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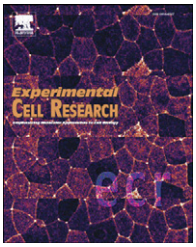

Research Article

Regulation of cellular actin architecture by S100A10

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ABSTRACT

Actin structures are involved in several biological processes and the disruption of actin polymerisation induces impaired motility of eukaryotic cells. Different factors are involved in regulation and maintenance of the cytoskeletal actin architecture. Here we show that S100A10 participates in the particular organisation of actin filaments. Down-regulation of S100A10 by specific siRNA triggered a disorganisation of filamentous actin structures without a reduction of the total cellular actin concentration. In contrast, the formation of cytoskeleton structures containing tubulin was unhindered in S100A10 depleted cells. Interestingly, the cellular distribution of annexin A2, an interaction partner of S100A10, was unaffected in S100A10 depleted cells. Cells lacking S100A10 showed an impaired migration activity and were unable to close a scratched wound. Our data provide first insights of S100A10 function as a regulator of the filamentous actin network.

Introduction

Dynamic alterations of the actin network are involved in several physiological processes in eukaryotic cells. Actin is associated with cell migration and cell adhesions as well as in the formation of cellular structures like cytoskeleton and membrane architecture. It has been proposed that some members of the S100 protein family are engaged in regulation of the cytoskeleton dynamics. The S100 protein family is considered as a group of multitasking proteins involved in several biological processes including the Ca^{2+} signalling network, cell growth and motility, cell cycle progression as well as cell differentiation [1,2]. In several studies, a complex composed of annexin A2 (ANX2) and S100A10 was detected which regulates cellular cytoskeleton [3–6]. S100A10 is expressed in several different cells and tissues such as the epidermis where it is present in both, the cytosol and in membranes [7]. In contrast to other members of the S100 protein family, S100A10 is insensitive towards Ca^{2+} because the protein always shows the equivalent of a Ca^{2+}-loaded structure and thus S100A10 is in a permanently active status [8]. Hence, S100A10 achieves biological functions, i.e. the well-described interaction with ANX2, in a calcium-independent manner [9]. S100A10 can be targeted to cell membranes due to the complex formation with ANX2. This interaction induces the formation of specific membrane domains accompanied by modulation of the actin cytoskeleton [10,11]. It is conceivable that S100A10 functions not only as a structural scaffold protein connecting the two ANX2 chains in sub-membranous regions but may also achieve protein interactions itself in the absence of ANX2 [12]. Recently, it was shown that S100A10 is able to interact with several subunits of different ion channels and that it is

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involved in the regulation of the traffic of plasma membrane proteins [13–16]. Additional interactions of S100A10 with cytosolic and membrane-associated proteins have also been described [17,18]. S100A10 seems to have an influence in formation of actin containing structures. It was shown that an interaction between ANX2/S100A10 and the actin-binding protein AHNAK is able to regulate cell membrane architecture [5]. Furthermore, the ANX2/S100A10 complex is recruited by phosphatidylinositol (4,5)-biphosphate to membrane areas which function as actin assembly sites [6]. It should be noted, however, that ANX2 does not generally co-localise with actin in cells [19]. Recently, it was shown that ANX2 is not required for association of S100A10 with the extracellular plasma membrane as detected in the colorectal cancer cell line CCL-222. Interestingly, S100A10 seems to be involved in the regulation of invasive processes in colorectal cancer as down-regulation of S100A10 by specific siRNA attenuates the invasiveness of CCL-222 cells [20].

In the present study, to get a deeper insight in an S100A10 mediated regulation of actin arrangement we knocked down S100A10 by RNA interference to assess its contribution in actin dynamics as well as in cellular migration.

Material and methods

Cell culture

The human epithelial squamous carcinoma cell line A431 was cultured in DMEM supplemented with 10% fetal bovine serum. Cells were grown to 80% confluence and were passaged at a split ratio of 1:4. Cells were harvested at 70–90% confluence and lysed in a buffer containing 100 mM sodium phosphate pH 7.5, 5 mM EDTA, 2 mM $MgCl_2$, 0.1% CHAPS, 500 μM leupeptin, and 0.1 mM PMSF. After centrifugation (15 min; 15,000 rpm) the supernatant was immediately processed further for Western blotting.

siRNA mediated knockdown of S100A10

Small interfering RNA (siRNA) duplex oligonucleotides used in this study are based on the human cDNAs encoding S100A10. S100A10 siRNA as well as a non-silencing control siRNA were obtained from QIAGEN GmbH (Hilden, Germany). The siRNA sequences applied to target S100A10 were 5′-UGA UAA GAG UUG UCC CAA AdTdT-3′ (sense) and 5′-UUU GGG ACA ACU CUU AUC AdGdG-3′ (antisense). The siRNA sequences employed as negative controls were 5′-UUC UCC GAA CGU GUC ACG UdTdT-3′ (sense) and 5′-ACG UGA CAC GUU CGG AGA AdTdT-3′ (antisense). A431 cells (1×10^5) were plated on 6-well plates 18 h prior to transfection and were 50% confluent when siRNA was added. The amount of siRNA duplexes applied was 1.5 μg/well for S100A10. Transfection was performed using the amphiphilic delivery system SAINT-RED (Synvolux Therapeutics B.V., Groningen, The Netherlands) as recently described [21]. Briefly, siRNA was complexed with 15 nmol of transfection reagent and added to the cells for 4 h. Subsequently, 2 ml of culture medium was added and incubation proceeded for 48 h. Cells were retransfected with the specific S100A10 siRNA or a non-silencing unspecific siRNA after 48 h for a stable down-regulation of S100A10 and grew for further 2 days.

Western blot

Proteins of interest in crude extracts of A431 cells treated with specific S100A10 siRNA or unspecific control siRNA, respectively, were verified using specific antibodies against S100A10 (mouse monoclonal, H21; Swant), actin (rabbit polyclonal, A266; Sigma), tubulin (rabbit polyclonal, ab18251; Abcam) and S100A11 (rabbit polyclonal, 10237-1-AP; Protein Tech Group) by Western blot assays as described.

Immunocytochemistry and confocal microscopy

Cells grown on coverslips were fixed by treatment with methanol at -20 °C for 5 min followed by acetone (prechilled to -20 °C) for 3 min, or by incubation in 4% paraformaldehyde for 10 min at room temperature followed by 25% Triton-X 100 for 3 min. Immunofluorescence was performed as previously described [22]. Samples were scanned with a Zeiss LSM 510 laser scanning confocal device attached to an Axioplan 2 microscope using a 63× Plan-Apochromat oil objective (Carl Zeiss, Jena, Germany). Fluorescein, Cy3 or Cy5 dyes were excited by laser light at a 488-, 552-, or 633-nm wavelength, respectively. To avoid bleed-through effects in double or triple staining experiments, each dye was scanned independently using the multitracking function of the LSM 510 unit. Single optical sections were selected either by eye-scanning the sample in *z*-axis for optimal fluorescence signals, or selected from *z*-stacks. Images were electronically merged using the LSM 510 (Carl Zeiss) software and stored as TIFF files. Figures were assembled from the TIFF files using Adobe Photoshop software.

Antibodies and agents for immunofluorescence-based microscopy

Anti-S100A10 mouse monoclonal antibody (H21; Swant), anti-ANX2 rabbit polyclonal antibody (sc-9061; Santa Cruz) and anti-tubulin rabbit polyclonal antibody (ab18251; Abcam) were used in two-color immunofluorescence staining as primary antibodies which were detected with species-specific secondary antibodies linked to fluorescein, Cy3 or Cy5 (Dianova). Actin was detected in immunocytochemistry experiments by Cy5-tagged phalloidin (647-33; Dyomics, Jena).

Scratch assay

Scratch wounds were created in confluent monolayers of A431 cells treated with specific S100A10 siRNA or unspecific control siRNA by using a sterile 0.1–10 μl pipette tip. After washing away suspended cells, cultures were re-fed with complete DMEM. Cell migration into the wound space was estimated visually at 0, 24, 48, 72 and 144 h after wounding using a microscope (Carl Zeiss) equipped with a digital camera.

Construction of the GFP-ANX2 plasmid

Human Annexin A2 was PCR amplified from A431 cDNA using following oligonucleotides: 5′-taatctcgagatatgggccgccagctagcg-3′ (sense) and 5′-tccggatccgtcatctccaccacacaggta-3′ (antisense). The PCR fragment was cloned between the *Xho*1 and *Bam*H1 restriction site of pEGFP-C1 (Clontech). Correct insertion of ANX2 was confirmed by sequencing.

Results

Down-regulation of S100A10 by specific siRNA

At first, we used RNA interference to manipulate the S100A10 protein level. Specific S100A10 siRNA was able to effectively deplete S100A10 in A431 cells 96 h after the first transfection (Fig. 1). The used S100A10 siRNA was specific since the protein level of S100A11, a further member of the S100 protein family, persisted unaffected by S100A10 down-regulation as assessed by Western blotting. Additionally, a signal derived from S100A10 was completely absent in A431 cells treated with the specific S100A10 siRNA compared to cells treated with non-silencing control siRNA as analysed by immunofluorescence experiments followed by confocal laser scanning microscopy (Fig. 1).

S100A10 depletion disturbs the structure of the actin skeleton

S100A10 controls in complex with annexin A2 (ANX2) the structure of cellular cytoskeleton elements [10]. To investigate whether S100A10 is directly involved in the regulation of different cytoskeleton proteins we analysed A431 cells treated with specific S100A10 siRNA in immunofluorescence experiments followed by confocal laser scanning microscopy (Fig. 2). Immunofluorescence microscopy revealed that S100A10 siRNA treated A431 cell population contained some unaffected cells but in a majority S100A10 was visually undetectable (Fig. 2A, bottom panel). In cells lacking S100A10, the structure of the actin skeleton was altered completely compared to control cells treated with unspecific control siRNA (Fig. 2A). Under this circumstance, cells showed an anomalous F-actin phenotype. In such cells, actin was no longer organised in prominent stress fibres and cortical actin structures. It is conceivable that actin cytoskeleton dynamics and polymerisation processes were influenced by cellular depletion of S100A10. In contrast, formation of tubulin structures were not disturbed by S100A10 depletion as the tubulin architecture was comparable in both A431 cells treated with specific S100A10 siRNA and control cells (Fig. 2B). Microtubuli showed a consistent distribution in typical fibre structures under both conditions. Notably, the total protein expression of actin is not influenced by S100A10 depletion as the actin concentration in both cells lacking S100A10 and control cells containing endogenous S100A10 was comparable (Fig. 2C). In control cells treated with the unspecific siRNA, filopodia formation is observed which sustains cell motility. These structures were completely absent in cells lacking S100A10.

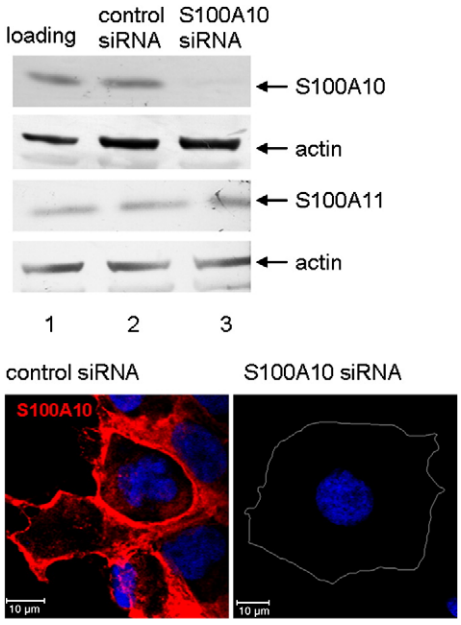

Fig. 1 – Down-regulation of S100A10 by specific siRNA. Upper panel: Protein extracts of A431 cells transfected with specific S100A10 siRNA (lane 3) or, as a control, non-silencing control siRNA (lane 2), respectively, were subjected to immunoblotting against S100A10 and S100A11 using specific antibodies. As a control for equal protein loading corresponding actin levels were shown by immunoblot. Untreated A431 cell extract is shown as loading control in lane 1. Bottom panel: Fixed A431 cells treated with specific S100A10 siRNA or unspecific control siRNA, respectively, were analysed by immunostaining using an anti-S100A10 antibody (red). Nuclei were labelled due to DNA staining by ToPro.3 iodide. S100A10 depleted cell was encircled for identification using a white line.

Cellular distribution of annexin A2 is unaffected by S100A10 down-regulation

S100A10 is mostly localised in complex with annexin A2 (ANX2) under normal cellular conditions [23]. We used a proteomic approach for the identification of interacting proteins of S100A10 in vivo [24]. Hereby, we were able to detect a complex containing S100A10 and ANX2 (suppl. Fig. S1). A co-localisation of S100A10 and ANX2 was predominantly detectable at the cell membrane in A431 cells by confocal microscopy (Fig. 3A). For further experiments, we generated an ANX2 construct tagged with a green fluorescence protein (GFP-ANX2). The cellular distribution of the GFP-ANX2 was similar to results shown in the literature [25]. When A431 cells were transfected with the GFP-ANX2, we also found a predominant co-localisation of S100A10 and the GFP-ANX2 in membranous regions of the cell which was comparable to the distribution of endogenous ANX2 (Fig. 3B). To assess if down-regulation of S100A10 induces an altered distribution pattern of GFP-ANX2 we treated A431 cells with specific S100A10 siRNA and analysed cellular appearance by confocal laser scanning microscopy (Fig. 3C). No alteration in the distribution of the GFP-ANX2 construct was observed in S100A10 siRNA treated cells, compared to controls. This result is comparable to a study using S100A10-knockout mice which shows an unaffected distribution of endogenous ANX2 [26]. Notably, we also observed a correlation between complete absence of an S100A10 signal and a disturbed actin structure (Fig. 3C).

Deregulation of actin structures prevents cell migration activity

Dynamic rearrangements of actin are involved in almost every aspect of eukaryotic cell biology. Beside its role in different cellular migration processes actin is also localised in invadopodia which are cytoskeleton structures involved in cell invasion [27,28]. To analyse possible effects of down-regulation of S100A10 regarding

cellular motility, we carried out scratch assays. A431 cells lacking S100A10 were restricted in migration activity and possessed no sufficient capacity to close the scratched wound within 144 h (Fig. 4). In contrast, control cells transfected with unspecific siRNA grew to nearly complete confluence 48 h after scratching by a pipette tip.

Discussion

In the present study we investigated the ability of S100A10 to regulate different factors of the cytoskeleton. We showed that distinguishable components of specific cytoskeleton structures like actin fibres and microtubuli, respectively, are differently influenced by S100A10. In contrast to the cytoskeletal tubulin network, it seems that the formation of the proper cellular actin filament structures is dependent on the occurrence of S100A10. In cells lacking S100A10 by treatment with specific S100A10 siRNA, actin polymerisation dynamics is disturbed although the total actin concentration was unaltered. The down-regulation of S100A10 was specific as the protein content of a further member of the S100 protein family, S100A11, was unaffected. Based on this observation, we suggest that the actin network can be directly and specifically regulated by S100A10. There are data available suggesting that S100A10 in complex with ANX2 organises the cortical actin cytoskeleton [29,30] and it was assumed that ANX2 is the critical component regulating actin dynamics [11]. Completely unexpected we show in the present study, that an alteration of the cellular S100A10 content is sufficient to cause a deregulation of the polymerisation ability of actin. Cytoskeletal actin structures were strongly dependent on the presence of S100A10 (Fig. 2A). In cells that were successfully transfected with the specific S100A10 siRNA, polymerised actin disappeared. The ANX2/S100A10 complex is probably able to bind filamentous actin due to the ANX2 fraction of the complex if the two ANX2 molecules bind to different actin filaments [10]. A possible explanation of these data might be that binding of S100A10 to ANX2 triggers a particular conformational change in ANX2 which allows both binding to actin and bundling of actin in directed filaments. If the ANX2/S100A10 interaction is disturbed by depletion of S100A10 the particular cross-linking of actin filaments by the ANX2/S100A10 complex is restricted.

We showed in this study a complex formation between endogenously expressed S100A10 and ANX2. The endogenous S100A10/ANX2 complex was mainly localised in the cellular plasma membrane consistent with literature data on ANX2 localisation [31]. The subcellular distribution of our fluorescence tagged ANX2 construct (ANX2-GFP) was identical to endogenous ANX2 (compare Figs. 3 and 4A), and also identical to previously used ANX2 fusion proteins [6,32]. Inevitable overexpression effects were minimised by selecting very low expressing cells. No signals corresponding to ANX2 or S100A10, respectively,

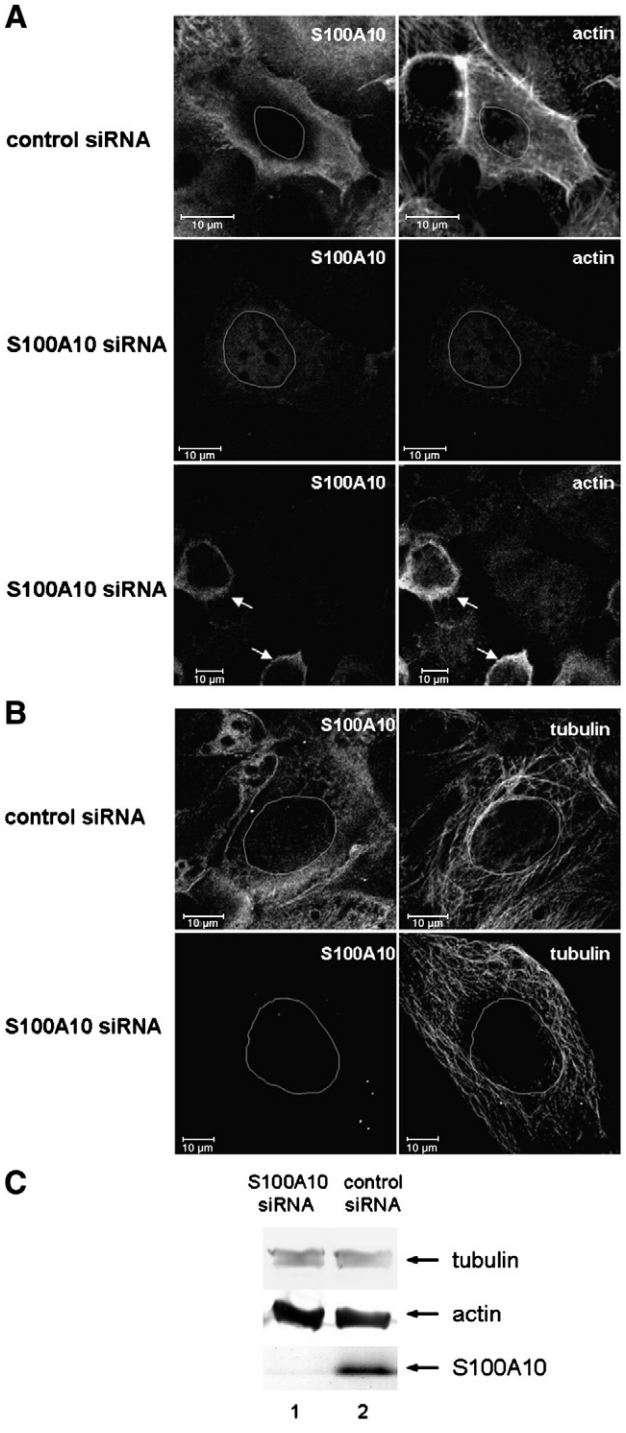

Fig. 2 – S100A10 is involved in regulation of actin polymerisation. (A) A431 cells were transfected with specific siRNA for depletion of S100A10 (S100A10 siRNA) or, as control, with unspecific non-silencing siRNA (control siRNA), respectively, following immunostaining against both S100A10 with a specific antibody and actin with Cy5 labelled phalloidin. In control cells treated with unspecific siRNA, filopodia were detectable in approaches by immunostaining against actin. In contrast to cells lacking S100A10, actin structures occurred in cells with non-adequate transfection of S100A10 siRNA (cells are labelled with white arrows). (B) Tubulin as well as S100A10 was detected by immunostaining in cells treated with specific S100A10 siRNA or unspecific control siRNA using a specific antibody against tubulin or S100A10, respectively. (C) Total protein content of tubulin, actin and S100A10 was detected using specific antibodies in whole crude extracts of A431 cells treated with specific S100A10 siRNA (lane 1) or unspecific control siRNA (lane 2), respectively, by Western blotting.

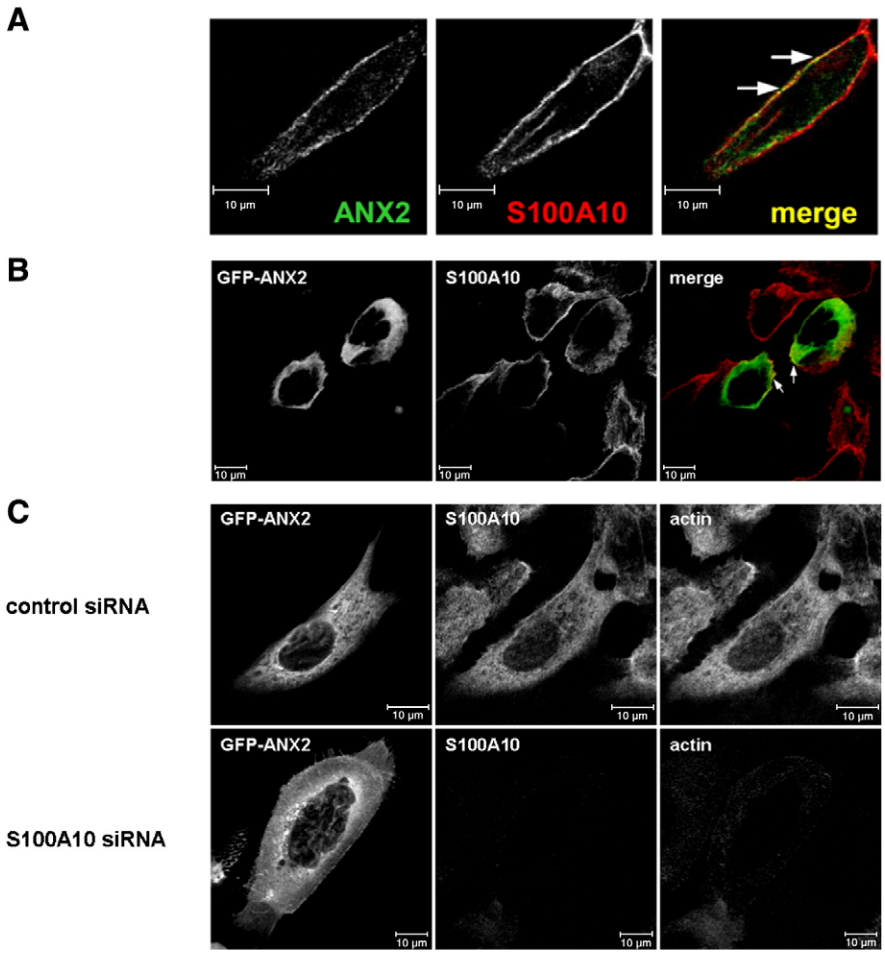

Fig. 3 – Cellular distribution of ANX2 is not influenced by S100A10 depletion in A431 cells. (A) Fixed A431 cells were co-immunostained with anti-ANX2 (green) and anti-S100A10 antibody (red). The merged image reveals significant co-localisation between S100A10 and ANX2 at the plasma membrane of A431 cells labelled by arrows. (B) A GFP-ANX2 construct expressed in A431 cells co-localised with endogenous S100A10 at the plasma membrane in the same manner like endogenous ANX2 as analysed by immunostaining using a specific anti-S100A10 antibody followed by laser scanning microscopy. Membranous areas of GFP-ANX2/S100A10 co-localisation are labelled by arrows. (C) A431 cells transfected with a GFP-ANX2 construct which were treated with specific S100A10 siRNA or unspecific control siRNA were analysed by immunostaining followed by laser scanning microscopy for GFP-ANX2, S100A10 and actin.

were detectable at other cellular membranous structures (i.e. vesicles or the nuclear membrane). Therefore, ANX2-GFP is an appropriate tool to analyse alterations of ANX2 subcellular distribution, as performed in our study. ANX2 does not require S100A10 for its association with the plasma membrane [20]. As our results show, it seems that the localisation of ANX2 is also

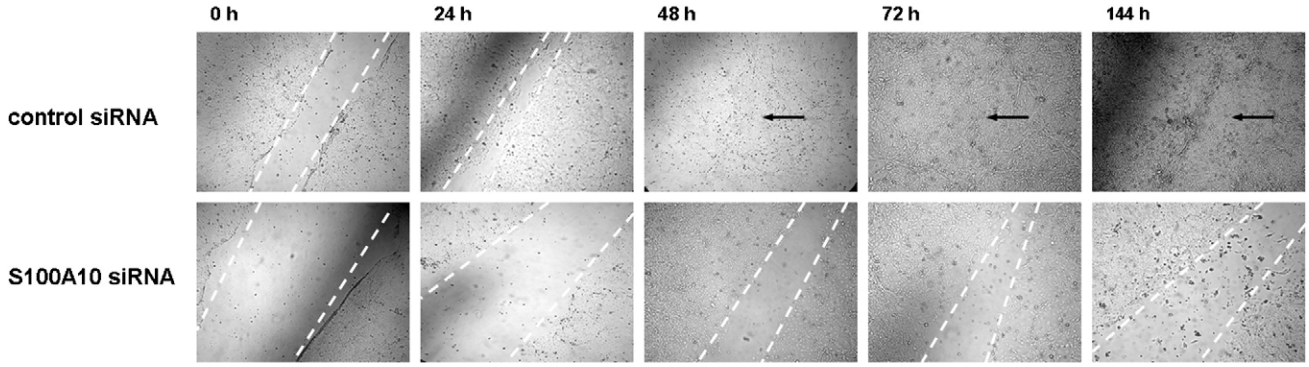

Fig. 4 – S100A10 depleted A431 cells show decreased migration capacity in scratch assays. Images of scratch wounded monolayer of A431 cells treated with specific S100A10 siRNA or unspecific control siRNA, respectively, immediately after scratching and up to 144 h after scratching. Marker lines (white) visualise scratched wound, arrows indicate closed wounds.

not dependent on the presence of S100A10 because the distribution of ANX2 is only little affected in S100A10 depleted cells compared to control cells. In cells lacking S100A10, ANX2 was also mainly localised at plasma membranes. These results are fully compatible with literature data which show that depletion of S100A10 is ineffective with respect to cellular distribution of ANX2 as well as ANX2 protein level [26,33].

The motility of eukaryotic cells is governed by the polymerisation of actin monomers into filaments [34]. The disruption of any one of the actin polymerisation regulatory pathway would likely have an effect on cellular motility. In the present study we used invasive A431 squamous cell carcinoma cells which possess numerous filopodia as motility promoting structures [35]. We showed that in S100A10 depleted A431 cells, filopodia were not detectable. Hence, the depletion of S100A10 caused an inhibited polymerisation of actin monomers in filaments and prevented cellular migration processes. Migration activity seems to be a precondition for the invasiveness of cancer cells. Recently, it was shown that down-regulation of S100A10 provoked a restricted invasion capacity of colorectal carcinoma cells [20]. In this case migration activity was not decreased. An explanation for this opposite results might be that S100A10 has specific effects on distinct types of tumours because S100A10 is expressed in a cell and tissue specific manner [36].

On the basis of the data presented here, we speculate that S100A10 might be considered as a regulator of actin polymerisation and cell motility processes.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.yexcr.2010.01.022.

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