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Cortisol-induced SRSF3 expression promotes GR splicing, RACK1 expression and breast cancer cells migration

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ABSTRACT

Recent data have demonstrated that triple negative breast cancer (TNBC) with high glucocorticoid receptor (GR) expression are associated to therapy resistance and increased mortality. Given that GR alternative splicing generates mainly GRα, responsible of glucocorticoids action, we investigated its role in the regulation of RACK1 (Receptor for Activated C Kinase 1), a scaffolding protein with a GRE (Glucocorticoid Response Element) site on its promoter and involved in breast cancer cells migration and invasion. We provide the first evidence that GRα transcriptionally regulates RACK1 by a mechanism connected to SRSF3 splicing factor, which promotes GRα, essential for RACK1 transcriptional regulation and consequently for cells migration. We also establish that this mechanism can be positively regulated by cortisol. Hence, our data elucidate RACK1 transcriptional regulation and demonstrate that SRSF3 involvement in cells migration implies its role in controlling different pathways thus highlighting that new players have to be considered in GR-positive TNBC.

1. Introduction

Triple-negative breast cancer (TNBC) lacks effective targeted therapies and consequently cytotoxic chemotherapy offers the only systemic treatment option. Approximately 25% of invasive TNBCs are glucocorticoid receptor (GR)-positive. Previous reports have found a significant association between high tumor GR expression and shortened relapse-free survival (RFS), suggesting that GR-mediated regulation of gene expression contributes to chemotherapy resistance [1–6]. It was found that physiological concentrations of glucocorticoids (GCs) decrease TNBC sensitivity to chemotherapy both *in vitro* and *in vivo* [7,8], suggesting that GR activation in TNBCs may contribute to chemotherapy resistance in tumor cells following GR activation by endogenous cortisol. Indeed, it was demonstrated that GR antagonism by mifepristone can counteract these effects of GC activation and increase paclitaxel cytotoxicity both *in vitro* and *in vivo* [5]. To identify GR-regulated genes, two chemically distinct GR antagonists, mifepristone and CORT108297 were used to perform global gene expression and GR ChIP-sequencing in MDA-MB-231cells, the TNBC cell line showing the highest GR expression, particularly GRα isoform, which mediates most of the known glucocorticoid actions [1,4,9]. The resulting subset of GR targeted genes were aberrantly expressed in TNBC patients and were associated with unfavorable clinical outcomes thus demonstrating how GR activity signature could be useful for patient stratification [6,4,9]. A deep mRNA sequencing of the transcriptional and post-transcriptional profiling of TNBC, Non-TNBC and HER2-positive breast cancers also allowed elucidating several modulators, including RACK1 [10] which, was involved in proliferation and invasion/metastasis both *in vitro* and *in vivo* and suggested as a predictor factor of poor outcome [11–13]. In addition, altered RACK1 expression was also reported in melanoma, non-small cell lung cancer and hepatocellular carcinoma [14–17].

RACK1 is a 36-kDa protein, recognized as a key element in multiple signalling pathways, partnering with proteins such as the oncoprotein Src [18–20], protein phosphatase 2A (PP2A) [21,22] and focal adhesion kinase (FAK) [23,24] thus playing a critical role in cancer cell migration and invasion [25]. Therefore, a deeper understanding of RACK1 transcriptional regulation is becoming of pivotal interest considering that RACK1 could be a GR target gene. Indeed, bioinformatics analysis of RACK1 gene promoter demonstrated the presence of a functional glucocorticoid responsive element (GRE) consensus sequence [26,27]. In

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THP-1 cells, we conducted a detailed analysis of GRE role in RACK1 promoter, demonstrating that cortisol and synthetic corticosteroids can target RACK1 by acting at transcriptional level [28–31]. Recently we also demonstrated that cortisol treatment in THP-1 cells induced a significant increase of GRα isoform [28]. Our data also demonstrated that cortisol-mediated GR splicing towards the α isoform was related to the up-regulation of SRSF3 (Serine/Arginine-Rich Splicing Factor 3 also known as SRp20), a splicing protein which was previously involved in GR splicing thus demonstrating that cortisol effect is also driven by modulation of GR splicing [32,33].

The purpose of this work was to investigate RACK1 transcriptional regulation in MDA-MB-231, a highly aggressive, invasive and poorly differentiated TNBC cell line characterized by a significant increase of GR expression compared to other similar cell lines [4,34] and consequently used as a model to investigate GR-positive TNBC. In fact, it was reported that MDA-MB-231 cells were used to identify gene expression profiles induced by dexamethasone (Dex), commonly associated with chemotherapy. This analysis demonstrated a striking association between Dex up- and down-regulated genes in MDA-MB-231 cells and in patients with invasive ductal breast carcinoma [9]. The same cell line was also used to study GR antagonism effects by mifepristone treatments [4,35–37] and differentially expressed genes forming GR activity signature in MDA-MB-231 cells were validated in two independent ER-negative BC cohorts [6].

To understand whether and how RACK1 could be a GR target gene in MDA-MB-231, we focused on the role of cortisol in the regulation of RACK1 expression and the consequent effect on cell migration. To elucidate cortisol mechanism of action, we investigated whether it was able to modulate SRSF3 expression and direct GR splicing towards GRα. Our work establishes that RACK1 is a GR target gene through a mechanism involving SRSF3 and can be positively regulated by cortisol. We have demonstrated that SRSF3-induced expression promotes GR splicing toward GRα, which in turn, up-regulated RACK1 determining a significant increase of MDA-MB-231 cells migration which is prevented by RACK1 or SRSF3 silencing. Therefore, our results not only confirm literature data about SRSF3 and RACK1 role in cell migration but also elucidate RACK1 transcriptional regulation and how this mechanism seems to be correlated with cell migration.

2. Materials and methods

2.1. Chemicals

Cortisol (PubChem CID:5754), mifepristone (PubChem CID:55245) and G418 were obtained from Sigma Aldrich (St. Louis, MO, USA). They were dissolved in DMSO at concentration of 1 mM and 10 mM and frozen (−20 °C) in stock aliquots. Stock aliquots were diluted at a final concentration in culture media at the time of use (final concentration of DMSO in culture medium < 0.1%). Cell culture media and all supplements were from Sigma Aldrich. Mouse monoclonal antibodies anti-RACK1 (sc-17754) and anti-GRα (sc-393232), anti-GAPDH (sc-32233) were obtained from Santa Cruz Biotechnology (Dallas, TX, USA). Mouse monoclonal antibodies anti α-tubulin and anti-SRP20 [SRSF3] (MABE 116) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Host specific peroxidase conjugated IgG secondary antibody (31,460) was purchased from Thermo Scientific (Waltham, MA, USA). All reagents were purchased at the highest purity available. Electrophoresis reagents were purchased from Bio-Rad (Richmond, CA, USA).

2.2. Cell culture and treatments

The TNBC cell line MDA-MB-231(ATCC®) was maintained at 37 °C in 5% CO_2 and cultured in DMEM medium supplemented with 10% heat inactivated FBS, 2 mM L-glutamine and 2 mM penicillin/streptomycin. In line with our previous results, cells were treated for 6 h in a medium containing vehicle or 0,1 μM cortisol [32]. To demonstrate the role of glucocorticoid receptor, cells were pretreated for 1 h with 30 μM mifepristone before addition of cortisol and for 7 h with mifepristone alone [37]. Other specific details of times and concentrations are given in figure legends.

2.3. Construction of GR minigene and generation of a stable MDA-MB-231 cell line (GRmini-MDA231)

GR minigene was obtained as described in Ref. [38]. Briefly, human genomic DNA was isolated from peripheral blood using Wizard® Genomic DNA Purification Kit (Promega, Madison, WI). The GR genomic region including exon 8, intron H exon 9α, intron J and exon 9β was amplified by polymerase chain reaction (PCR) using Phusion High-Fidelity DNA Polymerase (F530S -Thermo Fisher Scientific, Rockford, IL, USA) with specific primers located in exon 8 (GR forward including a BamHI site F: 5′-CGGGATCCAGGACGGTCTGAAGAGCCAA GAGCTATTTG-3′) and exon 9β (GR reverse including XhoI site R: 5′−CCGCTCGAGCCCAGAGCTCATCCCATGCTAATTATCCAG -3′) as indicated in Ref. [38]. PCR fragment was inserted into pcDNA3 vector (Invitrogen, USA) between BamHI and XhoI sites to generate GR minigene eukaryotic expression vector (pcDNA3-GR). The construct was sequenced by BMR Genomics (Padova, Italy) and subsequently MDA-MB-231 cells were transfected with pcDNA3-GR vector by using Lipofectamine 2000 following manufacturer's instructions. Stable clones obtained by limiting dilution in medium with 1 mg/ml G418 were tested for GR minigene expression by Reverse Transcriptase-PCR (RT-PCR).

2.4. Plasmid DNA preparation, transient transfections and luciferase assays

Plasmids for transfections were purified with the HiSpeed® Plasmid Midi Kit (Qiagen, Valencia, CA). DNA was quantified by Quantus™ Fluorometer (Promega, Madison, WI).

Transient transfections for luciferase assay were performed in 6 multi well culture plates; for each well $2{,}5 \times 10^5$ cells were seeded in DMEM medium without phenol red. Transfections were carried out using Lipofectamine 2000 following manufacturer's instructions. Each luciferase-reporter construct plasmid DNA was co-transfected with pRL-TK Renilla luciferase expressing vector to measure transfection efficiency (Promega, Madison, WI). During transfection MDA-MD-231 cells were incubated at 37 °C in 5% CO_2 and then treated with 0.1 μM cortisol for 6 h. Cells were lysed with Passive Lysis Buffer provide by Dual-Luciferase Reporter Assay System following manufacturer's specifications (Promega, Madison, WI). The luminescence was measured with a 20/20n Luminometer with 10 s of integration (Turner BioSystems, Sunnyvale, CA).

2.5. RT-PCR and real-time PCR

To analyze mRNA expression of our interest genes and GR minigene, 2×10^6 cells were plated in a Petri dish 60 mm. Total RNA was extracted using RNeasy Plus Mini Kit (Qiagen, Valencia, CA, USA) following manufacturer's instructions. RNA quantification was obtained by Quantus™ Fluorometer (Promega, Madison, WI). Following manufacturer's specifications, QuantiTect reversion transcription kit (Qiagen, Valencia, CA) was used for cDNA synthesis. To analyze alternative splicing of GR minigene we used specific primers; GRα and GRβ exogenous forward was F:(T7): 5′-AATACGACTCACTATAGGGAGACC-3′. GRα exogenous reverse was R:(9aR) 5′-GATGACGACTCAACTGCTT CTG-3′ whereas GRβ exogenous reverse was R:(9bR) 5′-TTGTCGATGA GCATCAGTTGAC-3′ also reported in ref. [38]. Amplification was obtained by polymerase chain reaction (PCR) using GoTaq®G2 DNA polymerase (Promega, Madison, WI). Real-time PCR was performed by QuantiTect Syber Green PCR kit and for gene expression analysis of SRSF3 (SRp20), RACK1, NR3C1 (GR) and RpL6, primers were provided by Qiagen as indicate in Ref. [32]. GRα and GRβ primers were custom

designed and synthesized by Primm (Milan, Italy) and the nucleotide primer sequences are indicated in ref [31]. The RpL6 (ribosomal protein L6) RNA transcription was used as endogenous reference [32,39] and the quantification of the transcripts was performed by the $2^{-\Delta\Delta CT}$ method [40].

2.6. Subcellular fractionation

Cellular fractionation protocol was obtained as described in ref [39]. Briefly, 3×10^6 MDA-MB-231 were seeded in 100 mm2 dishes and treated for 6 h with 0,1 μM cortisol, 30 μM mifepristone or pretreated for 1 h with 30 μM mifepristone and subsequently 0,1 μM cortisol was added; afterwards the medium was removed, and cells were washed with PBS. These cells were subsequently homogenized 15 times using a Teflon glass homogenizer in 0.32 M sucrose buffered with 20 mM Tris−HCl (pH 7.4) containing 2 mM EDTA, 10 mM EGTA, 50 mM β-mercaptoethanol, 0.3 mM phenylmethylsulfonyl fluoride, and 20 μg/ml leupeptin. The homogenate was centrifuged at 3600 × g for 5 min to obtain the nuclear fraction. The supernatant was centrifuged at 100,000 × g for 30 min; the supernatant obtained represented the cytosolic fraction. The pellet was sonicated in the same homogenization buffer supplemented with 0.2% (vol/vol) Triton X-100. The sample was incubated at 4∘C for 45 min and centrifuged at 100,000 × g for 30 min. The supernatant was separated and represents the membrane fraction. The pellet represents cytoskeleton, which was resuspended in fractioning buffer. Aliquots of the fractions were used for protein assay by the Bradford method and the remaining was boiled for 5 min after dilution with sample buffer and subjected to polyacrylamide gel electrophoresis and immunoblotting as described.

2.7. Western blot analysis

The expression of GRα, SRSF3, RACK1 and α-tubulin in cell homogenates was assessed by Western blot analysis. Briefly, cells were treated and then collected, washed twice with PBS, centrifuged and lysed in 100 μL of homogenization buffer (50 mM Tris−HCl pH 7.5, 150 mM NaCl, 5 mM EDTA, 0.5% Triton X-100 and protease inhibitor mix). The protein content was measured using the Bradford method (Bradford, 1976). Western blotting samples were prepared mixing the cell lysate with sample buffer (125 mM Tris−HCl pH 6, 8.4% SDS, 20% glycerol, 6% β-mercaptoethanol, 0.1% bromophenol) and denaturing at 95 °C for 5 min. Equivalent amounts of extracted protein (10 μg) were electrophoresed into an appropriate % SDS-PAGE under reducing conditions. The proteins were then transferred to a PVDF membrane (Amersham, Little Chalfont, UK) which was blocked in 5% w/v BSA, 1X TBS, 0.1% Tween-20 for 1 h with gentle shaking. The proteins were visualized using primary antibodies diluted in 5% w/v BSA, 1X TBS, 0.1% Tween-20 for RACK1 (1:1000), GRα (1:1000), SRSF3 (1:500) and α-tubulin (1:1000) as indicated in Refs. [31,32]. In all experiments, immuno-reactivity was measured using host specific secondary IgG peroxidase conjugated antibodies (1:7000 diluted) and developed using enhanced chemiluminescence reagent (Pierce, Thermo Fisher Scientific, Rockford, IL, USA).

2.8. Small interference RNA (siRNA)

To evaluate the role of SRSF3 in GRα and RACK1 expression, silencing experiments were conducted. The effect of inducing RNA interference on SRSF3 and RACK1 was assessed using commercially available reagents from Life Technologies [29,32]. siRNA transfection was performed with Lipofectamine RNAiMAX Transfection Reagent following manufacturer's instructions. Forty-eight hours after transfection cells were treated as previously described.

2.9. Scratch wound healing assay

Scratch wound healing assay was performed as described in Ref. [41]. Briefly, cells were seeded in a six-well plate and grown to confluence. Confluent cells were grown in serum-free DMEM medium for 24 h before experimentation. The monolayer cells were scratched and plates were washed twice to remove floating cells and then cells were incubated with medium containing 5% dextran-coated charcoal-treated fetal bovine serum (DCC-FBS) with cortisol (0.1 μM) or vehicle. Cells migrating from the leading edge were photographed at 0, 3, 6 and 24 h. Percentage open wound area (percentage of an image that is not considered as occupied by cells) was calculated using ImageJ (NIH).

2.10. Statistical analysis

Following acquisition of the Western blot or RT-PCR images, the optical density of the bands was calculated and analyzed with Scion Image program for digital image processing (W. Rasband, Research Service Branch, National Institute of Mental Health, National Institutes of Health, Bethesda, MD). The relative densities of the bands were expressed as arbitrary units and normalized to data obtained from control sample run under the same conditions. All experiments were performed at least three times, with representative results shown. Statistical analysis was performed using GraphPad Prism version 7 (GraphPad Software, San Diego, CA, USA). The data were analyzed by analysis of variance (ANOVA) followed by an appropriate post-hoc comparison test as indicated in figure legend. Effects were indicated as significant if $p < 0.05$.

3. Results

3.1. Cortisol induces RACK1 expression in MDA-MB-231 cells

The presence of a GCs responsive GRE site in RACK1 promoter prompted us to investigate the transcriptional regulation of cortisol on RACK1 gene promoter. MDA-MB-231 cells were transiently transfected with three luciferase reporter constructs, Δ1, Δ6 and Δ9 (Fig. 1A) and treated for 6 h with the physiological concentration of cortisol, according to our data showing that increasing concentration of cortisol (0.1, 1 and 10 μM) significantly up-regulated RACK1 (Fig. S1A). Luciferase reporter constructs containing GRE site, Δ1 and Δ9, showed a significant increase in luciferase activity (Fig. 1B and C) whereas in Δ6, cortisol had no effect and luciferase activity was comparable to transfected control cells (Fig. 1D). Data concerning transcriptional effect on Δ1 and Δ9 reporter constructs are paralleled by mRNA and protein expression results (Fig. 1E and F) suggesting that in MDA-MB-231 cells, RACK1 expression is significantly up-regulated by glucocorticoids. Cortisol role in RACK1 expression was confirmed by GR inhibitor mifepristone (RU486), which abolished cortisol-induced RACK1 mRNA and protein up-regulation (Fig. 1E and F). The same results were also obtained with low serum level (1% DCC-FBS) (data not shown) thus highlighting that RACK1-increased expression involves GR-induced gene transcription. Moreover, the presence of only mifepristone induced a modest reduction of RACK1 mRNA expression thus suggesting that RACK1 basal regulation could be dependent to the high level of GR expression, as previously discussed. Indeed, after a 24 h treatment with different concentrations of mifepristone, RACK1 protein expression was significantly down-regulated in a dose-dependent manner (Fig. S1B).

MDA-MB-231 cells treated with cortisol also showed a significant up-regulation of GRα at both mRNA and protein level, which was prevented by mifepristone treatment (Fig. 1G and H). Mifepristone also reduced GRα expression suggesting that GRα could be involved in RACK1 regulation. Suppressed GR expression could be part of mifepristone mechanism of action as reported by literature [42].

Data presented here demonstrate that RACK1 is a GR target gene and this regulation is a cell specific event. In our previous work we

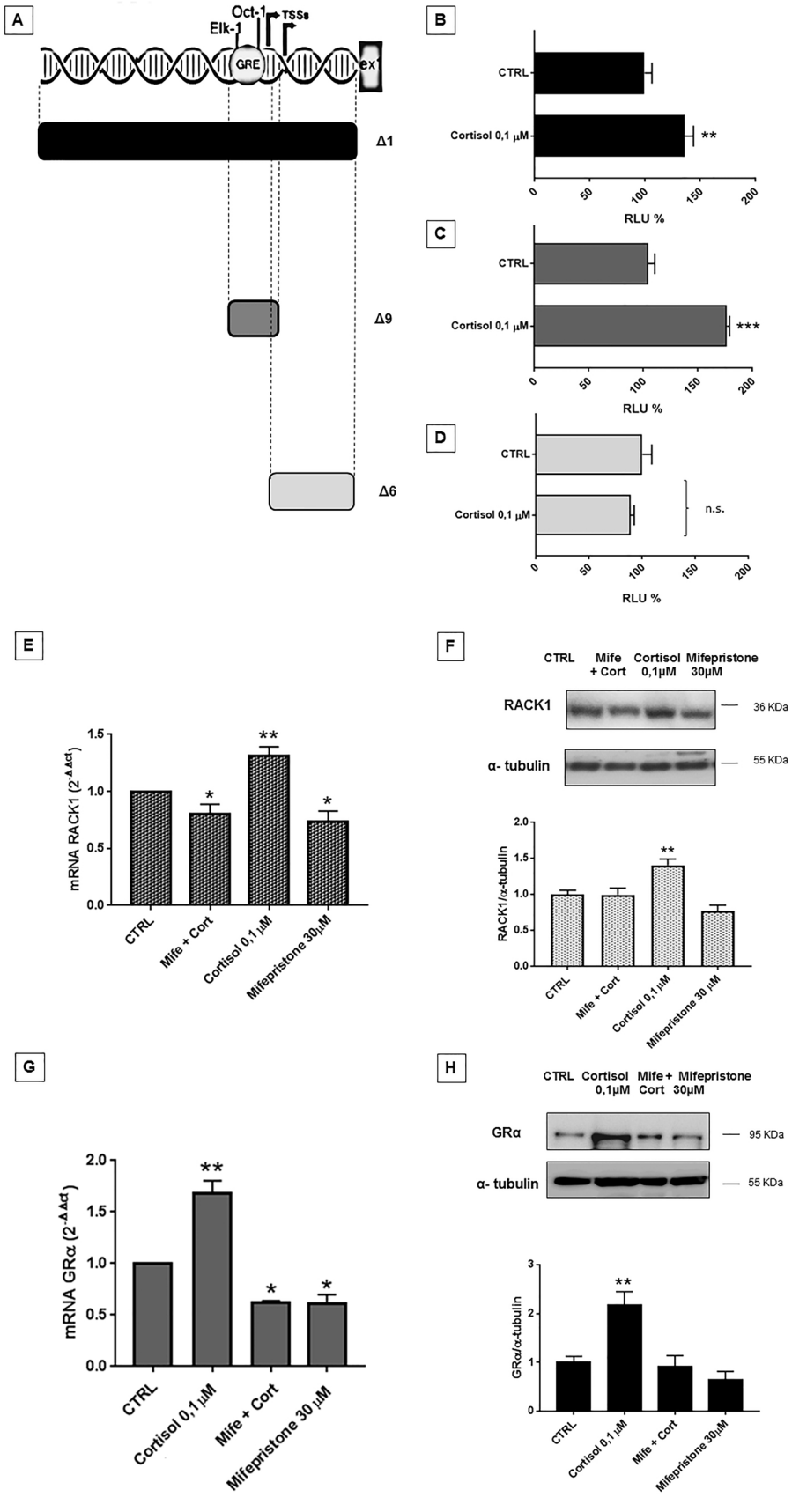

Fig. 1. Effect of cortisol on RACK1 expression through GRα modulation.

A. Scheme of the genomic promoter region of the human RACK1 gene modified from Ref. [26]. The figure also presents the luciferase reporter constructs of RACK1 promoter region used for this study and described in Ref. [28]; Δ1 represented the entire 2-kb region 5′ of the human *rack1* gene, Δ6 was a promoter fragment that did not include the GRE sequence whereas Δ9 construct included only the GRE sequence. **B–D.** MDA-MB-231 cells were transfected with Δ1, Δ9 or Δ6 reporter constructs, and subsequently treated with 0.1 μM cortisol for 6 h. For each reporter construct, luciferase activity was expressed as RLU% and compared to CTRL values assumed at 100%. Each bar represents the mean ± SEM of three independent experiments, in triplicate. Significance was set at $p < 0.05$ by the Student's *t*-test (**$p < 0.01$, ***$p < 0.001$). In **E-H**, MDA-MB-231 cells were treated for 6 h with 0,1 μM cortisol, 30 μM mifepristone or pretreated for 1 h with 30 μM mifepristone and subsequently 0,1 μM cortisol was added. **E–F.** RACK1 mRNA and protein expression. Value bars represent the mean ± SEM of six independent experiments. **G–H.** GRα mRNA and protein expression. Value bars represent the mean ± SEM of three independent experiments. In E and G, real-time PCR for RACK1 and GRα was performed as described in materials and methods. In F and H, immunoblotting was performed with the indicated antibodies described in Refs. [31,32]. In **E-H**, statistical analysis was performed with Dunnett's multiple comparison test, with *$p < 0.05$, **$p < 0.01$

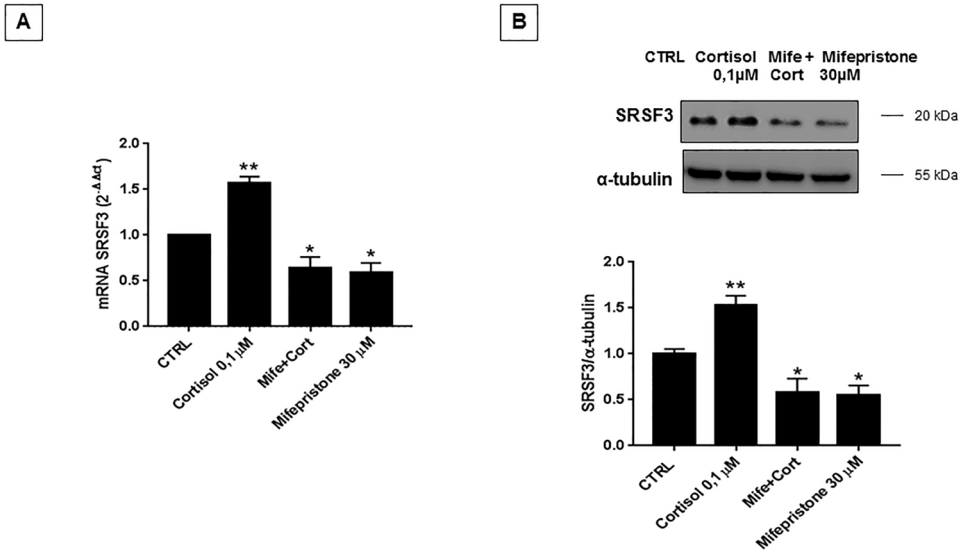

Fig. 2. Effect of cortisol on SRSF3 expression. MDA-MB-231 cells were treated for 6 h with 0,1 μM cortisol, 30 μM mifepristone or pre-treated for 1 h with 30 μM mifepristone and subsequently 0,1 μM cortisol was added. RNA total extracts and cellular extracts were analyzed by real-time PCR (A) and Western blot (B) respectively. In A–B value bars represent the mean ± SEM of three independent experiments. Statistical analysis was performed with Student's *t*-test, with $^{**}p < 0.01$.

demonstrated that, in THP-1 cells used as models of monocyte immune functions, cortisol and other corticosteroids induce RACK1 down-regulation. This is paralleled by significant reduction of cytokine release following cell stimulation and affecting basal immune responses [29]. Therefore, GR-depended RACK1 transcriptional regulation seems to be related to cellular environment and to specific cofactors that determine the final net effect of GR on gene transcription. This matter will require further specific investigation to understand the complex involvement of RACK1 in different forms of cancer and cancer cell types.

3.2. Cortisol modulates GRα expression through SRSF3 involvement

Recently, we have demonstrated that in THP-1 cells cortisol induced GR up-regulation with specific increase of GRα isoform *via* induction of splicing and SRSF3-induced expression [32]. Cortisol also promoted a significant increase of SRSF3 at mRNA and protein level in MDA-MB-231, which was abolished by mifepristone treatment (Fig. 2A and B) thus suggesting cortisol involvement in GR alternative splicing and in GRα up-regulation. To investigate the role of SRSF3 in cortisol-induced GRα splicing, its expression was silenced for 48 h. SRSF3 knockdown blocked cortisol effect on SRSF3 expression (Fig. 3A and B) and consequently on GRα mRNA and protein expression (Fig. 3C and D). Therefore, cortisol effect is driven by SRSF3 modulation, which in turn, influences GR splicing in favor of α isoform. SRSF3 down-regulation also affected cortisol-induced RACK1 expression (Fig. 3A and E) thus highlighting a possible interpretation of a chain of events that link the effect of cortisol on RACK1 expression, depending on GRα expression levels, which are induced by the effect of the splicing factor SRSF3. GRα and RACK1 expression was also affected in untreated SRSF3 silenced cells (see Fig. 3A) suggesting a basal level of control of this splicing factor over their expression, similar to the data obtained with mifepristone treatment (Fig. S1B).

Considering that dexamethasone in MDA-MB-231 cells was able to increase total GR expression [1], we investigated whether this cortisol-induced GRα over-expression could be the results of a GRα/GRβ ratio increase [43]. Our data showed that cortisol significantly promoted GR total expression (Fig. 4A) without affecting GRβ mRNA levels, which remained similar to control cells (Fig. 4B). Therefore, we observed a GRα/GRβ ratio increase, which is mainly due to the up-regulation of GRα mRNA (Fig. 4C). A similar mechanism of action was also observed in THP-1 cells treated with dehydroepiandrosterone (DHEA) where the hormone increased total GR expression. In this case the effect was through the induction of SRSF9, another splicing factor which increased GRβ isoform without affecting GRα expression [31,32].

3.3. Characterization of cortisol effect on GRmini-MDA231 cells

To confirm the involvement of cortisol in the alternative splicing of exon 9 in GR pre-mRNA, we constructed a GR minigene vector in order to generate a stable MDA-MB-231 cell line (GRmini-MDA231) (Fig. 5A). Cortisol caused a significant increase of GRα exogenous mRNA, which was abolished in GRmini-MDA231 SRSF3-silenced cells thus confirming that SRSF3 is a critical splicing factor to generate GRα (Fig. 5B). Hence, cortisol treatment induces an over-expression of SRSF3, suggesting that modulation of the spliceosome proteins involved in GR mRNA splicing can be a significant mechanism for regulation of glucocorticoid activities. However, GRβ exogenous splicing trend (data not shown) is comparable to not transfected cells (Fig. 4B). We can speculate that splicing factors involved in GRβ generation are significantly down-regulated in order to prevent GRβ action as, in different cell lines, GRβ was demonstrated to acts as a dominant negative on GRα [44,45]. Consequently, it is also possible that more time is necessary to observe a significant modulation of GRβ. However, we observed that GRα/GRβ ratio was increased in untransfected-minigene cells (Fig. 4C) where cortisol did not influence GRβ mRNA levels (Fig. 4B). However, considering the possible GRβ antagonist role towards GRα, we recognize that future studies are need to better understand GRβ modulation and expression in this cell line.

3.4. Cortisol-induced RACK1 expression promotes MDA-MD-231 cell migration

Considering the established role of RACK1 in cancer cell migration and invasion [25] we decided to evaluate whether cortisol-induced RACK1 could influence MDA-MB-231 cell motility. Indeed, MDA-MB-231 is a highly invasive cell line, used in the identification of genes and pathways that are potential mediators of metastasis to specific sites, lung, brain and bones [46–50].

First, we evaluated the subcellular localization of cortisol-induced RACK1 protein. Cells were treated with cortisol (in the absence or presence of mifepristone) and then we performed a fractionation protocol separating cytosol and cytoskeletal compartments. In cortisol-treated cells, we found a significant increase of RACK1 in cytoskeleton compartment, which is consistent with a possible effect on cell migration (Fig. 6A and B). We next performed a scratch wound healing assay. As shown in Fig. 6C, cells incubated with cortisol exhibited a significant increase in cell migration of about 45% at 6 h while migration slope is constant. After 24 h, in cells treated with cortisol we could observe that 80% of wound area was healed whereas only 60% was covered in

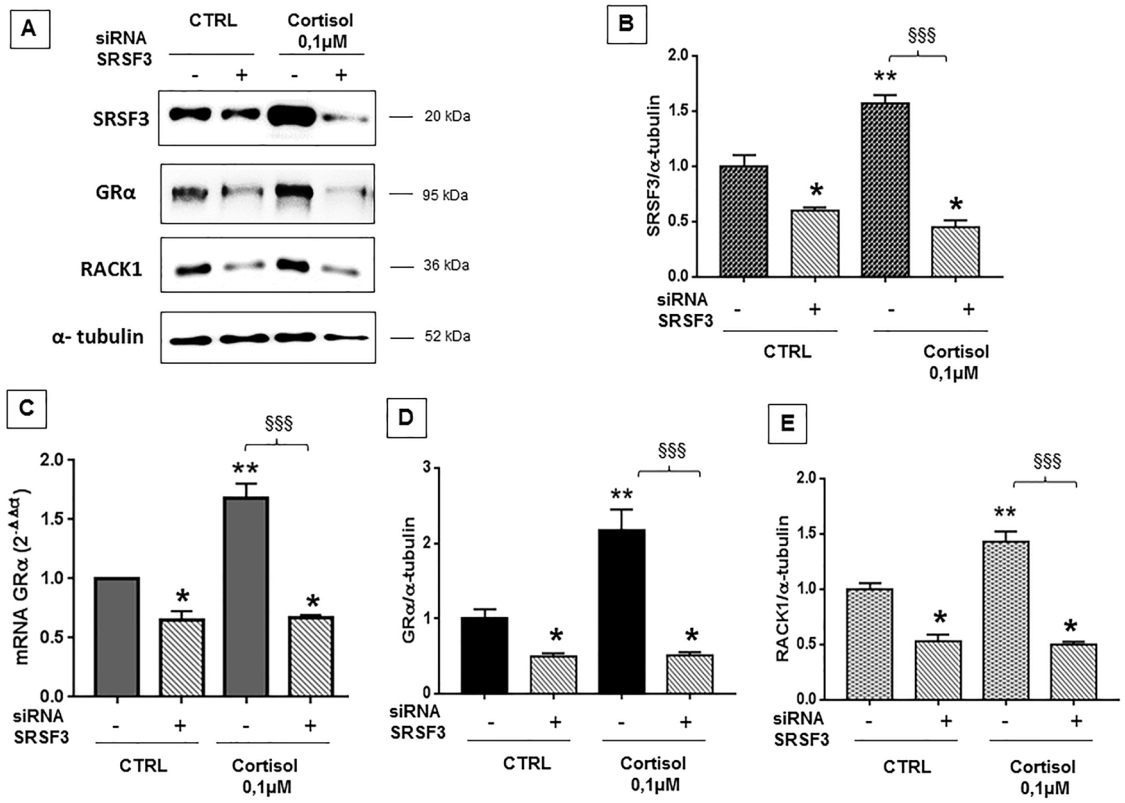

Fig. 3. Prevention of cortisol effect on SRSF3, GRα and RACK1 expression in SRSF3 silenced cells **A.** MDA-MB-231 cells silenced for 48 h with SRSF3 siRNA were treated for 6 h with 0,1 μM cortisol. The image is a representative Western Blot result. **B.** Results are shown as ratio SRSF3/α-tubulin ± SEM of four independent experiments. Tukey's multiple comparison test with *p < 0.05, **p < 0.01 vs CTRL and with §§§ p < 0.001 vs SRSF3 siRNA. **C**. GRα mRNA expression analysis was performed by real-time PCR as described in materials and methods. Statistical analysis was performed with Tukey's multiple comparison test with *p < 0.05, **p < 0.01 vs CTRL and with §§§ p < 0.001 vs SRSF3 siRNA. Each value in the graph represents the mean ± SEM of three independent experiments. In **D-E**, quantitative analysis of GRα and RACK1 protein. Each value in the graph represents the mean ± SEM of at least three independent experiments (GRα n = 3; RACK1 n = 4). Statistical analysis was performed with Tukey's multiple comparison test with *p < 0.05, **p < 0.01 vs CTRL and with §§§ p < 0.001 vs SRSF3 siRNA.

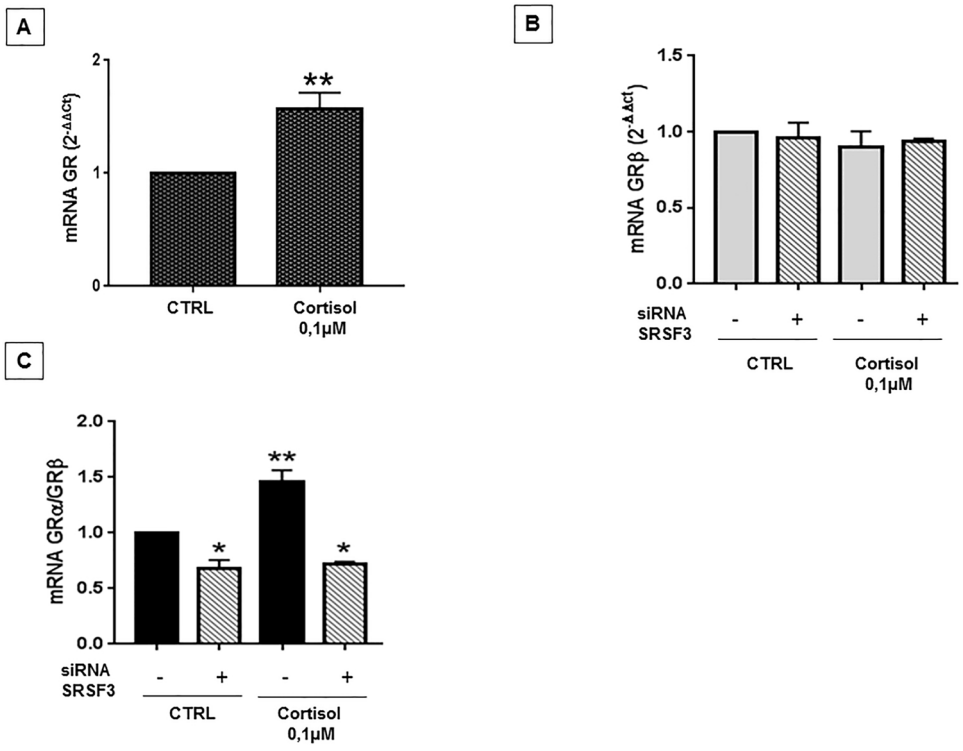

Fig. 4. Cortisol-induced increase GRα/GRβ ratio through the regulation GR total mRNA expression **A.** MDA-MB-231 cells were treated for 6 h with 0,1 μM cortisol and subsequently RNA total extracts were analyzed by real-time PCR to evaluate GR total mRNA expression. Value bars represent the mean ± SEM of three independent experiments. Statistical analysis was performed with Student's *t*-test, with ** p < 0.01. **B**. MDA-MB-231 cells silenced with SRSF3 siRNA were treated with for 6 h with 0,1 μM cortisol and subsequently RNA total extracts were analyzed by real-time PCR by specific primer as detailed in materials and methods. Each value in the graph represents the mean ± SEM of three independent experiments. Statistical analysis was performed with Tukey's multiple comparison test, not significant. In **C**, statistical analysis was performed with Dunnett's multiple comparison test with *p < 0.05, **p < 0.01.

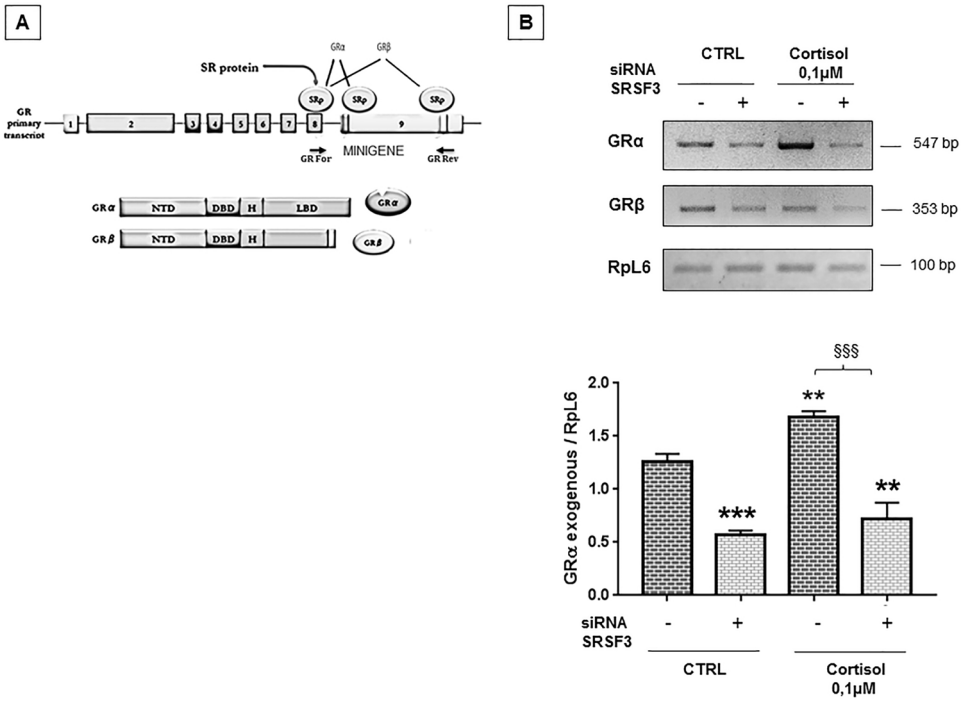

Fig. 5. Depletion of SRSF3 to prevent cortisol-induced GRα splicing in GR minigene **A.** Schematic representation of GR alternative splicing to generate α and β isoforms. Black arrows indicate GR forward and reverse primers used to construct GR minigene in order to amplify GR genomic region including exon 8, intron H exon 9α, intron J and exon 9β as described in materials and methods. Alternative splicing in exon 9 generates two highly homologous isoforms, GRα and GRβ. The two kinds of receptors share the first 727 amino acids at their N terminus coded by first eight exons (NTD, N-terminal domain, DBD, DNA binding domain and H, hinge region), and differ only at their carboxyl-terminus (LBD, ligand binding domain): GRα has an additional 50 amino acids coded by exon 9α but GRβ has only another 15 amino acids coded by exon9β. As a result of these differences, GRβ is unable to bind GC and can not *trans*-activate GC-sensitive genes. **B.** GRmini-MDA231 cells silenced with SRSF3 siRNA were treated for 6 h with 0,1 μM cortisol. To analyze alternative splicing of GR minigene we used GRα and GRβ exogenous specific primers, reported in Ref. [35]. The image is a representative RT-PCR result. Each value in the graph represents the mean ± SEM of four independent experiments for GRα minigene splicing. Statistical analysis was performed with Tukey's multiple comparison test with $^{**}p < 0.01$, $^{***}p < 0.001$ vs CTRL and with §§§ $p < 0.001$ vs SRSF3 siRNA.

untreated control cells (Fig. 6C). In line with literature data, we also demonstrated that mifepristone blocked MDA-MB-231 cells migration both in presence and in absence of cortisol (data not shown). Finally, cell migration was almost completely blocked in cells where SRSF3 or RACK1 expression was down-regulated by specific siRNA. (Fig. 6C–D and E for quantitative analysis).

Overall, we confirm the important role of SRSF3 in MDA-MB-231 cell migration [45] and demonstrate that GRα isoform is one of the products of the activity of this splicing factor. We show a mechanism in which in MDA-MB-231, SRSF3 modulates GRα splicing, which is essential for RACK1 transcriptional regulation, a scaffolding protein that is important in cell migration according to literature data [12]. Finally, we also established that this mechanism can be positively controlled by cortisol administration with consequent cell migration increase.

4. Discussion

GR seems to have a pivotal role in TNBC progression and drug resistance, as previously discussed, these data give a further support in considering SRSF3 and RACK1 as potential target for TNBC therapy [45]. Our data demonstrated that in MDA-MB-231 cells, SRSF3 is essential for GRα splicing and consequently for cells migration; therefore, in line with literature, these results confirm that SRSF3 is involved in splicing events correlated with cell migration [45]. Moreover, we give the first evidence that GRα isoform is one of the products of SRSF3 activity; indeed, SRSF3 promotes GRα isoform, which is essential for RACK1 transcriptional regulation thus highlighting that SRSF3 involvement in cell migration implies its role in controlling different pathways. According to our data, it has been shown that the potent GR inhibitor mifepristone inhibits migration of MDA-MB-231 cells suggesting GR-regulated genes involvement on cell migration [37]. Therefore, considering that mifepristone down-regulated RACK1 in a concentration-dependent manner (Fig. S1B) and that, this protein was involved in cell migration, our data further support the relationship among SRSF3, GRα and RACK1 in MDA-MB-231 cells. RACK1 has been identified as one of the major transcript in TNBC [10] and subsequent investigations in breast cancer patients suggested that RACK1 can be proposed as a highly powerful predictor of poor outcome [13]. It was also observed that multiple genes involved in cell migration and invasion can be modulated by glucocorticoids [51]. More specifically GR antagonism can reverse expression of these genes and it was proposed that GR antagonism could be exploited as additive chemotherapy to improve the likelihood of response in patients at high risk [6].

The data presented here also contribute to the evidence about the negative effect of stress hormones reported by literature data [1]. We demonstrated that SRSF3 up-regulation induced by cortisol was responsible of cell migration increase through its effect on RACK1 expression and localization. We found that in cortisol treated cells, RACK1 protein was significantly increased in cytoskeleton where it could improve focal adhesion assembly [24,37]. It is interesting to note that GCs were reported to be able to promote fibronectin deposition, focal adhesion-dependent activation of Src and the remodeling of the actin cytoskeleton [52] thus giving a further support to our finding. In MDA-MB-231 cells, stress hormones also reduced paclitaxel efficacy through induction of DNA damage, which blocked the cell cycle in G1 phase [1]. Since we observed that cortisol induced cell migration, we suggest that MDA-MB-231 cells increased migration could be another mechanism activated by GCs to induce drug resistance. Accordingly, it has been observed that breast cancer cell migration is faster in the G1 phase of the cell cycle [51,53].

Our data provide the first evidence that RACK1 can be transcriptionally regulated by GR in MDA-MB-231 cells, the most widely used model for GR-positive TNBC as previously discussed. This also demonstrate that cortisol-induced RACK1 regulation is correlated with the migration potential of MDA-MB-231 cells and may lead to possible drug targeting considering that GR antagonism is being advocated as addition to chemotherapy and in a more distant perspective considering that many of the RACK1 signalling partners have been identified. Finally, we provide the demonstration that RACK1 expression modulation is connected to the expression of SRSF3 splicing factor thus highlighting that, in GR-positive TNBC, new players have to be considered in cell migration and drug resistance.

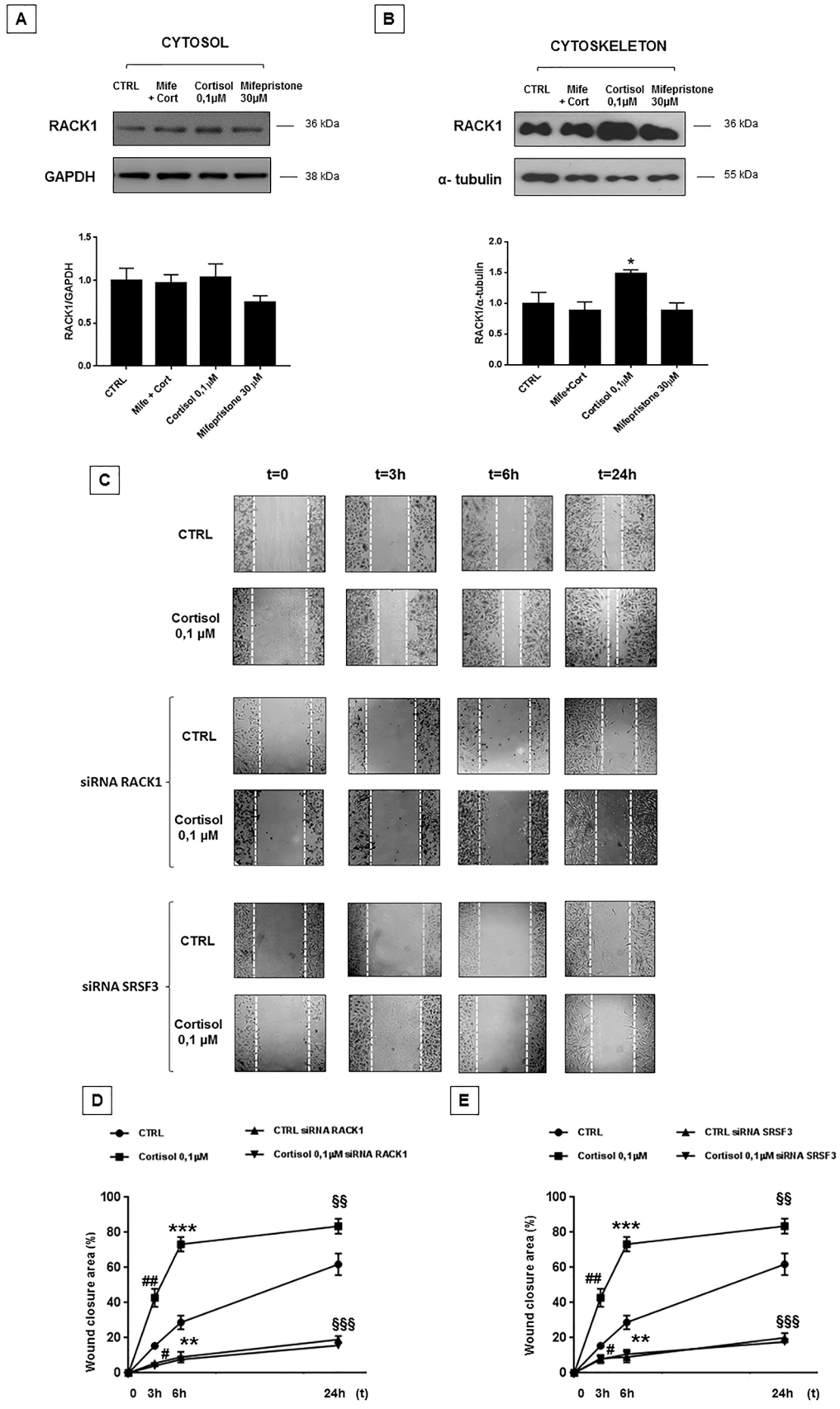

Fig. 6. Inhibition of cortisol-induced cell migration in SRSF3 and RACK1 silenced MDA-MB-231 cells.**A–B.** Analysis of RACK1 expression in cytosolic and cytoskeleton fractions of MDA-MB-231 cells treated for 6 h with 0.1 μM cortisol or 30 μM mifepristone or pretreated for 1 h with 30 μM mifepristone before 0,1 μM cortisol addition. The image is a representative Western blot result. Each value in the graph represents the mean ± SEM of five independent experiments. Statistical analysis was performed with Dunnett's multiple comparison test, with $^{**}p < 0.01$. **C-E**. Effect of cortisol on cellular migration in RACK1 or SRSF3 silenced and unsilenced cells by scratch wound healing assay, performed as described in materials and methods. Silenced and unsilenced cells were cultured without (CTRL) or with 0.1 μM cortisol and subsequently migration was evaluated at 3, 6 and 24 h. In C, the image is a representative result. In **D-E**, value bar in the graph represents the mean ± SEM of three independent experiments, in duplicate of wound healing area in RACK1 and SRSF3 silenced and unsilenced MDA-MB-231 cells. The analysis was performed by two-way ANOVA with Bonferroni's multiple comparisons test with # $p < 0.05$, ##$p < 0.01$ vs CTRL at t = 3 h, $^{***}p < 0.01$ vs CTRL at t = 6 h, §§ $p < 0.01$, §§§ $p < 0.001$ vs CTRL at t = 24 h.

5. Conclusion

Breast cancers (BCs) lacking expression of estrogen receptor (ER), progesterone receptor (PR) and HER2 are termed triple-negative breast cancers (TNBCs) and are classified in six subtypes: basal-like-1, basal-like-2, immunomodulatory, mesenchymal, mesenchymal stem-like and luminal androgen receptor (LAR) [54]. Patients with TNBC are the subgroup with the worst outcome: no specific targeted therapy is currently available and consequently cytotoxic chemotherapy offers the only systemic treatment option [55,56]. However, development of multidrug resistance has led to the search of chemosensitizer drugs in order to enhance the efficacy of standard chemotherapy [57]. Glucocorticoid receptor (GR) antagonism was demonstrated to sensitize cells to chemotherapy-induced cytotoxicity in TNBC [4,9]. A phase I clinical trial conducted in advanced breast cancer patients suggests that GR can be a useful biomarker and a promising target in TNBC [5,58]. Indeed GR is a corticosteroid receptor, with both transcription factor and chromatin remodeling functions, involved in the regulation of genes involved in cell survival and migration functions [59]. Indeed, it has been demonstrated that in ER negative BC patients, ligand-dependent genomic binding of GR to GRE is the predominant mechanism in the regulation of genes associated with drug resistance and unfavorable clinical characteristics and outcomes [6,7,13]. Hence, to improve clinical outcome, the network of GR target genes, including RACK1, may be a better indicator of GR activity in TNBC rather than GR expression alone. Therefore, GR activity signature may be useful for patient stratification in order to identify individual ER-negative early-stage patients with a relatively increased risk of relapse that could benefit from adding GR antagonism to adjuvant chemotherapy. The observation that RACK1 expression is modulated as a consequence of GRα activation by SRSF3 opens the opportunity to further study about the connections between glucocorticoids, GR positive-TNBC and SFSF3 splicing factor. Therefore, our work allows not only to confirm and integrate literature data but also highlight that new players have to be considered in cell migration and drug resistance.

Conflict of interest

The authors declare that they have no affiliations with or involvement in any organization or entity with any financial interest or non-financial interest in the subject matter or materials discussed in this manuscript.

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Appendix A. Supplementary data

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