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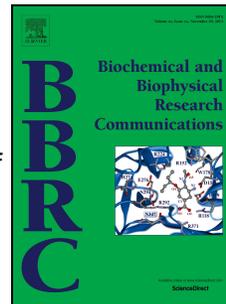
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*Title*

**Extracellular S100A4 affects endothelial cell integrity and stimulates  
transmigration of A375 melanoma cells**

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**Abstract**

High extracellular S100A4 level proves a specific characteristic of some cancer cases, including malignant melanoma. Concerning the latter, extracellular S100A4 in an autocrine manner was shown to promote prometastatic activation of A375 cells by interaction with the receptor for advanced glycation endproducts (RAGE). We hypothesized that interaction of extracellular S100A4 with RAGE in a paracrine manner will affect endothelial cell (EC) integrity thus further promoting melanoma metastasis. We investigated the influence of recombinant and cell (A375)-derived S100A4 on junction protein expression and EC (hCMEC/D3) integrity by measuring transendothelial electrical resistance (TEER). Decrease of TEER and diminished expression of both occludin and VE-cadherin revealed the loss of EC integrity. Transmigration of transgenic A375 cells (A375-hS100A4/A375-hRAGE) through the EC monolayer was significantly higher compared to wild-type A375 cells, and was substantially decreased by sRAGE. A pilot study in mice, intracardially injected with A375-hS100A4 or A375-hRAGE cells, showed lower survival rates and a higher incidence of metastases compared to wild-type A375 cells. Tumor development was mostly located in the brain, bones, and ovaries. These findings provide further evidence on extracellular S100A4 as paracrine mediator of prometastatic endothelial dysfunction involving its interaction with RAGE.

**Keywords**

hCMEC/D3 • prometastatic endothelial dysfunction • receptor for advanced glycation endproducts (RAGE) • soluble RAGE (sRAGE) • S100 proteins • transendothelial electrical resistance (TEER)

## Introduction

The S100 protein family is suggested to play key role in melanoma metastasis [1,2]. One member of this family, S100A4, is overexpressed in several tumor entities, including melanoma [3,4]. The role of intracellular and extracellular S100A4 in regulation of tumorigenic processes has been described in detail in the literature [5,6,7]. Explicitly referring to extracellular S100A4 in melanoma, the multiligand receptor for advanced glycation endproducts (RAGE) is considered as prominent binding partner [8,9,10]. Interaction of both recombinant and cell-derived extracellular S100A4 with RAGE activates the proinflammatory NF- $\kappa$ B axis [10,11,12]. In this regard, we demonstrated that macrophage-derived S100A4 interacts in a paracrine manner with RAGE in melanoma cells and promotes their invasiveness and metastatic lung colonization [11]. In those melanoma cells, which are able to actively secrete S100A4, S100A4-RAGE interaction in an autocrine manner prompts prometastatic activation [13]. Moreover, interaction of S100A4 with RAGE also has been demonstrated in non-tumor cells, e.g., pulmonary artery ECs and smooth muscle cells, and potentially contributes to vascular remodeling in the development of pulmonary arterial hypertension [14].

However, the influence of S100A4 on ECs is poorly understood. Recently, Fazakas et al. reported that selected melanoma cells were able to transmigrate through an EC monolayer by disrupting the tight and adherence junctions via release of serine proteases [15]. This observation suggested a novel concept on how melanoma cells blast their way through the tight endothelial layer. However, it can be assumed that some melanoma's armory is more comprehensive. We hypothesized that extracellular S100A4 belongs to it and will promote melanoma metastasis by leading to a S100A4-RAGE induced endothelial dysfunction.

Therefore, the human malignant melanoma cell line A375, two derived transgenic A375 cell sublines, which are characterized by overexpression/-secretion of S100A4, and

hCMEC/D3 were used to investigate influence of recombinant or cell-derived S100A4 on EC integrity and transmigratory behavior of melanoma cells. As the groups of Nakai et al. and Hiraga et al. already reported appropriate metastasis models by intracardially injected A375 cells [16,17], we further investigated, in a pilot study, S100A4- and RAGE-dependent metastasis by intracardially injecting wild-type and transgenic A375 cells in athymic nude mice.

## **Material and methods**

### **Cell culture**

Human cerebral microvascular endothelial cells (hCMEC/D3) were cultured on rat collagen-type-I-coated (R&D Systems, Germany) culture ware and using the EC growth medium kit enhanced obtained from PELOBIOTECH GmbH (Germany). The human melanoma cell line A375 (ATCC, CRL-1619) and, additionally, stably transfected S100A4-overexpressing (A375-hS100A4) and RAGE-overexpressing A375 (A375-hRAGE) cells were cultured as published elsewhere [10]. Introduced plasmid of transgenic cells includes the sequence for the expression of the green fluorescent protein (GFP).

### **Measurement of transendothelial electrical resistance (TEER)**

For measurement of TEER hCMEC/D3 were seeded onto cell culture inserts at a density of 300,000 cells/cm<sup>2</sup> for 3 days. Afterwards, astrocyte-conditioned medium (ScienCell Research Laboratories, USA) was added for 24 h in order to tighten the cellular junctions [18]. TEER was measured using an epithelial voltohmmeter (EVOM) with a STX2 electrode (World Precision Instruments, USA).

### **Influence of recombinant and cell-derived S100A4 on EC integrity**

TEER and protein expression of selected tight and adherence junction proteins (occludin, claudin-5, VE-cadherin) were analyzed after incubation of hCMEC/D3 with recombinant human S100A4 (300 and 1500 ng/ml; 10× and 50× molar excess of secreted S100A4) [13], with cell culture supernatants (CCS) of wild-type and transgenic A375 cells, and after co-culture with A375 cells. ECs were seeded in 6-well plates ( $5 \times 10^5$  cells/well). After reaching confluency, hCMEC/D3 cells were washed with PBS and fresh medium with recombinant S100A4 or CCS of melanoma cells were added for 24 h. For co-culture experiments  $5 \times 10^4$  melanoma cells/well were loaded onto confluent endothelial monolayer. After 24 h co-cultured cells were separated via magnetic cell separation system (MACS) using anti-Melanoma microbeads (Miltenyi Biotec, Germany) according to manufacturer's instruction.

### **SDS-PAGE and Western blotting**

The synthesis of tight junction proteins occludin and claudin-5, adherence junction protein VE-cadherin, and RAGE were detected in EC lysates via Western immunoblot assay as reported previously [10]. Membranes were incubated with primary antibodies anti-occludin (sc-8145, Santa Cruz Biotechnology, Germany), anti-claudin-5 (sc-374221, Santa Cruz Biotechnology), anti-VE-cadherin (PA5-17401, Thermo Scientific Pierce Antibodies, Germany), anti-RAGE (ab37647, abcam, UK), or anti- $\beta$ -actin (Sigma-Aldrich, Germany) followed by the appropriate secondary horseradish peroxidase-conjugated antibodies (Sigma-Aldrich). Densitometric analysis of those Western blots showing substantial changes of protein synthesis was performed using TotalLab software (UK) as reported before [19].

### **Transmigration of A375 melanoma cells**

ECs were seeded onto cell culture inserts accordingly to the procedure of TEER measurement. After 4 days 50,000 *CellTracker green*-labeled (Invitrogen, Germany)

melanoma cells were plated into the upper compartment and allowed to transmigrate through the EC monolayer for 24 h. Transmigrated cells were quantified in the lower compartment by photometrical analysis. Influence of S100A4 was studied by addition of 1500 ng/ml recombinant soluble RAGE (sRAGE) as blocking agent or 1500 ng/ml recombinant S100A4 [13].

### **Intracardiac injection of melanoma cells in NMRI (nu/nu) mice**

In order to examine metastatic spread of melanoma cells *in vivo* in a pilot experiment A375, A375-hS100A4, and A375-hRAGE cells ( $0.5 - 2 \times 10^6$  cells) were intracardially injected into female NMRI (*nu/nu*) mice (8-10 weeks old; Harlan, USA). Animals were anesthetized under air flow of 0.7 L/min with 40% oxygen and 7-9% desfluran. Mice were kept under standardized pathogen-free conditions with access to standard food and tap water *ad libitum*. Experiments were performed in accordance with the guidelines of the German Regulations for Animal Welfare after approval by the local Ethical Committee for Animal Experiments. Kaplan-Meier curves were captured for 40 days and mice with physical defects, other conspicuous behavior or visible tumors/metastases were further characterized after sacrificing by macroscopic observation or optical imaging of GFP expression, originated from transgenic A375 cells, in whole body and organ slices.

For pathohistological investigation organs were surgically removed and fixed in 4% (w/v) PFA. Organs were washed with PBS to remove the PFA and stored in PBS with 0.1% (w/v) sodium azide until paraffin blocks were prepared. Paraffin-embedding was prepared as described elsewhere [20]. Organs were cut into 100  $\mu\text{m}$  sections and presence of metastases was investigated by optical imaging [21]. Preparation of whole body slices (100  $\mu\text{m}$ ) was performed as described elsewhere [21].

## Statistical analysis

Descriptive data were expressed as mean  $\pm$  standard error of mean (S.E.M.). Nonparametric statistical analyses (Wilcoxon-Mann-Whitney test) were calculated by using the SPSS 20 software package (IBM, Germany). For all analyses a value of  $P < 0.05$  was considered as statistically significant.

## Results

### Extracellular S100A4 induces endothelial dysfunction

#### *Recombinant S100A4*

In order to investigate the influence of S100A4 on EC integrity, we incubated hCMEC/D3 with recombinant S100A4 in 10- up to 50-fold molar excess (300 ng/ml up to 1500 ng/ml). S100A4 is actively secreted by wild-type and transgenic A375 melanoma cells and prompts prometastatic behavior as reported previously [13]. A375-hS100A4 cells secreted maximum amounts of 30 ng/ml S100A4 being measured in CCS of these cells [13]. Furthermore, hCMEC/D3 cells showed significant RAGE expression (Figure 1A) that was comparable to HAEC [10]. Addition of S100A4 resulted in a significant decrease of TEER (Figure 1B), indicating a loss of EC integrity caused by S100A4. To further elucidate this influence, we detected synthesis of tight junction proteins occludin and claudin-5, as well as synthesis of adherence junction protein VE-cadherin in hCMEC/D3 (Figure 1C). Densitometric analysis of Western blots showed that synthesis of occludin was significantly diminished after addition of S100A4 compared to untreated control cells (Figure 1D). However, synthesis of VE-cadherin was not affected (Figure 1C). Claudin-5 was neither influenced by recombinant nor by cell-derived S100A4 (data not shown).

### ***Melanoma cell-derived S100A4***

Regulative effects on EC might also be caused by proteins secreted from surrounding melanoma cells. Therefore, we incubated hCMEC/D3 with CCS of wild-type and transgenic A375 cells and investigated TEER value and synthesis of intercellular proteins. Compared to addition of recombinant S100A4, basal S100A4 levels in CCS amounted up to 8 ng/ml in CCS of A375 cells, 30 ng/ml in those of A375-hS100A4 cells, and 2 ng/ml in those of A375-hRAGE cells indicating substantial differences in S100A4 concentration in CCS. One day after addition of CCS, TEER values significantly decreased (Figure 2A). In contrast to addition of recombinant S100A4, synthesis of occludin was not affected by CCS (Figure 2B). Here, expression of VE-cadherin was significantly diminished by addition of CCS of wild-type A375 and A375-hS100A4 cells (Figure 2C). In order to detect an interaction between melanoma cells and ECs, we further co-cultivated them and analyzed TEER and protein synthesis. TEER was significantly decreased after 2 days of co-cultivation (Figure 3A), being paralleled by a diminished expression of occludin and VE-cadherin (Figure 3B). Densitometric analysis of Western blots has revealed significant losses of occludin (Figure 3C) and VE-cadherin (Figure 3D) expression after co-cultivation with wild-type and transgenic A375 melanoma cells. Therefore, presence of both wild-type and transgenic cells increased permeability of EC monolayer.

### **S100A4 stimulates melanoma cell transmigration**

After biochemical investigations concerning EC integrity we looked on physiological changes on transmigratory behavior of melanoma cells *in vitro* and *in vivo*. Firstly, transmigration of melanoma cells through an EC layer was investigated using an *in vitro* blood-brain barrier model. Transgenic A375 melanoma cells showed a significantly faster transmigration compared to wild-type A375 cells (Figure 4A). Addition of recombinant S100A4 and sRAGE increased or decreased transmigration rate in A375 cells, respectively (Figure 4B). Incubation

with sRAGE significantly diminished transmigration rate of A375-hS100A4 cells by 29% compared to untreated control. According to this, in A375-hRAGE cells addition of S100A4 resulted in an increased transmigration rate by 19% compared to untreated cells.

### **S100A4 stimulates metastatic spread in NMRI (nu/nu) mice**

Objective of the *in vivo* experiment was to investigate a disseminated metastasis of melanoma cells in a S100A4- or RAGE-dependent manner in athymic mice in a pilot study. Mice intracardially injected with transgenic A375 cells earlier became cachectic and died earlier because of a higher incidence of tumors compared to wild-type A375 cells (Figure 4C). The average survival time was 28.8 days for mice injected with wild-type A375 cells and 24.5 or 24.0 days for those mice injected with A375-hS100A4 or A375-hRAGE cells, respectively.

Metastatic spread in this pilot study was histopathologically investigated in those mice being injected with transgenic cells by optical imaging. There, brain metastases were detected in 7% or 15% of mice injected with A375-hS100A4 or A375-hRAGE cells, respectively (Figure 4D). Interestingly, paresthesia, originating from metastases near the spinal column, was observed in 44% of mice injected with A375-hS100A4 cells but not in mice injected with A375-hRAGE. Tumors in adrenals were detected in 20% and 23% of mice with S100A4- and RAGE-overexpressing cells, respectively. Ovarian tumor lesions were mostly caused by A375-hRAGE cells (54%) but less by A375-hS100A4 cells (27%). Therefore, S100A4- or RAGE overexpression enabled the melanoma cells to cross different blood-tissue barriers possibly depending on amounts of extracellular secreted S100A4. Other tumor lesions rarely were detected in lungs and lymph nodes but not in kidneys and spleens. Disseminated metastasis was detected in 81% of all injected mice whereas 19% remained unaffected.

## Discussion

Recent investigations provided experimental evidence *in vitro* on interaction of melanoma cells with cerebral ECs resulting in disturbed EC integrity and paracellular transmigration of melanoma cells through the blood-brain barrier [15,22,23]. Furthermore, other investigations suggested that extracellular S100A4 may induce disruption of tight junctions and disturbed integrity of the blood-brain barrier by RAGE-dependent cellular alterations in ECs [24]. In the light of these previous findings we deduced our working hypothesis that extracellular S100A4 belongs to those molecular players, which will promote melanoma metastasis by affecting EC integrity. In this study we investigated the influence of recombinant or melanoma cell-derived S100A4 on cell integrity of a tight EC monolayer and trans migratory behavior of melanoma cells *in vitro* and *in vivo*.

Both recombinant and cell-derived S100A4 led to reduced TEER values revealing first evidence for a destruction of EC integrity. Furthermore, decrease of TEER through S100A4 addition was paralleled by a reduced expression of the tight junction protein occludin as it was previously reported for mouse brain ECs [25]. However, in our study expressions of claudin-5 and VE-cadherin were not affected by recombinant S100A4. Tai and colleagues also demonstrated a higher permeability of hCMEC/D3 and a reduced expression of occludin but not of claudin-5 in response to amyloid beta peptides [26]. In their study, even a claudin-5 knockdown did not impede formation of tight endothelial monolayer [26]. Although claudin-5 is supposed to be a key tight junction protein, there seems to be reorganization of junction complexes while affecting EC monolayer [27]. Furthermore, a comparative study with HUVEC and HDMEC revealed an inconsistent regulation of claudin-5 and VE-cadherin in these cells [28]. The same group also supposes instable phenotypes of immortalized cell lines that lead to altered regulations of protein expressions. Therefore, regulation of the junctional complex might also depend on cultivation conditions.

Looking more closely on the influence of cell-derived S100A4, the tightness of EC monolayer was comparably diminished by all three cell lines. This effect was paralleled by a significantly reduced expression of both VE-cadherin and occludin in almost all experiments with wild-type and transgenic A375 cells. In contrast to this, recombinant S100A4 merely diminished occludin expression. Obviously, the EC monolayer was influenced by recombinant or cell-derived S100A4 to a different manner and extent. However, it must be noted that in experiments with melanoma CCS and co-culture extracellular S100A4 concentration was lower than in experiments with recombinant S100A4. Therefore, influence on ECs was not comparable in these experiments.

As S100A4 binds extracellular calcium, incubation with high concentrations of recombinant S100A4 leads to a reduction of calcium concentration in the cell culture medium. In this context, lower calcium levels were reported to be paralleled by the appearance of inter-endothelial gaps in rat ECs [29]. Therefore, S100A4 overexpression might induce the dissociation of ECs. Next to the S100A4 induced regulation of the occludin expression, extracellular S100A4 might also act as calcium chelator which in turn impedes the homophilic binding of junction proteins. In contrast to this, melanoma cells also secrete matrix degrading enzymes like MMP-9 or others. Thus, incubation with CCS or co-cultivation results in shedding of cell surface proteins like VE-cadherin to a higher amount compared to incubation with recombinant S100A4.

Furthermore, we showed an enhanced transmigration of A375 cells in the presence of higher concentration of extracellular S100A4. On the one hand, S100A4 seems to act as an autocrine regulator on melanoma cells, as it was previously reported by us [13]. Hsieh and colleagues demonstrated S100A13-stimulated NF- $\kappa$ B activation through RAGE in human ECs which could be inhibited by addition of sRAGE [30]. In our study, incubation with sRAGE also diminished trans migratory behavior of A375-hS100A4 cells. Therefore, S100A4-RAGE interaction clearly influences transmigration of melanoma cells secreting high

amounts of S100A4. However, having in mind that S100 proteins are multireceptor ligands, contribution of other S100A4-recognizing receptors, like toll-like receptors should also be considered. In this regard, activation of TLR2/6 was described to be involved in regulation of EC permeability [31].

Moreover, in an *in vivo* pilot study we investigated metastasis of human A375 melanoma cells in NMRI (*nu/nu*) mice administrated by intracardiac injection. Mice injected with transgenic cells overexpressing S100A4 or RAGE showed a higher tumor burden, developing metastases in the brain, bone, ovaries, spleen, and lung, and showed more severe paraneoplastic symptoms. This is consistent with former own investigations showing that macrophage-derived extracellular S100A4 led to increased metastatic lung colonization of intravenously injected murine B16-F10 melanoma cells being paralleled by lower survival rates [11]. Other studies also reported brain metastasis in balb/c mice after intracardiac injection of YDFR.CB3 cells, originating from human melanoma brain metastases [32]. Valle and others reported that mouse B16 melanoma cells first metastasize to the lungs and secondarily to the adrenals and ovaries, and frequently colonize the bones after intracardiac injection [33]. Formerly, others also reported osteolytic lesions and hypercalcemia caused by intracardially injected A375 cells [16,17]. Sabatini et al. demonstrated that A375 cells produce granulocyte-macrophage colony-stimulating factor, which in turn causes production of tumor necrosis factor leading to some of the paraneoplastic syndromes like hypercalcemia and cachexia [34]. S100A4 also influences calcium homeostasis and, therefore, might promote osteolysis.

In summary, extracellular S100A4 stimulates permeability of ECs by decreasing occludin and VE-cadherin expression and, therefore, facilitates transmigration of melanoma cells through EC monolayer *in vitro*. *In vivo*, cell-derived S100A4 as well as RAGE overexpression led to a high tumor burden, particularly, in brain, bones, and ovaries. This supports our hypothesis that extracellular S100A4 and its subsequent interaction with

endothelial RAGE in a paracrine manner is a further prometastatic attribute in those melanoma cases characterized by high secretion rates of S100A4. This possibly provides an additional molecular target for therapeutic approaches to melanoma metastasis, e.g., in the brain.

### **Conflict of interests**

There is no conflict of interest.

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## Figure legends

### Figure 1: Influence of recombinant S100A4 on EC integrity

(A) Representative Western blots showing protein synthesis of RAGE in hCMEC/D3.  $\beta$ -actin was used as loading control. (B) Relative TEER was measured for two days in untreated hCMEC/D3 (=100%) and after addition of 10- up to 50-fold molar excess (10 $\times$  or 50 $\times$ ) of recombinant S100A4 (n = 12 of 3 independent experiments, \*P < 0.05 *versus* untreated). (C) Representative Western blots showing protein synthesis of occludin and VE-cadherin in hCMEC/D3 after 24 h incubation with S100A4 or without (-).  $\beta$ -actin was used as loading control. (D) Densitometric analysis of occludin expression in hCMEC/D3 related to  $\beta$ -actin expression after treatment with S100A4. Untreated control was set as 100% (n = 3, \*P < 0.05 *versus* untreated).

### Figure 2: Influence of melanoma CCS on EC integrity

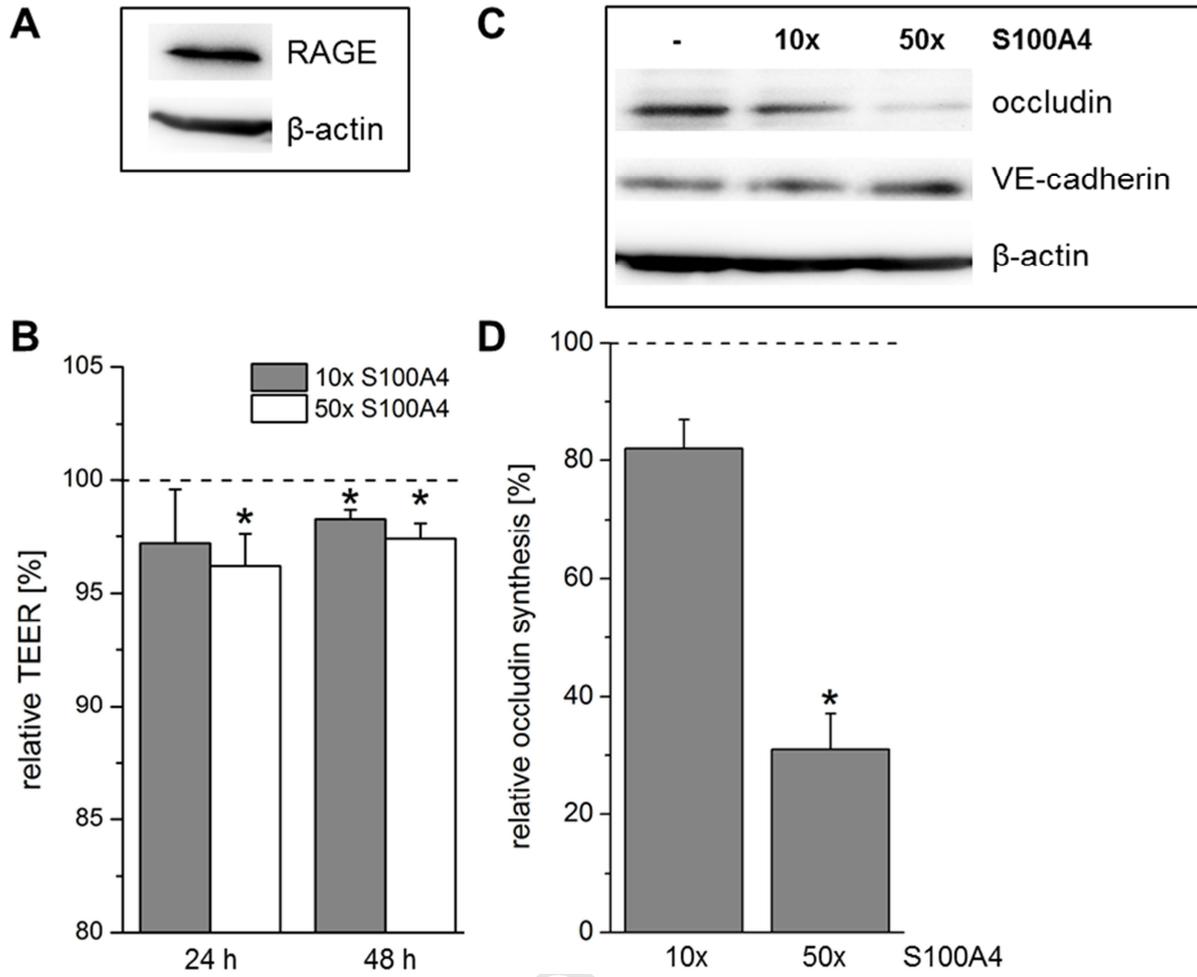
(A) Relative TEER was measured for two days in untreated hCMEC/D3 (=100%) and after addition of CCS (n = 12 of 3 independent experiments, \*P < 0.05 *versus* untreated). (B) Representative Western blots showing protein synthesis of occludin and VE-cadherin in hCMEC/D3 after addition of melanoma CCS or untreated cells (-).  $\beta$ -actin was used as loading control. (C) Densitometric analysis of VE-cadherin expression in hCMEC/D3 related to  $\beta$ -actin expression after addition of CCS. Untreated control was set as 100% (n = 3, \*P < 0.05 *versus* untreated).

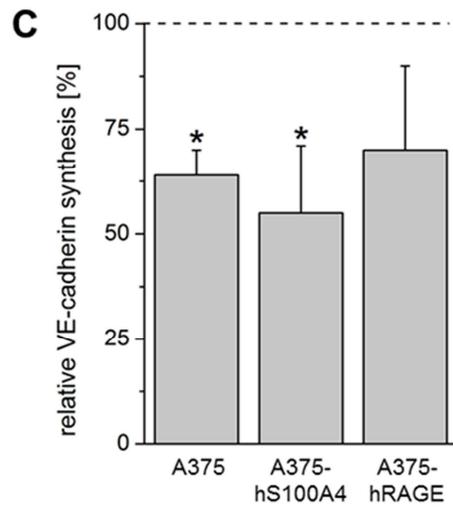
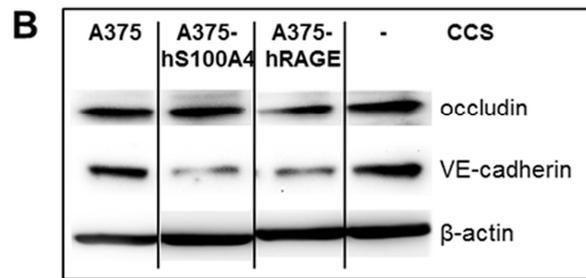
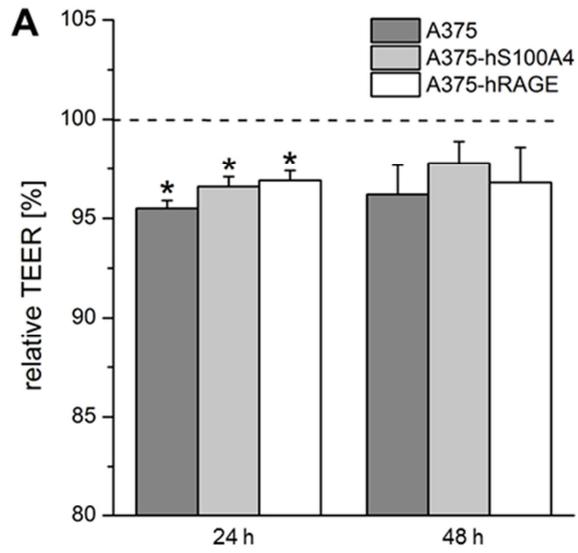
### Figure 3: Influence of melanoma cells on EC integrity

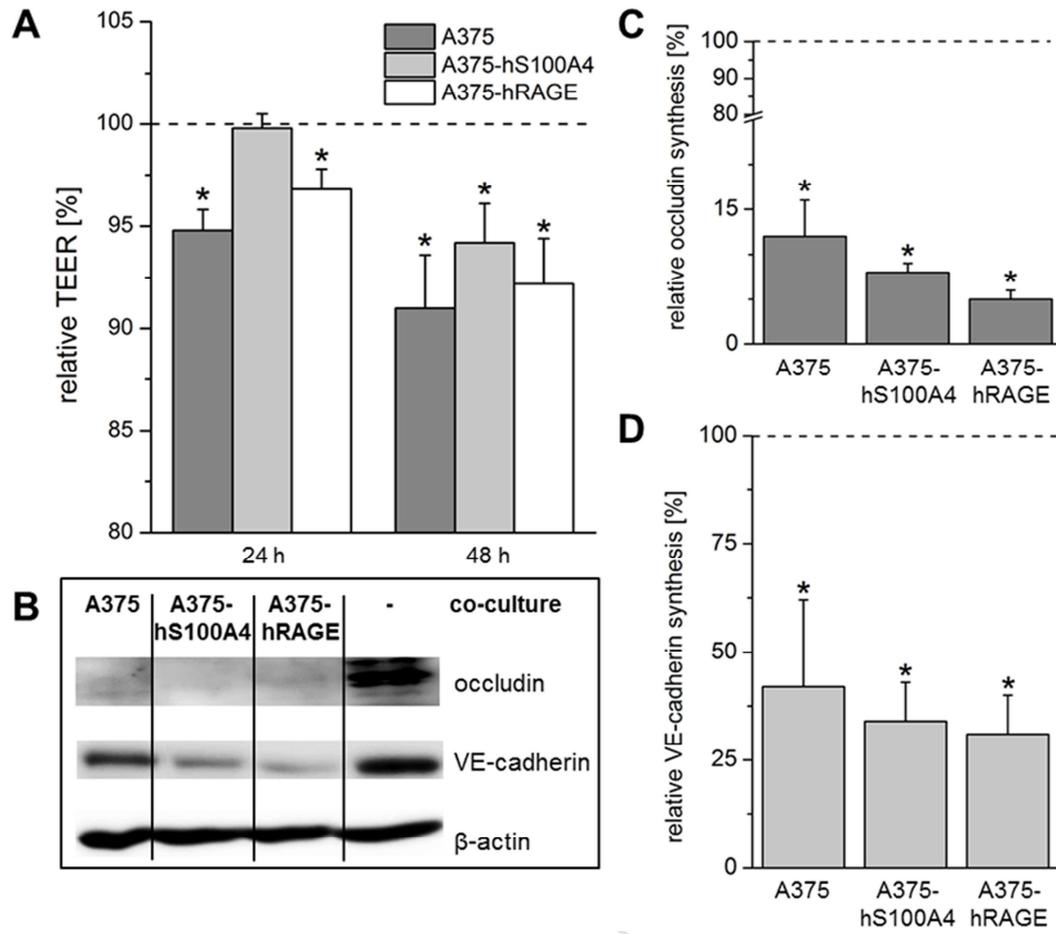
(A) Relative TEER was measured for two days in untreated hCMEC/D3 (=100%) and after co-culture with A375 melanoma cells (n = 12 of 3 independent experiments, \*P < 0.05 *versus* untreated). (B) Representative Western blots showing protein synthesis of occludin and VE-cadherin in hCMEC/D3-cells after co-culture with A375 cells.  $\beta$ -actin was used as loading control. Densitometric analysis of occludin (C) and VE-cadherin (D) expression in hCMEC/D3 related to  $\beta$ -actin expression after co-culture. Untreated control was set as 100% (n = 3, \*P < 0.05 *versus* untreated).

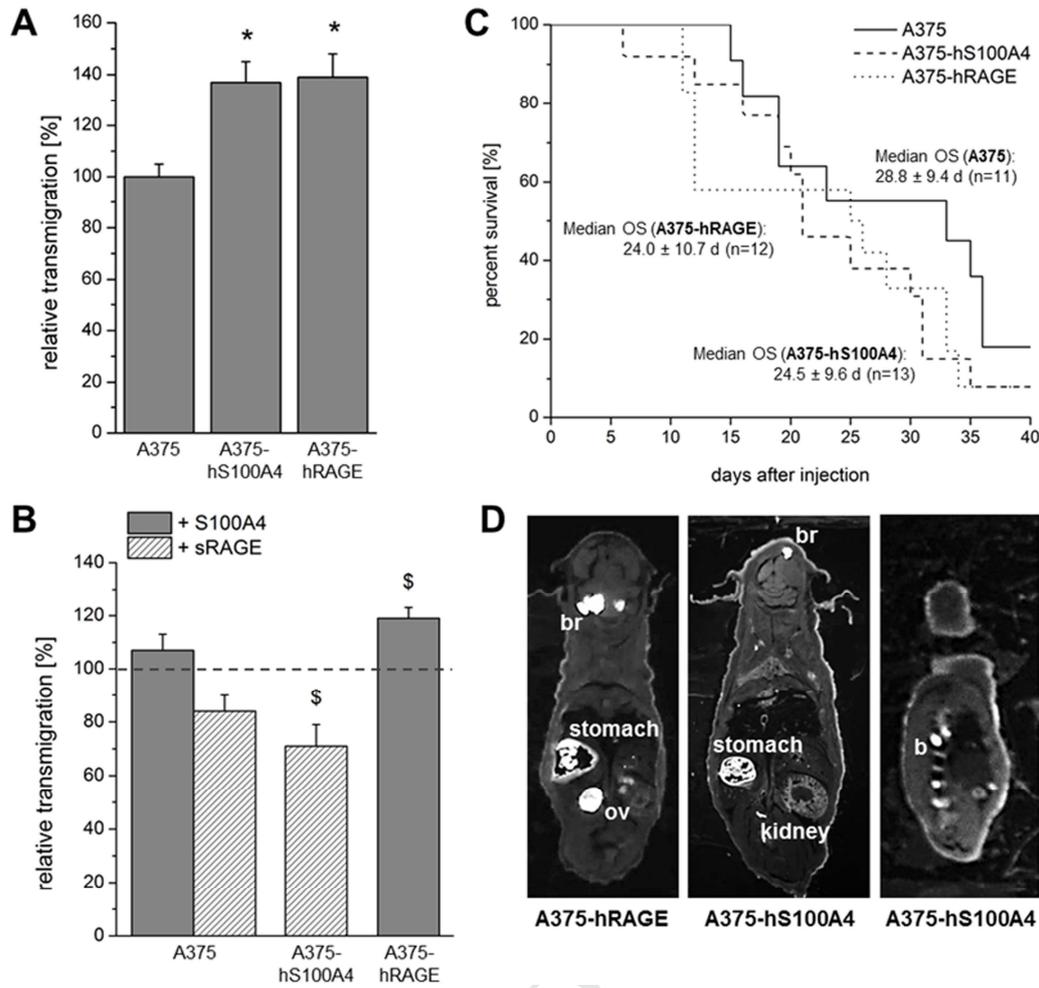
**Figure 4: Physiological effects mediated by S100A4/RAGE interaction**

Transmigration was measured in (A) untreated melanoma cells and (B) after addition of recombinant S100A4 and sRAGE. Untreated control was set as 100% (n = 12 of 3 independent experiments, \*P < 0.05 versus A375 cell, <sup>s</sup>P < 0.05 versus untreated). (C) Kaplan-Meier analysis shows overall survival (median OS ± S.D.) of NMRI (*nu/nu*) mice after intracardiac injection of wild-type and transgenic A375 melanoma cells. (D) Detection of brain (br), bone (b) or ovarian (ov) tumor lesions in NMRI (*nu/nu*) mice after injected A375-hRAGE and A375-hS100A4 cells via optical imaging of GFP synthesis, originating from transgenic A375 cells. The signal of the stomach is related to auto-fluorescence of the content of the stomach.









- Studies on recombinant and melanoma cell-derived human S100A4
- S100A4 interacts with endothelial receptor of advanced glycation endproducts
- S100A4 as paracrine mediator of prometastatic endothelial dysfunction

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