RAGE overexpression confers a metastatic phenotype to the WM115 human primary melanoma cell line

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Abstract

The formation of melanoma metastases from primary tumor cells is a complex phenomenon that involves the regulation of multiple genes. We have previously shown that the receptor for advanced glycation end products (RAGE) was upregulated in late metastatic stages of melanoma patient samples and we hypothesized that upregulation of RAGE in cells forming a primary melanoma tumor could contribute to the metastatic switch of these cells. To test our hypothesis, we overexpressed RAGE in the WM115 human melanoma cell line that was established from a primary melanoma tumor of a patient. We show here that overexpression of RAGE in these cells is associated with mesenchymal-like morphologies of the cells. These cells demonstrate higher migration abilities and reduced proliferation properties, suggesting that the cells have switched to a metastatic phenotype. At the molecular level, we show that RAGE overexpression is associated with the up-regulation of the RAGE ligand S100B and the down-regulation of p53, ERK1/2, cyclin E and NF-kB. Our study supports a role of RAGE in the metastatic switch of melanoma cells.

1. Introduction

The receptor for advanced glycation end products (RAGE) is expressed during early development, but repressed in most adult tissues, except in the lungs, and is found up-regulated in a large number of pathologies such as diabetes, Alzheimer's disease and in many cancers [1, 2]. RAGE is activated by structurally unrelated ligands such as the advanced glycation end products (AGEs), HMGB1, or members of the S100 protein family, that are associated with tissue damage and pathological conditions [3–10]. Due to the diversity of its ligands, RAGE is classified as a pattern recognition receptor. RAGE signaling is complex and depends on ligands and cell types [11]. For instance, in colon, gastric, breast, pancreatic and liver cancer tissues RAGE is found up-regulated and suppression of RAGE expression or RAGE signaling reduces cellular proliferation and/or migration [12–17]. However, in lung carcinomas and rhabdomyosarcoma, it is the down-regulation of RAGE that results in increased cellular proliferation and/or migration [18, 19], suggesting that the role of RAGE is cancer specific and depends on the tissue and tumor environment [20–22].

The goal of this study was to elucidate the role of RAGE upregulation in melanoma. In melanoma, RAGE has been detected at high levels in a subset of metastatic tumor samples, both at the transcription and protein levels [12, 23], suggesting that RAGE might contribute to tumor development only in certain melanoma tumors [23]. It was also shown that the metastatic human melanoma cells G361 showed higher levels of cellular proliferation and migration in the presence of RAGE activating AGE ligands [24] and that cellular proliferation could be blocked by anti-RAGE antibodies [24]. Similarly, anti-RAGE antibodies have been shown to reduce the growth of melanoma tumor xenografts in mice implanted with G361 melanoma cells [24]. In a different study, RAGE was shown to promote the formation of B16F10 melanoma metastases in mice [25].

Based on these data, we hypothesized that RAGE may contribute significantly to melanoma tumor cell proliferation and/or metastases formation in the population of melanoma tumors where RAGE is up-regulated. To test our hypothesis, we overexpressed RAGE in a melanoma cell line (WM115) that originates from a primary melanoma tumor and compared the cellular proliferation and migration of populations of WM115 cells that differed only by their level of RAGE. We observed that the cells expressing high levels of RAGE presented an altered morphological phenotype with an elongated mesenchymal-like shape that is a typical feature of metastatic cells. Interestingly, we observed that the RAGE overexpressing cells migrated at higher levels and showed reduced cellular proliferation compared to control cells suggesting that RAGE upregulation led to a metastatic switch [26]. At the molecular level, we showed that the RAGE overexpressing cells produced higher levels of S100B, which is used as a prognostic marker in melanoma patients [27–29]. In addition, we found lower levels of the tumor suppressor p53 protein, cyclin E as well as of ERK1/2 in the cells overexpressing RAGE, which supports the reduced cellular proliferation observed.
2. Materials and methods

2.1. Cell lines and transfection

The human melanoma cell lines WM115 and WM266 were purchased from ATCC and grown in Opti-MEM (Invitrogen, Carlsbad, CA) supplemented with 4% FBS (Invitrogen) in the presence of penicillin and streptomycin (JR Scientific, Woodland, CA), 5% CO₂ and at 37 °C. The cells were stably transfected with pcDNA3 expressing full-length RAGE (a gift from Prof. C.W. Heizmann, Children’s Hospital, Zürich, Switzerland) using the SatisFection (Stratagene, Santa Clara, CA) or the X-tremeGENE 9 (Roche Applied Science, Indianapolis, IN) transfection reagents according to the manufacturer’s protocols. Cells transfected with the empty pcDNA3 vector were used as negative control cells (WM115-MOCK and WM266-MOCK). Prior to transfection, all plasmids were linearized with MfeI. The transfected cells were selected by limited dilution in the presence of 1 mg/ml G418 (WM-115-RAGE and WM115-I) or 0.5 mg/ml G418 (WM15-MOCK).

Transfection of WM15-RAGE and WM115-MOCK with RAGE siRNA (sc-37007) or S100B siRNA (sc-43356) (Santa Cruz Biotechnology, Dallas, TX) was performed according to procedures recommended by the manufacturer. Briefly, cells were seeded and grown to 60–80% confluence, in 6 well plates in Opti-MEM (Invitrogen) and 4% FBS. The transfection was performed in the absence of serum. The cells were collected after 48 h and cell lysates were prepared for RT-PCR analysis (Section 2.2) or ELISA (Section 2.5).

2.2. Real-time PCR (RT-PCR)

RNAs were extracted using a commercial kit (Ambion, Invitrogen) according to the manufacturer’s instructions. The quality of the RNA was assessed by absorbance spectroscopy and by agarose gel electrophoresis. The RNA was reverse transcribed into cDNA using the Reverse Transcription System from Promega (Madison, WI).

The real time PCR (RT-PCR) was run with 20 ng cDNA per well on a Stratagene Mx3000p thermocycler using the Brilliant II SYBER Green QPCR Master mix (Stratagene). The genes of β-actin and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were used as housekeeping genes. The primers were used to detect the transcripts of RAGE, S100B, S100A2, S100A6, S100A10 and β-actin and are listed in a previous publication [23]. The other primers were as follows: GAPDH-Forward: GAAG GTGAAAGTGGACGT; GAPDH-Reverse: GAAGATTGGTAGGGATTC; p53-Forward: CACGGCCAGCATTG; and p53-Reverse: CCCTGCTA TGCTGCTGATCG. The following RT-PCR program was used: 10 min at 95 °C followed by 40 cycles of 30 s at 95 °C, 30 s at 58 °C, and 30 s at 72 °C. A melting curve was recorded at the end of the cycles to evaluate the quality of the amplified products.

The Ct values for each gene were calculated and normalized to β-actin and GAPDH ($\Delta Ct = Ct_{\text{gene}} - Ct_{\text{actin or GAPDH}}$). Since similar ΔCt values were obtained with the housekeeping genes β-actin and GAPDH, only the delta Ct normalized with β-actin are shown. The ΔCt obtained from RAGE transfected cells were then compared to MOCK transfected cells ($\Delta \Delta Ct = \Delta Ct_{\text{RAGE}} - \Delta Ct_{\text{MOCK}}$). For each gene, the fold of change ($2^{\Delta \Delta Ct}$) of gene expression was calculated using $f = 2^{\Delta \Delta Ct}$.

The experiments were performed in triplicate using cDNAs obtained from three independent RNA preparations. The standard deviations were calculated from the folds of changes in gene expression.

2.3. Immunofluorescence

For staining the actin filaments, cells were seeded on microscope chamber slides (BD Biosciences, San Jose, CA) overnight, fixed with 3.7% paraformaldehyde followed by permeabilization with 0.1% Triton X-100. The cells were then blocked with 3% BSA in PBS prior to incubation with sulfurhodamine 101 conjugated phalloidin (1:40 dilution) (Biotium, Hayward, CA). Nuclear staining was performed by adding Hoechst 33342 (Invitrogen) in PBS. The microscope slides were examined using a Zeiss AxioObserver Z1 inverted microscope with a 40× magnification.

The ellipticity of the cells and nuclei of the WM115-MOCK and WM115-RAGE cells was assessed by calculating the ratio of the length over the width of at least 30 cells and their corresponding nuclei.

2.4. Flow cytometry

Flow cytometric measurements were performed on a C6 Accuri flow cytometer (Accuri, Ann Arbor, MI). The cells were gently detached with a cell scraper and incubated on ice with a serial dilution of a mouse anti-RAGE antibody (Mab 1145, R&D Systems, Minneapolis, MN). After PBS washes, the cells were further incubated with a FITC-conjugated secondary anti-mouse antibody (10 μg/ml, Jackson ImmunoResearch, West Grove, PA). The mean fluorescence of 5000 events per sample was plotted as a function of the antibody concentration. The data were fitted with a 1:1 binding model using the KaleidaGraph software (Synergy). The experiments were performed in triplicate.

Cell cycle analysis was performed on the same instrument using propidium iodide (PI) (Calbiochem) and an antibody against cyclin E (H12, Santa Cruz). Briefly, 60–80% confluent WM115-MOCK and WM115-RAGE cells were fixed with 70% alcohol, and permeabilized with Triton X-100 prior to incubation with cyclin E antibody and PI, according to the published procedures [30]. A FITC-conjugated secondary antibody (Jackson ImmunoResearch) was used to detect the bound primary anti-cyclin E antibody. The fluorescence of PI was used to identify the G1, S and G2/M phases of the cell cycle and to gate the cells. To compare the level of cyclin E in the different phases of the cell cycle, the mean fluorescence of FITC was reported for each population of gated cells. The experiment was performed independently twice using triplicate samples in each experiment.

2.5. ELISA

Cell extracts were prepared using a commercial kit (Paris, Invitrogen) and the protein content was determined with the Pierce BCA protein assay kit (Pierce/Thermo Scientific, Rockford, IL). RAGE protein levels in the cell extracts were measured using the Quantikine human RAGE Immunoassay kit (R&D Systems) according to the manufacturer’s procedure, and expressed in picogram of RAGE per mg of total protein.

S100B levels in the cell extracts and the conditioned media were determined with the help of a calibration curve obtained from a sandwich ELISA. For the sandwich ELISA, we used a polyclonal anti-S100B antibody (DakoCytomation, Denmark) to capture S100B. A calibration curve was performed using recombinant human S100B (0.01 nM to 150 nM), which was purified according to the standard procedures [31]. To detect bound S100B, we used a second anti-S100B antibody (MAb1820, R&D Systems). The detection was performed using an alkaline phosphatase-conjugated (AP) secondary antibody and para-nitrophenyl phosphate as substrate. The cell lysate and conditioned media samples from two independent experiments were run in duplicate.

2.6. Western blots

Cell extracts were prepared using a commercial kit (Paris, Invitrogen) and the protein concentration in the cell extracts was determined as described above. The proteins (20–100 μg) were resolved on 10 or 15% SDS PAGE and blotted against nitro-cellulose membranes. The blots were blocked with 3% BSA in TBS (50 mM Tris pH 7.4, 150 mM NaCl) and incubated with the primary antibody diluted in 1% BSA in TBS. HRP conjugated secondary antibodies (donkey anti-rabbit, goat anti-mouse and rabbit anti-goat) were all from Jackson ImmunoResearch and used at dilutions recommended by the manufacturer. The primary antibodies against β-actin (#4970), Akt (#4691), phospho-Akt (Ser473, #4060S), SAPK/JNK (#9258), phospho-SAPK/JNK (#4668, Thr183/Tyr185), p44/p42 (#4695)
formed in triplicate.

addition of the cells. The migration and invasion experiments were performed with the inclusion of collagen I (Santa Cruz Biotechnology, Santa Cruz, CA) prior to the exception that the insert was described in our previous publications [34,35]. The blots were developed using a chemoluminescent substrate (Pierce ECL Western Blotting Substrate, Thermo Scientific). The blots were scanned and the intensities of the scanned bands were determined using the ImageJ software [32]. Following the staining for S100 proteins, p53 and cyclin E, the blots were stripped using standard conditions, which consisted of 45 min incubation at 50 °C in the presence of 200 mM glycine buffer at pH 2.2, and re-stained for actin. For the staining of AKT/P-AKT, JNK, p-JNK, and ERK/p-ERK, the blots were first stained with the antibodies recognizing the phosphorylated form of the proteins and, when indicated in the figure legend, the blot was stripped and re-stained with the antibody recognizing the total forms (phosphorylated and non-phosphorylated) of the protein. Alternatively, samples from the same cellular extracts were loaded onto different wells of SDS-PAGE gels. After electroblotting, the blot was cut into parts that were used for separate Western blot analysis.

2.7. Cell assays

2.7.1. Alamar Blue assay

The cells were seeded (4 × 10^4 cells/well) in 24 well plates and incubated in Opti-MEM as described above. When the cells reached 70–80% confluency, Alamar Blue (1/10 dilution, Nalgene) was added to the wells and the plate was further incubated for 3 h or 4 h at 37 °C. The reduced form of Alamar Blue was detected by fluorescence spectroscopy (Ex: 540 nm; Em: 590 nm) [33] and corrected for the fluorescence emission of AB in the cell culture medium only. The experiments were performed in triplicate.

2.7.2. Migration assay

The cells were added (10^4 cells/well) to the top of 8 μm filter inserts (24 well plate, Greiner Bio-One). The inserts were then added into the wells of a 24 well plate containing Opti-MEM and 4% FBS. After 24 h incubation at 37 °C, the cells on top of the filter were gently removed using cotton swabs. Alamar Blue (1/10 dilution, Nalgene) was added to the wells and its fluorescence emission (Ex: 540 nm; Em: 590 nm) was measured after 9 h or 10 h further incubation. The wells containing either the cell culture media alone or the seeded cells without insert were used as negative and positive controls, respectively. When indicated, a polyclonal anti-S100B antibody (DakoCytomation) or polyclonal anti-RAGE antibody (100 nM) was added to the cells at the time point of seeding into wells. The polyclonal anti-RAGE antibody consisted of an equimolar mixture of anti-V, anti-C1 and anti-C2 antibodies as described in our previous publications [34,35].

2.7.3. Invasion assay

The assay was performed similarly to the migration assay with the exception that the insert filters were coated with a solution of bovine collagen I (Santa Cruz Biotechnology, Santa Cruz, CA) prior to the addition of the cells. The migration and invasion experiments were performed in triplicate.

2.7.4. Soft agar colony formation assay

Cell suspensions (1 × 10^4 cells/well) were mixed (1:1) with 1.4% agarose in Opti-MEM containing 8% FBS and added on top of the wells containing solidified 1% agarose. After solidification of the soft agarose, the cell culture medium (Opti-MEM containing 4% FBS) was added to the wells. The plate was then incubated for 4 to 5 weeks at 37 °C and fresh medium was added to the plate every 3 to 4 days. The colonies formed were visualized by staining with crystal violet. The colonies in ten different fields were counted.

2.7.5. Wound healing assay

The cells were seeded in 96 well plates and incubated until reaching 90% to 100% confluence. A wound was performed using the tip of sterile P10 pipet tip. The wells were imaged at the indicated time points using a CCD camera attached to an Olympus Fluoview microscope.

2.8. NF-κB assay

The cells were seeded (10^4 cells/well) in 24 well plates and incubated until they reached 60–70% confluence. The cells were then co-transfected with the NF-κB cis-Reporting System (# 219077; Stratagene) containing a luciferase encoding gene and the monster GFP plasmid (SA Biosciences) that was used to estimate the transfection efficiency. The transfection was performed using the X-tremeGENE 9 transfection reagent (Roche Applied Science, Indianapolis, IN) according to the manufacturer’s protocols. The pF-C-MEKK plasmid was used in co-transfection experiments with the NF-κB plasmid and served as positive control.

Following transfection (24 h–48 h), the cells were lysed using the Reporter lysis buffer (Promega, Madison, WI). The activity of the luciferase was determined using a commercial luciferase detection system (# E1500, Promega) on a Quick-Pak Quantifier luminometer.

2.9. Statistical analysis

The migration and invasion assays were replicated at least three times using 6 wells for each condition. All other experiments were also performed in duplicate or triplicate, as indicated. The mean ± standard deviation is reported. Statistical significances between groups were determined using the Student’s t-test or one-way ANOVA using Bonferroni Post-Hoc test.

3. Results

3.1. Overexpression of RAGE in WM115 cells and characterization of the transfected cell lines

To determine the effect of RAGE up-regulation in melanoma cells, we stably transfected the WM115 melanoma cell line with a vector coding for full-length RAGE. WM115 cells stably transfected with the empty vector (WM115-MOCK) were used as negative control. To characterize the transfected cells, we determined the levels of RAGE mRNA and protein by RT-PCR and ELISA, respectively. From two independent transfection procedures, we selected two clones WM115-RAGE and WM115-RAGE-L, which overexpressed RAGE, at the protein levels, 94-fold and 7-fold, respectively, when compared to the WM115-MOCK controls. WM115-MOCK cells expressed 11.5 ± 0.1 pg RAGE per mg of total protein and WM115-RAGE cells expressed 1081.3 ± 53.0 pg RAGE per mg total protein (Fig. 1A). The second transfected cell line WM115-RAGE-L expressed 81.0 ± 2.0 pg RAGE per mg total protein. The expression of RAGE at the protein level reflected the levels of transcripts that were determined by RT-PCR. We also measured the level of RAGE in WM-266 cells. This cell-line (WM266) originates from the same patient as the WM115, but was established from a metastatic tumor [36]. RAGE protein levels in WM266-MOCK were 2.4 higher than in WM115-MOCK cells and were equal to 24.4 ± 5.4 pg per mg of protein.

To ensure that in the transfected cells RAGE was properly processed and exported to the cell surface, we measured the binding of anti-RAGE antibodies to cell surface RAGE by flow cytometry (Fig. 1B). We found that the antibodies bound to larger numbers of RAGE receptors in RAGE overexpressing cells than in control cells.
3.2. RAGE overexpression is accompanied with changes in cell morphology and actin remodeling

We observed large differences in morphologies between the WM115-MOCK, WM115-RAGE-I and WM115-RAGE cell lines. Whereas the WM115-MOCK cells presented the classical epithelial morphology as previously described for this cell line (Fig. 1C) [37,38], the RAGE transfected cells showed a more elongated morphology resembling that of fibroblasts (Fig. 1E). We also measured the length and width of the cells and found that the ratio \( R \) of the length over the width was significantly larger \((p < 0.001)\) in RAGE transfected cells than in MOCK control cells \((R_{\text{MOCK}} = 1.77 ± 0.68 \text{ versus } R_{\text{RAGE}} = 9.85 ± 3.8)\). Interestingly, the WM115-RAGE-I cells that expressed RAGE at an intermediate level also showed an “intermediate” morphology between those of WM115-MOCK and WM115-RAGE (Fig. 1D). We also compared the morphologies of WM115-RAGE and WM266-MOCK, the “sister” cell line of WM115 [36]. We observed that WM266-MOCK presented similar elongated shape than WM115-RAGE (Fig. 1F). Since the architecture of cells is under the control of actin filaments, we also investigated the distribution of F-actin filaments in the transfected cells by fluorescence microscopy, using sulforhodamine conjugated phalloidin. We observed differences in organization of the actin filaments between the two cell lines. In the WM115-MOCK cells, many structures resembled short cortical bundles (Fig. 1G), these structures appeared to be absent in the WM115-RAGE cells (Fig. 1H). Instead in the RAGE-overexpressing cells, longer parallel fibers were observed as well as structures that resemble collapsed F-actin (Fig. 1H). These changes in actin organization resemble the changes occurring during the epithelial mesenchymal transition (EMT) of cancer cells [39,40].

3.3. RAGE overexpression causes changes in cell proliferation and migration

In order to determine the effect of RAGE overexpression on the cellular behavior of the WM115 melanoma cells, we compared the proliferation and migration properties of these cells compared to MOCK controls. To our surprise, we did not observe an increase but rather a significant 30% decrease in cellular proliferation in the WM115-RAGE cells compared to the MOCK cells (Fig. 2).

We tested several metastatic properties of the transfected cells and as a first step, we studied the growth of the cells in the absence of support or anchor, a phenomenon which is also referred as anoikis [41–44]. In these conditions, we observed that the WM115-RAGE cells formed a significantly larger number of colonies \((19 ± 5 \text{ colonies; } p < 0.01)\) than the WM115-MOCK cells \((7 ± 3 \text{ colonies})\) (Fig. 3A, B).

We next compared the migration properties of the melanoma cells using standard Boyden chamber assays. We found that after 24 h, a significantly larger number of WM115-RAGE cells had migrated through the filter \((33 ± 4.5\%; p < 0.01)\) compared to the WM115-MOCK cells \((14 ± 1.9\%)\) (Fig. 4A). We also observed that the percentage of migrated WM115-RAGE cells was not significantly different from the percentage of migrated WM266-MOCK cells \((27.9 ± 4.2\%)\).
We also investigated whether the migration abilities of the WM115 cells correlated with the level of RAGE in these cells. We indeed observed that the WM115-RAGE-I cells migrated to a level (19 ± 3.2%) that was intermediate between that of the WM115-MOCK cells (14 ± 1.9%) and that of the WM115-RAGE cells (33 ± 4.5%), the difference of percentage of migrated cells being statistically significant between the three groups (p < 0.05) (Fig. 4A).

In addition, we investigated the invasion properties of these cells and observed that a significantly larger number of WM115-RAGE cells had invaded collagen-coated filters (18.5% ± 1%, p < 0.05) than the WM115-MOCK cells (8% ± 4.5%) (Fig. 4B).

To complement our findings, we compared the migration abilities of the melanoma cells after the formation of a wound on a layer of confluent cells (Fig. 5). In agreement with the Boyden chamber assay results, we observed that the WM115-RAGE cells (Fig. 5, lower row) recovered the wounded area significantly faster than the MOCK control cells (Fig. 5, upper row).

To further support our hypothesis that RAGE overexpression in the WM115 cell line was responsible for the observed changes in cellular migration, we tested whether RAGE suppression by siRNA in the WM115-RAGE cells could revert the increased migration. We observed that suppression of RAGE by specific siRNA significantly reduced (p < 0.05) the percentage of migrated cells from 49% ± 1% (control siRNA) to 39% ± 5% (RAGE siRNA) (Fig. 6A). The level of suppression at the protein level was measured by ELISA and showed a 3.2 fold reduction (±1.5) after transfection with siRNA (Fig. 6B). In addition, we observed that RAGE suppression resulted in a significant (p < 0.01) increase in cellular proliferation (Fig. 6C).

3.4. RAGE overexpression is accompanied with the up-regulation of S100B

Since we showed that the RAGE overexpressing cells acquired several metastatic properties, our next goal was to interrogate the levels of RAGE ligands in the transfected cells. We chose to compare the levels of several S100 proteins that are relevant to melanoma and therefore measured the levels of S100A2, S100A4, S100A6, S100A10 and S100B in cell extracts of the different cell-lines. Among those S100 proteins,
we could only detect significant changes in S100B levels. S100B mRNA levels in WM115-RAGE cells were (4.6 ± 0.7) higher than in the control WM115-MOCK cells. At the protein level, S100B levels were also significantly higher in WM115-RAGE cells than in WM115-MOCK cells as determined by Western blot (2.5 ± 0.7) and ELISA (2.7 ± 1.4) (Fig. 7). In addition, we found that WM115-RAGE cells secreted higher levels of S100B in the media (8.2 ± 1.5 fold) than the WM115-MOCK cells (Fig. 7B).

Interestingly, suppression of RAGE by siRNA in the WM115-RAGE cells did not result in a significant reduction in the levels of intracellular S100B protein (data not shown). To test the hypothesis that S100B participates in the increased migration of WM115-RAGE cells, we compared the migration of these cells after suppressing extracellular RAGE/S100B interaction with anti-S100B antibodies (DakoCytomation) or suppressing intracellular S100B using siRNA (Fig. 8). S100B suppression by siRNA resulted in a 8.5 (±3.3) fold decrease in S100B transcript levels. We observed no changes in migration in the WM115-RAGE cells after treatment with either S100B antibodies or S100B specific siRNA suggesting that the changes in migration in the WM115-RAGE cells occurred in a S100B independent manner (Fig. 8).

To test the hypothesis that other RAGE ligands might stimulate the migration of the WM115-RAGE cells, we compared the migration of these cells after treatment with anti-RAGE antibodies. We could not detect changes in migration of either WM115-MOCK or WM115-RAGE cells after treatment with polyclonal anti-RAGE antibodies (data not shown), suggesting that the increase in migration of the WM115-RAGE cells occurred in a RAGE-ligand independent manner.

3.5. Overexpression of RAGE results in decreased levels of the tumor suppressor p53 and cyclin E

Following the observation that S100B levels were up-regulated in the WM115-RAGE cells, we next investigated the levels of possible intracellular targets of S100B relevant in cancer and melanoma. One of these targets is the tumor suppressor protein p53 and the mechanism of interaction of S100B with p53 has been well documented [28,45,46]. We observed that the level of p53 was significantly lower in the WM115-RAGE cells than in the MOCK control cells, both at the transcription (2.6 ± 0.3 fold) and protein levels (1.9 ± 0.4 fold) (Fig. 9A). We also measured the levels of the cell cycle regulator cyclin E, and found significantly lower protein levels of cyclin E (4.0 ± 1.2 fold) in RAGE overexpressing cells than in MOCK controls (Fig. 9B). To further support our findings, we analyzed the level of cyclin E during the different phases of the cell cycle (G1, S and G2/M) by flow cytometry. Our data shows that cyclin E was significantly reduced (p < 0.01) in all phases of the cell cycle (Fig. 9C).

Fig. 5. Wound healing assay. Top row: Assay with WM115-MOCK cells. Bottom row: Assay with WM115-RAGE cells. After wounding the confluent cells with a pipet tip, the RAGE transfected cells (bottom row) recovered the wounded area faster than the MOCK transfected cells (top row). Three independent experiments were performed and show similar results. The figure shows a representative experiment. The pictures of the wounded area were taken at t = 0 h; t = 14 h and t = 24 h.

Fig. 6. Effect of RAGE blockage by siRNA on cellular migration and proliferation of WM115-RAGE cells. A: Migration of WM115-RAGE following transfection with control and RAGE specific siRNA. B: Protein RAGE levels following suppression with RAGE siRNA. C: Changes in cellular proliferation of WM115-RAGE following transfection with control and RAGE specific siRNA.
3.6. Overexpression of RAGE is accompanied by a decrease in NF-κB activity

The transcription factor NF-κB has been shown to promote multiple processes in cancer such as proliferation, invasion, metastasis, angiogenesis, and chemoresistance [47–49]. We hence determined whether RAGE overexpression influenced the activity of NF-κB. For this purpose, we transfected both WM115-MOCK and WM115-RAGE with a luciferase/NF-κB reporter system and observed that the basal activity of NF-κB was significantly reduced in the RAGE-transfected cells and was only 37% of the level in the control cells (Fig. 10A).
3.7. Overexpression of RAGE is accompanied by a decrease in ERK1/2 phosphorylation

We next analyzed the activity of several signaling kinases that are relevant in cancer and melanoma and we investigated the levels of ERK1/2, AKT and JNK/SAPK in cell extracts. We observed a 1.8 (±0.5) fold reduction of the levels of phosphorylated ERK1/2 in WM115-RAGE cells compared to the control cells (Fig. 10B). However, we did not observe differences in phosphorylated Akt or JNK between the two cell lines (Fig. 10B).

4. Discussion

The mechanisms by which RAGE contributes to cancer are not clear and may differ between cancer types [20–22]. Indeed, although RAGE up-regulation correlates with increased cellular proliferation and migration in many cancers (colon, gastric, breast, pancreatic and liver cancer) [12–17], it is the down-regulation of RAGE that correlates with increased proliferation and migration in lung carcinomas and rhabdomyosarcoma [18,19].

We previously showed that RAGE transcript levels varied greatly among stage III and IV melanoma tumor samples, suggesting that the role of RAGE in melanoma may be tumor specific [23]. To better understand the function of RAGE in melanoma progression, we chose to compare the proliferation and migration properties of the WM115 primary melanoma cell line following overexpression of RAGE. We chose the WM115 cell line because i) it was established from a primary tumor of a melanoma patient, ii) it has low basal levels of RAGE expression and iii) the metastatic cell line WM266, which was established from the same patient, was also available. Therefore, we were able to compare the metastatic properties of the WM115 cell line, after RAGE overexpression, to those of the sister metastatic cell line WM266.

We first observed that RAGE overexpression in WM115 cells resulted in changes in both cell morphology and actin remodeling, resembling the epithelial mesenchymal transition (EMT) characteristic of metastatic cells [39]. In addition to the change in cell morphology and the increase in cell ellipticity (Fig. 1), we noticed that the nuclei of the RAGE overexpressing cells were also notably more elongated than those of the control MOCK cells. As observed with the cells, comparison of the ratio (R) of the length over width of the nuclei showed a significant (p = 0.001) difference in ellipticity between the two cell lines. (R_{Nuclei-MOCK} = 1.4 ± 0.3 versus R_{Nuclei-RAGE} = 1.8 ± 0.5). Studies have shown that the nuclear shape is tightly regulated by the cell morphology and that the nuclear morphology could reflect changes in cell health [50,51]. In addition, defects in nuclear shape are also used in clinical settings as markers of disease [51]. The changes in nuclear shape that we observed following RAGE overexpression are thus consistent with the observed changes in cell morphology and the accompanied changes in cellular proliferation and migration properties. We also observed that RAGE overexpression increased significantly the mobility of the melanoma cells in all assays used by us, i.e. migration, invasion, anchorage independent colony formation and wound healing assays (Figs. 3 and 5). It thus appears that high levels of RAGE confer the WM115 cells a metastatic phenotype by inducing an epithelial–mesenchymal like transition. Our results are in agreement with earlier studies by Huttunen et al. who showed that B16 mouse melanoma cells overexpressing full-length RAGE produced a larger number of metastases than the B16 cells overexpressing a signaling incompetent form of RAGE that lacked the intracellular signaling domain [52]. Although RAGE overexpression in WM115 cells correlated with increased migration properties, other genes and proteins are involved in this complex process as demonstrated by many studies aiming at identifying metastatic melanoma gene expression signatures [53–56]. Indeed, we observed that in our experimental conditions, the WM266-MOCK cells had similar migration properties to the WM115-RAGE cells. However, in the WM266-MOCK cells, the level of RAGE is only 2.4 fold higher than in WM115-MOCK cells (24.4 ± 5.4 pg RAGE per mg of protein versus 11.5 ± 0.1 pg RAGE per mg of total protein, respectively). Our results demonstrate that overexpression of RAGE was sufficient to induce metastatic properties in the WM115 cell line, however, the WM266 cell line displays a metastatic phenotype despite only minimal increase in RAGE expression, suggesting that other genes are also involved in this process.

RAGE overexpression also resulted in decreased proliferation abilities of the cells, suggesting that RAGE triggered a phenomenon described as the metastatic switch, where the migration properties of cells are increased at the expenses of the proliferative capabilities of the cells [26]. The observed reduction in cell proliferation in RAGE overexpressing cells correlated with a decrease in levels of cyclin E in all cell cycle phases (Fig. 9C). Cyclin E can regulate cell proliferation by cyclin dependent kinase 2 (cdk2) dependent and independent processes resulting in cell cycle progression [57]. Overexpression of cyclin E has been previously observed in WM115 melanoma cells and was associated with the hyper-proliferative nature of this cell line [58]. To demonstrate that RAGE overexpression correlated to reduced cellular proliferation of the WM115 cells, we investigated if these effects were reversible, using specific RAGE siRNA. We observed that suppression of RAGE resulted in a significant (p = 0.01) increase in cellular proliferation (Fig. 6C), supporting our hypothesis that in melanoma cells, RAGE overexpression can control the metastatic switch.

One of the current prognostic markers for metastatic melanoma patients is S100B [27–29]. We show here that the WM115-RAGE cells express and release significantly larger amounts of S100B than the control cells. This finding is another indication that the WM115-RAGE cells have developed metastatic features and led us to investigate the level of the tumor suppressor p53, which is expressed as wild type in WM115 cells [59]. We observed lower levels of p53 in the WM115-RAGE cells than in control cells, in agreement with earlier studies that showed that S100B down-regulates p53 in melanoma cells [60–62]. Interestingly, a recent study has shown that p53 deficiency in the A375P metastatic melanoma cells resulted in increased migration and invasion of these cells and is in line with our results [63].

Downstream RAGE signaling is complex and not yet fully understood. Several intracellular proteins such as ERK1/2, Diaphanous-1 (Dia-1), TIRAP and MyD88, have been suggested to play the role of adaptor proteins of RAGE [64–66]. While Dia-1 was shown to promote cellular migration of C6 glioma through the activation of Rac-1 and cdc42 [65], it is not known if this interaction is relevant in melanoma cells. Sakagushi et al. recently described that they could not immunoprecipitate Dia-1 from HEK-293 cells overexpressing RAGE [66]. To test a possible effect of RAGE overexpression on Dia-1, we measured Dia-1 protein levels in cell lysates of WM115-MOCK and WM115-RAGE cells. Western blot analysis did not reveal differences in Dia-1 expression levels between the two cell lines (data not shown).

The activation of the Ras/BRAF/ERK signaling pathways is a central mechanism leading to cell proliferation and survival and promoting anchorage independent growth, migration and invasion [41,67–71]. In melanoma, the Ras/RAF/ERK signaling pathway is constitutively activated in more than 60% of melanoma cells, including WM115 and WM266 cells, due to gain of function mutations in B-Raf (V600E) [72–74]. Our analysis of signaling pathways that were modulated upon overexpression of RAGE, showed a significant decrease in the phosphorylated form of ERK1/2. We propose that the decrease of ERK1/2 activity reflects the decrease in proliferation of WM115-RAGE cells. Interestingly, a decrease in phosphorylated levels of ERK1/2 also correlated with a decrease in cellular proliferation in MC3T3-E1 osteoblast cells overexpressing RAGE [75].

In melanoma, cellular proliferation is also under the control of PI3K/AKT [76,77]. In our experimental conditions, we could not detect changes in activity of the AKT kinase, suggesting that the PI3K/AKT pathway is not involved in the changes of proliferation or migration observed when RAGE is overexpressed. Since JNK has also been shown to be activated in melanoma, at times through ERK signaling, we also determined the
levels of activated JNK in the two cell lines but could not find significant changes in the WM115-RAGE cells compared to the control cells [78]. These data suggest that RAGE overexpression modulates the ERK pathways without affecting the JNK and AKT pathways.

The observed increases in migration, invasion and anchorage independent cellular proliferation of melanoma cells occurred in the absence of exogenous ligand and correlated with the level of RAGE expression. Recent evidence strongly suggests that RAGE oligomerization is required for activation of the receptor [79–86], and for example, it has been shown that on the surface of HEK293 and HeLa cells, RAGE exists as constitutive dimers [87,88] and that oligomerization of the receptor could be further enhanced following stimulation with S100B or AGEs [88]. We propose that when overexpressed in the melanoma WM115 cells, RAGE spontaneously forms higher order oligomers which are sufficient to trigger RAGE signaling. In this case, RAGE oligomerization could thus result in ligand-independent RAGE activation. This hypothesis is also supported by our observation that the anti-RAGE antibodies, used in this study, were not able to suppress increases in cellular migration whereas treatment of the cells with RAGE targeting siRNA did reduce cellular migration. RAGE oligomerization is currently an active area of research and several mechanisms of oligomerization-dependent RAGE activation have been proposed: RAGE oligomerization could occur either through the V domains [84], C1 domains [84,89] or the C2 domains of the receptor [85,86] in a ligand dependent manner [83]. We propose here that RAGE oligomerization could also be a means for RAGE to generate intracellular signaling in the absence of interaction with its ligands. Although ligand-induced receptor oligomerization is a common mechanism of signaling, as observed with the epithelial growth factor receptor [90], several studies have also reported ligand-independent receptor signaling caused by receptor oligomerization [91–93]. For example, overexpression of CD30 was found to be sufficient to mediate signaling in Hodgkin–Reed–Sternberg cells through oligomerization of the receptor [92], RAGE could therefore represent a new paradigm of receptor that exhibits ligand-independent activation through receptor oligomerization. Further studies would be necessary to elucidate the mechanisms of RAGE oligomerization in the RAGE overexpressing melanoma cells.

In conclusion, our data support the hypothesis that RAGE up-regulation in primary melanoma cells triggers a metastatic switch with increase migration and reduced proliferation. Our data also suggest that RAGE overexpression can lead to ligand independent RAGE activation, possibly through oligomerization of the receptor. We will further investigate the mechanisms of ligand independent RAGE activation in model cells.

**Conflict of interest**

The authors have no conflict of interest.

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