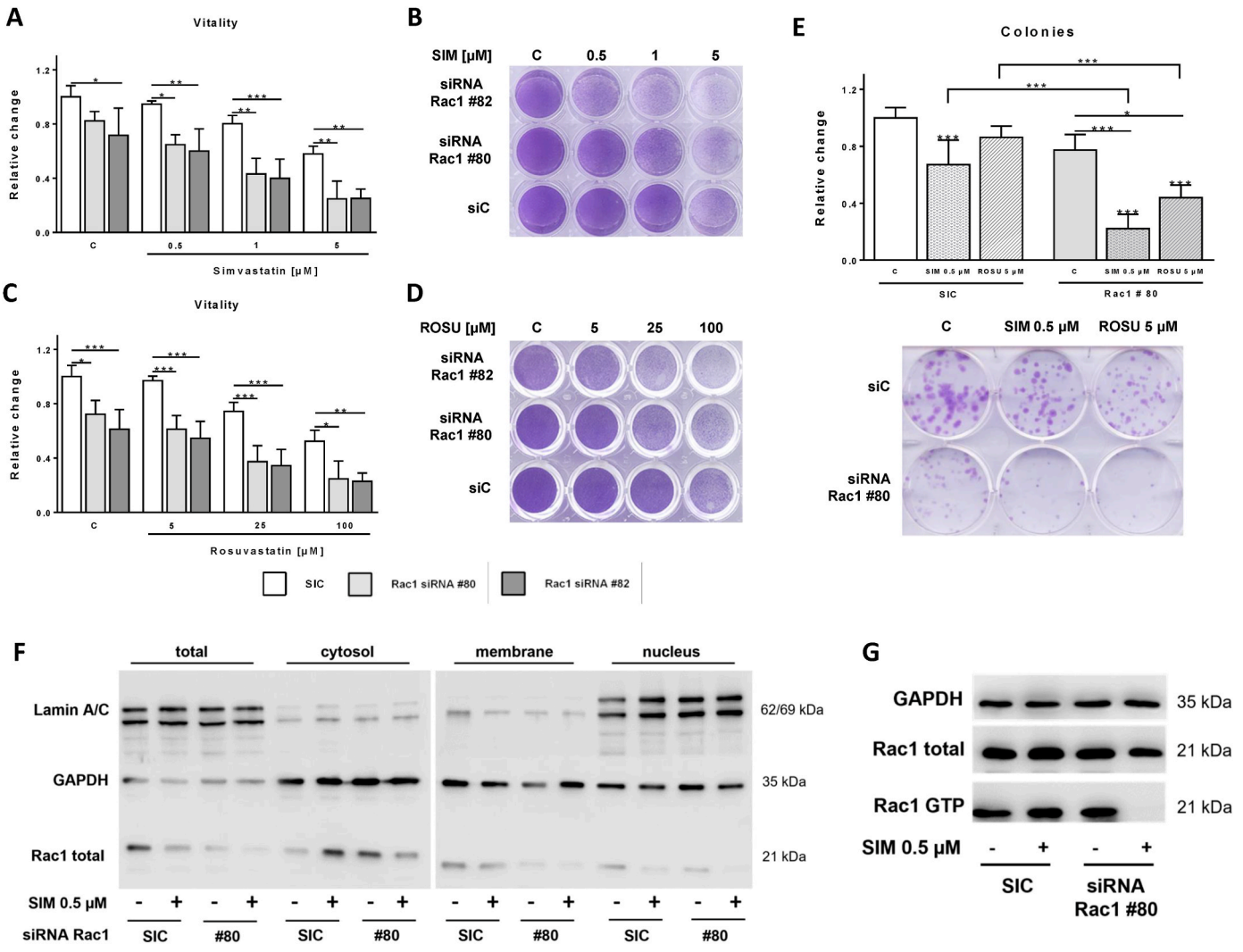

Fig. 2. Genetic knockdown of Rac1 potentiates individual statin-induced antitumor effects in triple-negative MDA-MB-231 BC cells. Knockdown of Rac1 was induced using two siRNAs (#80 and #82) in MDA-MB-231 for 6 h, which were subsequently treated with SIM **(A-B)** or ROSU **(C–D)** for 48 h. **(A–D)** Viability was assessed by Cell Titer Blue assay and cells stained by crystal violet staining. 250 MDA-MB-231 cells/well were treated with 0.5 µM SIM or 5 µM ROSU for 48 h 6 h after knockdown of Rac1. The clonogenic potential was determined after 13 days **(E)** Colonies were quantified by crystal violet staining and cell numbers were calculated. Dimethyl sulfoxide (DMSO) was used as a control (C). Data are shown as mean ± standard deviation of at least three independent experiments (*$p \leq 0.05$; **$p \leq 0.01$; ***$p \leq 0.001$). Subcellular protein fragmentation was performed 48 h after combined treatment with SIM and Rac1-specific siRNA #80. Western blots show subcellular localization of Rac1 in MDA-MB-231 cells (**F**). Total and GTP-bound Rac1 protein was eluted after 72 h by pulldown assay and analyzed in subsequent Western blot (**G**). Representative Western Blots are shown. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

treatments (Fig. 3C). The combinatory effects on cell vitality were confirmed in MDA-MET and MDA-BONE cells, respectively (Fig. 3D). In conclusion, the combination of statins with siRNA-mediated knockdown of Rac1 potentiated individual antitumor effects in triple-negative BC cells.

3.3. Compensatory effects of RhoA and CDC42 knockdown on antitumor effects upon dual inhibition of Rac1 and the mevalonate pathway

Previous data from our group have shown that the Rho-GTPases RhoA and CDC42 accumulate in their GTP-bound form upon statin treatment whereas GTP-bound Rac1 is reduced [19]. We therefore hypothesized that the additional knockdown of RhoA and CDC42 would attenuate the potentiated antitumor effects by the combination of Rac1 knockdown and statin treatment. First, we confirmed a significant potentiation of statin-mediated loss of vitality and induction of apoptosis by Rac1-specific siRNA in MDA-MB-231 cells ($p \leq 0.001$; Fig. 4A+B). We observed a tendency of reduced statin-induced apoptosis by the individual knockdown of RhoA or CDC42 (Fig. 4A + B). Of note, triple-knockdown of all Rho-GTPases partially prevented the antitumor effects that were induced by the individual combination of Rac1 knockdown and SIM ($p \leq 0.01$; Fig. 4A). Comparable results were achieved by the triple-knockdown of all Rho-GTPases upon ROSU treatment ($p \leq 0.001$; Fig. 4B). These results point to a role of RhoA and CDC42 in the potentiation of the antitumor effects observed by combining Rac1 knockdown and statin treatment.

4. Discussion

Although the prognosis of BC has improved, new therapeutic strategies for advanced BC, especially TNBC, are necessary [23]. This work, as well as previous studies, shows significant antitumor effects of statin treatment in TNBC cells, but at physiologically inaccessible doses [19,24,25]. Some clinical studies on the potential of statins in BC have demonstrated risk reduction of disease recurrence and mortality, whereas other investigations failed to confirm these observations [5,8,

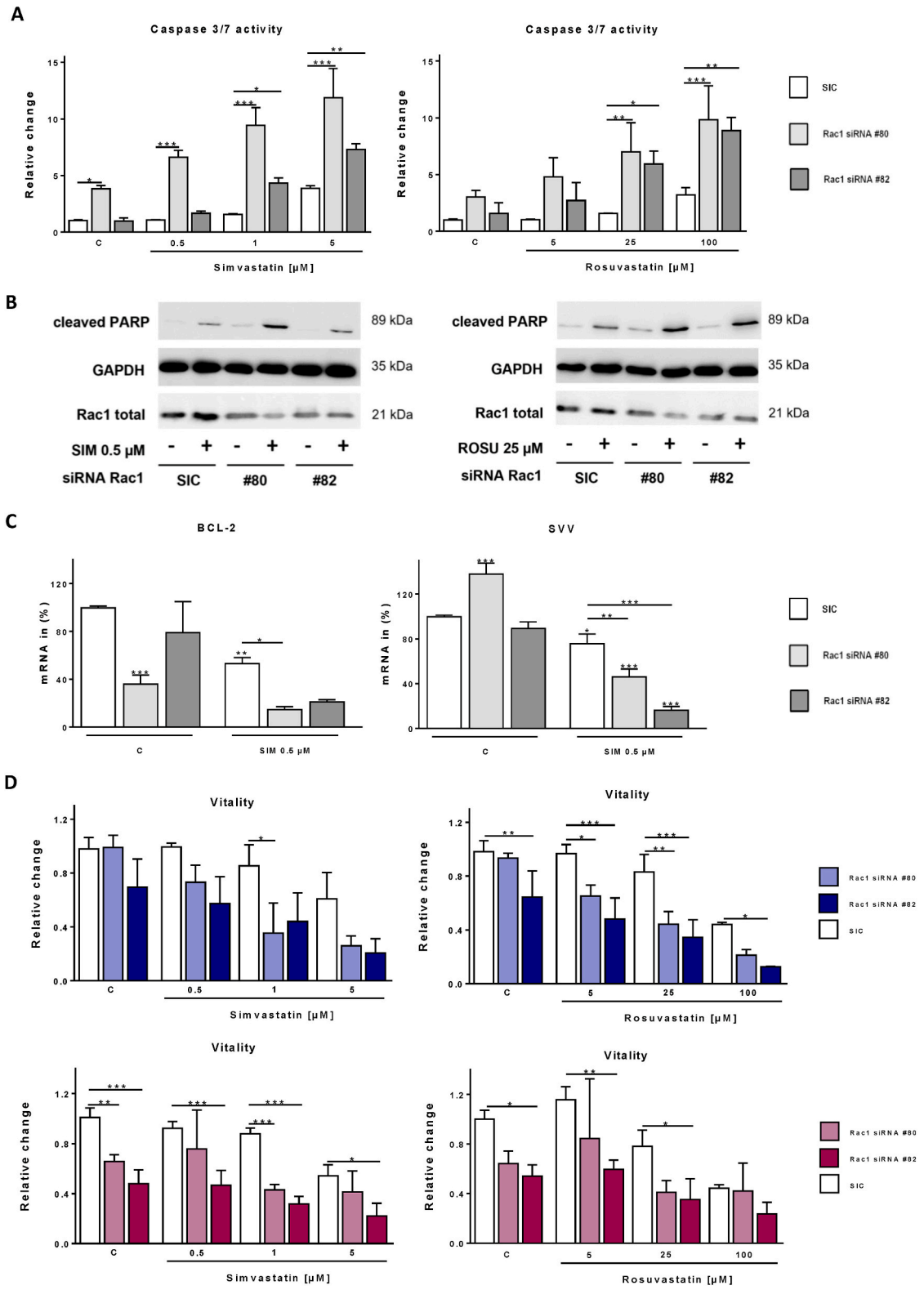

Fig. 3. Genetic knockdown of Rac1 potentiates individual statin-induced antitumor effects in triple-negative MDA-MB-231, MDA-MET, and MDA-BONE BC cells. Knockdown of Rac1 was performed by two siRNAs (#80 and #82) in MDA-MB-231 cells for 6 h. Cells were treated with SIM or ROSU for 48 h, and apoptosis detected by measuring caspases 3/7 activity **(A)** and detection of cleaved poly-ADP-ribose-polymerase (cPARP) **(B)**. Expression of the anti-apoptotic genes B-cell lymphoma 2 (BCL-2) and survivin (SVV) was analyzed by real-time PCR (**C**). Combinatory approaches using statins and Rac1-specific siRNAs were conducted in MDA-MET (blue) and MDA-BONE (red) cells, respectively (**D**). Vitality in all experiments was quantified using Cell Titer Blue assay. Dimethyl sulfoxide (DMSO) was used as a control (C). Data are shown as mean ± standard deviation of at least three independent experiments (*p ≤ 0.05; **p ≤ 0.01; ***p ≤ 0.001). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

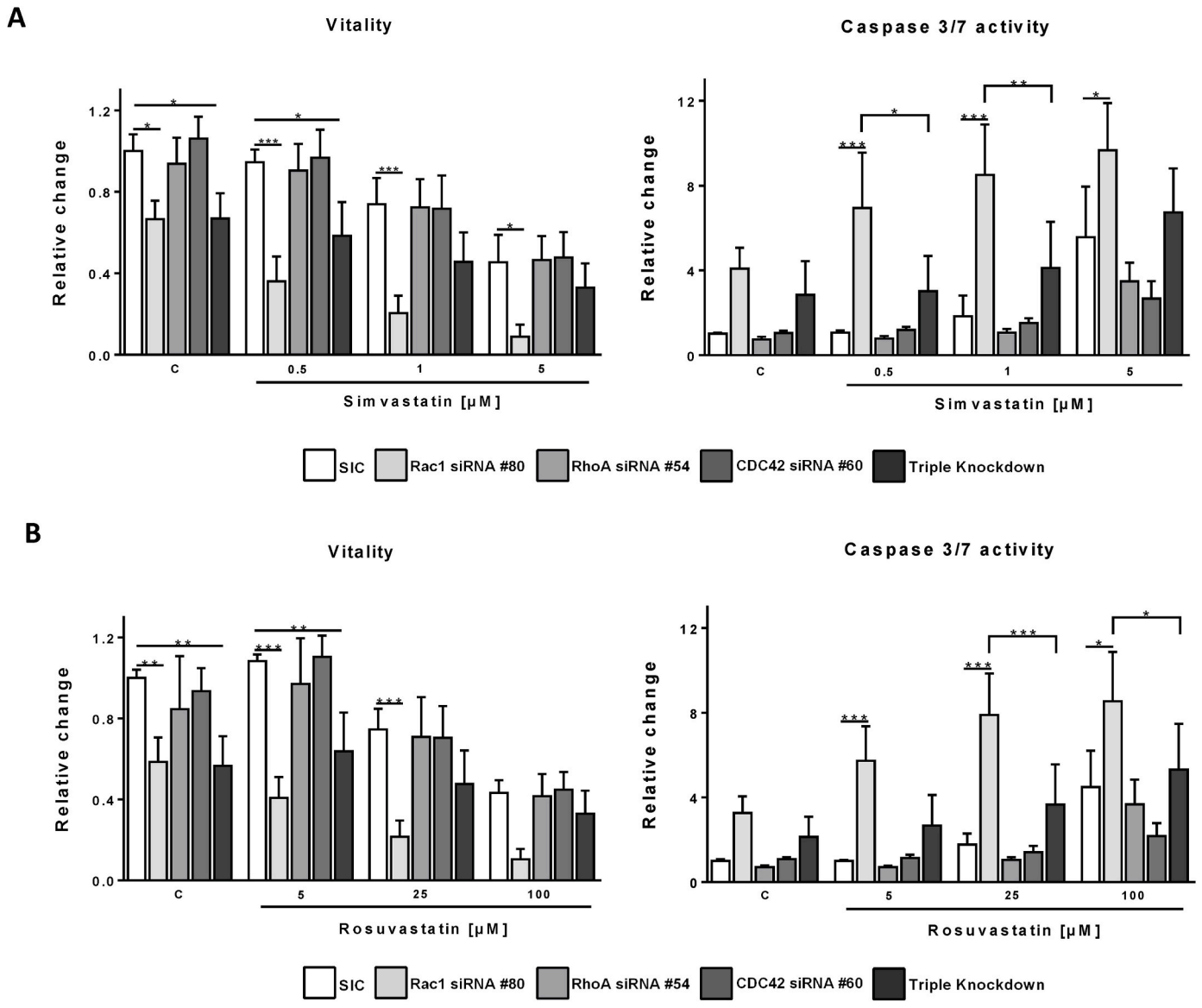

Fig. 4. Compensatory effects of Rho GTPases RhoA and CDC42 on antitumor effects of combined inhibition of Rac1 and statin treatment. Individual or triple knockdown of Rac1, RhoA, CDC42 was performed in MDA-MB-231 cells for 6 h. Cells were then treated with SIM **(A)** or ROSU **(B)** for 48 h. Viability was assessed by Cell Titer Blue assay, and apoptosis was detected by measuring caspases 3/7 activity. Dimethyl sulfoxide (DMSO) was used as a control (C). Data are shown as mean ± standard deviation of at least three independent experiments (*p ≤ 0.05; **p ≤ 0.01; ***p ≤ 0.001).

26,27]. The contradictory results may originate from a heterogeneous sensitivity of tumors to specific statins [11,28], resistance mechanisms and pharmacodynamic aspects such as their tissue accumulation [29]. Statins are inhibitors of the mevalonate pathway that is crucial for the prenylation of a wide spectrum of proteins, including Rho-GTPases. Several studies have demonstrated that the Rho-GTPase Rac1 plays a central role in BC tumorigenesis, however, the specific targeting of Rho-GTPases in cancer including BC remains challenging [15,30–33].

Here, we successfully demonstrate that siRNA-mediated knockdown of Rac1 potentiates the individual antitumor effects of statins in TNBC cells, whereas pharmacological Rac1 inhibitors failed to induce similar effects. SiRNAs decrease the available amount of mRNA resulting in reduced protein production, whereas pharmacological inhibitors block the catalytical activity of the protein at different levels depending on their binding affinity and inhibition kinetics. Thus, while some inhibitors may result in a lack of catalytic activity, Rac1 may still interact with some binding partners or assemble into macromolecular complexes, further impairing cellular uptake and resulting in serious off-target effects [34]. Future studies need to compare the potential of Rac1 inhibitors vs. siRNA on Rac1 GTP-loading, subcellular localization, regulation, and downstream effector interaction such as Rho-GDP-dissociation inhibitors (GDIs), the most important regulator proteins of Rac1 [35,36]. Moreover, the role of different Rac1 mutations in BC regarding their statin-sensitizing potential requires evaluation. Additional Rac1 inhibitors need to be tested for their statin sensitization potential [15].

In our study, we observed less membrane-bound and nuclear Rac1 after Rac1 knockdown and statin treatment. This is in line with the loss of membrane binding by isoprenoids, which is required for Rac1 enzymatic activity. Isoprenylation is necessary for functional subcellular Rac1 localization [13]. We hypothesize that statins reduce Rac1 activity by loss of prenylation, thereby influencing the subcellular localization of Rac1 rather than affecting its loading with GTP. As Rac1-GTP is still detectable after 0.5 µM simvastatin treatment, it might still be functional, accounting for the low antitumor effects by low statin concentrations. Altered subcellular localization of Rac1 might diminish the

potential of pharmacological Rac1 inhibitors to block its activity. The discrepant effects of pharmacological Rac1 inhibitors and siRNA may therefore be also due to differences in the subcellular localization of Rac1 and other Rho GTPases, to a varying uptake of the inhibitors into the cells or due to differential effects on Rac1 mutations.

Previous studies demonstrated that RhoA and CDC42, in contrast to Rac1, accumulate in their GTP-bound form upon statin treatment and, at least partially, mediate their antitumor effects in BC cells [21]. Notably, triple knockdown of RhoA, CDC42 and Rac1 resulted in a reduction of the sensitization to statins achieved by the single knock-down of Rac1. These findings are in line with other studies where statins caused an increase of GTP-loading of Rac1 and RhoA with reversed antitumor effects after their knockdown [37,38]. These effects also suggest that these Rho-GTPases might have complementary functions. Additional studies describe pro-apoptotic effects of RhoA and CDC42 in murine fibroblasts, in cardiomyocytes, and Jurkat T cells [39–41]. To comprehensively determine the role of Rac1, CDC42, and RhoA in the complexity of statin antitumor effects, further investigations need to unravel their precise dynamics of activation, subcellular localization and interactions with downstream signaling pathways, especially upon statin treatment [42].

Our study has potential limitations. First, the concentrations of statins used *in vitro* cannot be achieved physiologically. Maximum serum concentrations of 31 nmol/l are clinically achievable upon a therapy with simvastatin of 40 mg, whereby only the free fraction of the 96 % protein-bound statins is pharmacologically active [43]. Moreover, our study was performed in three TNBC cell lines only and needs validation in additional cell lines of human BC, and appropriate *in vivo* models of primary and bone-metastatic BC, especially as persistent tumor cells show a reduced sensitivity to primary therapies. Such models would also allow a better understanding of the importance of tumor microenvironment.

5. Conclusion

Our study implies that genetic suppression of Rac1, but not pharmacological inhibition of its GTPase activity, is an approach to potentiate the antitumor effects of statins in TNBC cell lines. A variety of concepts are currently being developed to apply siRNA molecules directly into tumor tissue or to deliver them into tumor cells via liposomes or plasmids [44]. Since our work shows that genetic depletion of Rac1 by siRNA sensitizes BC cells to statins, establishing local Rac1 siRNA treatment may be a future way to sensitize cancer cells to statins. Potentiation of statin antitumor activity depends at least in part on the two GTPases CDC42 and RhoA. Future studies concerning the subcellular localization, activity, and interaction with additional signaling mediators of Rho-GTPases upon statin treatment may improve our understanding of the underlying mechanisms.

CRediT authorship contribution statement

Romy M. Anger: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Project administration, Resources, Software, Validation, Visualization, Writing – original draft, Writing – review & editing. **Andy Göbel:** Conceptualization, Formal analysis, Funding acquisition, Project administration, Supervision, Writing – original draft, Writing – review & editing. **Lorenz C. Hofbauer:** Conceptualization, Supervision, Writing – original draft, Writing – review & editing, Funding acquisition. **Tilman D. Rachner:** Conceptualization, Data curation, Funding acquisition, Investigation, Project administration, Supervision, Writing – original draft, Writing – review & editing.

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Declaration of competing interest

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