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HepG2.2.15 as a model for studying cell protrusion and migration regulated by S100 proteins

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ABSTRACT

Much of the difficulty in elucidating the precise function of S100 protein family has been attributed to functional redundancy and compensation by its conserved family members. In this study, we showed that seven S100 family members were almost totally undetectable in HepG2.2.15 cells, while all of them were highly expressed in its parental HepG2 cells. Re-expression of S100 proteins in HepG2.2.15 cells can partially rescue their defects in cell protrusion and migration through the regulation of cytoskeletons and adhesions. Thus, HepG2.2.15 can serve as a useful model for studying cell protrusion and migration regulated by S100 proteins.

1. Introduction

The S100 protein family is the largest subgroup within the superfamily of proteins carrying the Ca^{2+}-binding EF-hand motif which consists at least 25 members. The majority of the S100 proteins (S100A1–S100A16) are clustered at the chromosomal locus1q21. The genes encoding some of the remaining known S100 proteins, S100B, S100G, S100P or S100Z, are found on chromosomes 21, X, 4 and 5, respectively. S100 proteins are small acidic proteins (10–12 kDa) expressed in vertebrates exclusively. Each member of S100 family exhibits a unique pattern of tissue/cell type specific expression and they can exist as monomers, homo-, heterodimers or even multimers [1,2].

Via interaction with different target proteins, S100 family members can function both intracellularly and extracellularly and are involved in the regulation of diverse cellular processes such as contraction, motility, cell growth, differentiation, cell cycle progression, transcription, and secretion [1–3]. Till now various diseases such as cardiomyopathies [4], neurodegenerative [5] and inflammatory disorders [6], and cancer [7,8] are associated with altered S100 protein levels. However, the precise function of this family is still largely unknown.

Much of the difficulty in elucidating the precise function of S100 protein family has been attributed to functional redundancy and compensation by its conserved family members. The degree of similarity is averagely around 50% when looking across all the different members, with S100A7 and S100A15 share the highest identical or similar sequences (95%).Targeted deletions of some known multi-functional members of S100 proteins (such as S100A4, S100A9 and S100B) in mice failed to demonstrate any overt anomalies, possibly because other S100 proteins can compensate for the loss of one family member [3].Thus, a cell/animal model with many S100 members simultaneously deleted will definitely help in exploring their complex molecular functions.

In our previous proteomic study, several S100 family members were found to be dysregulated in HepG2.2.15 cells compared with its parental HepG2 cells by two-dimensional electrophoresis (2-DE) analysis [9]. HepG2.2.15 is a widely used HBV producing cell line derived from the human hepatoblastoma cell line HepG2 [10,11]. In the present study, we confirmed the silencing of seven S100 members (S100A4, S100A6, S100A10, S100A11, S100A13, S100A16 and S100P) in HepG2.2.15 cells by immunoblot assays. Compared with its parental HepG2 cells, HepG2.2.15 cells exhibit a rounded morphology, and are defect in cell cilia, protrusion, spreading and migration. Of the seven S100 proteins, four (S100A4, S100A6, S100A11 and S100P) have been reported to be implicated in cell cytoskeleton regulation and migration [12–15].Recently, we also found that S100A6 was up-regulated

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in human liver cancer and had the ability to promote cell proliferation and migration in HepG2.2.15 cells [16]. So it is hypothesized that the lost of these S100 proteins may contribute to the rounded morphology and the defects in protrusion and migration of HepG2.2.15 cells, and the other three S100 proteins (S100A10, S100A13 and S100A16) may have the same effects.

To verify the hypothesis and to facilitate further analyze the potential extracellular/intracellular effects of the three S100 proteins (S100A10, S100A13 and S100A16), we constructed and purified histidine-tagged recombinant proteins and proteins tagged with cell-penetrating peptide (CPP, poly-arginine) in this study. As a result, treatment with each one of the three S100 members (S100A10, S100A13 or S100A16) with CPP-tag was able to partially rescue the defects in cell protrusion and migration of HepG2.2.15, while His-tagged proteins cannot. Because recently dysregulation of S100A10 have been reported to be associated with several human diseases, such as depression [5], virus infection [17], inflammation [18] and cancer [19], we chose it for further study. As a result we showed that S100A10 may interact with β-actin, β-tubulin and annexin A2 (ANXA2) proteins, and was able to regulate the expression and activation of Cdc42, Rac1 and ROCK1 in HepG2.2.15 cells. To our knowledge, S100A13 and S100A16 have not yet been reported to be involved in cell protrusion and migration till now. Thus, HepG2.2.15 can serve as a useful model for studying the molecular mechanisms underlying the regulation of cell protrusion and migration by S100 proteins.

2. Materials and methods

2.1. Cell treatment and membrane protrusion assay

Cells were seeded in a 24-well plate at a confluency of 30%, and allowed to adhere overnight in DMEM medium containing 10% FBS. Then the culture medium were replaced with serum-free DMEM in the presence of S100 recombinant protein (10 μg/ml), or equal molar poly-arginine peptide, or PBS control. Images were taken at 48 h after treatment using a microscope from Carl Zeiss.

2.2. Wound healing assay

Cells were seeded into a 24-well plate and left to grow for 24 h when they reached 90–100% confluency. Confluent cell layer was scratched with a sterile pipette tip to produce a wound gap. Then cells were treated and pictured.

2.3. Pull-down and IP analysis

Total cellular proteins were extracted by using a kit for Protein Extraction and Pull-down Assay (Beyotime Institute of Biotechnology, Beijing, China). 50 μl SP Sepharose beads were firstly saturated with 1 mg purified S100A10-arg proteins or polyarginine peptide, and then incubated with 300 μl protein samples from HepG2.215 cell lysates for 2 h. Bound proteins were washed and eluted with sodium chloride solution with different concentration (200 mM, 600 mM and 1 M). The collected samples were separated by 12% SDS–PAGE. Candidate bands were subjected to Mass Spectrometry analysis.

For Co-IP analysis, cells were firstly transfected with S100A10-EGFP and EGFP plasmids using Lipo2000. 48 h post transfection, cells were harvested and lysed in ice-cold lysis buffer (Co-IP kit from Beyotime). The extract was centrifuged at full speed for 10 min at 4 °C and incubated for 2 h with anti-EGFP antibodies, then agarose-conjugated protein G was added and incubated for another 1 h. Beads were then washed five times with cold lysis buffer. The immunoprecipitate was separated by SDS–PAGE and visualized by Western blot. Extracts of transfected cells were used as inputs.

2.4. RhoGTPase activation and inhibition assays

Total levels of Cdc42, Rac1 and ROCK1 were examined by Western blot 24 h post-stimulated with 10 μg/ml S100A10-arg or equal molar quantity poly-arginine peptide in HepG2.2.15 cells. Activation of Cdc42 and Rac1 were analyzed by using the Rac1/Cdc42 Activation Assay Kit from Millipore (17–441) according to the manuscript's instructions.

After seeded in the 24-well plate overnight, cells were pretreated with S100A10-arg or control groups for 24 h in serum-free DMEM (as described above), and then inhibitors of ROCK (Y-27632, 10 μM), Rac1 (NSC23766, 10 μM) and Cdc42 (ML141, 10 μM) were added into the cell culture. Images were photographed by a microscope from Carl Zeiss.

2.5. Statistical analysis

Data are presented as means ± SD. In all cases, they represent at least three independent determinations. The significance of the results was calculated by Student's t test utilizing Excel. P values $\leqslant 0.05$ were considered significant.

3. Results

3.1. Re-expression of S100 proteins partially rescue the defects in cell protrusion, migration and promote obvious F-actin polymerization in HepG2.2.15 cells

Western blot analysis showed that seven members of S100 proteins decreased dramatically or almost undetectable in HepG2.2.15 cells, while all of them were highly expressed in HepG2 cells (Fig. 1A, a). However, in RNA level, only S100A4, S100A6, S100A11 and S100A16 decreased obviously, and we did not detect any changes as to S100A10, S100A13 and S100P (Fig. 1A, b). HepG2.2.15 cells exhibit a rounded morphology and are defect in cell protrusion, spreading and migration. Through treatment with CPP-tagged recombinant S100 proteins (Fig. S1), it was obvious that each one of the three S100 members (S100A10, S100A13 and S100A16) was able to promote membrane protrusion (Fig. 1C) and cell migration (Fig. 1D) in HepG2.2.15 cells, while equal molar CPP peptide (poly-arginine) had no apparent effect. Spatially controlled polymerization of actin is at the origin of cell motility and is responsible for the formation of cellular protrusions like lamellipodia. Through immunofluorescence staining, we showed that an obvious polymerization of F-actin occurred upon CPP-tagged S100 proteins treatment compared with polyarginine peptide control (Fig. 1E). To examine if the three S100 members may function extracellularly, we also treated cells with His-tagged S100 proteins (Fig. S2). As shown, His-tagged S100 proteins failed to promote membrane protrusion. The intracellular delivery assays showed that polyarginine-tagged S100A10 protein was able to enter cells 2 h post treatment, while His-tagged S100A10 cannot enter cells even after treatment for 8 h (Fig. S3). The results of intracellular delivery assays of polyarginine- or His-tagged S100A13 and S100A16were similar to S100A10 (data not shown). These results suggested that the effects of the three S100 proteins on cell protrusion and migration may depend on their intracellular functions. By contrast, it showed that none of the three S100 members (S100A10, S100A13 and S100A16) with CPP-tag could influence the morphology of HepG2 cells (Fig. S4).

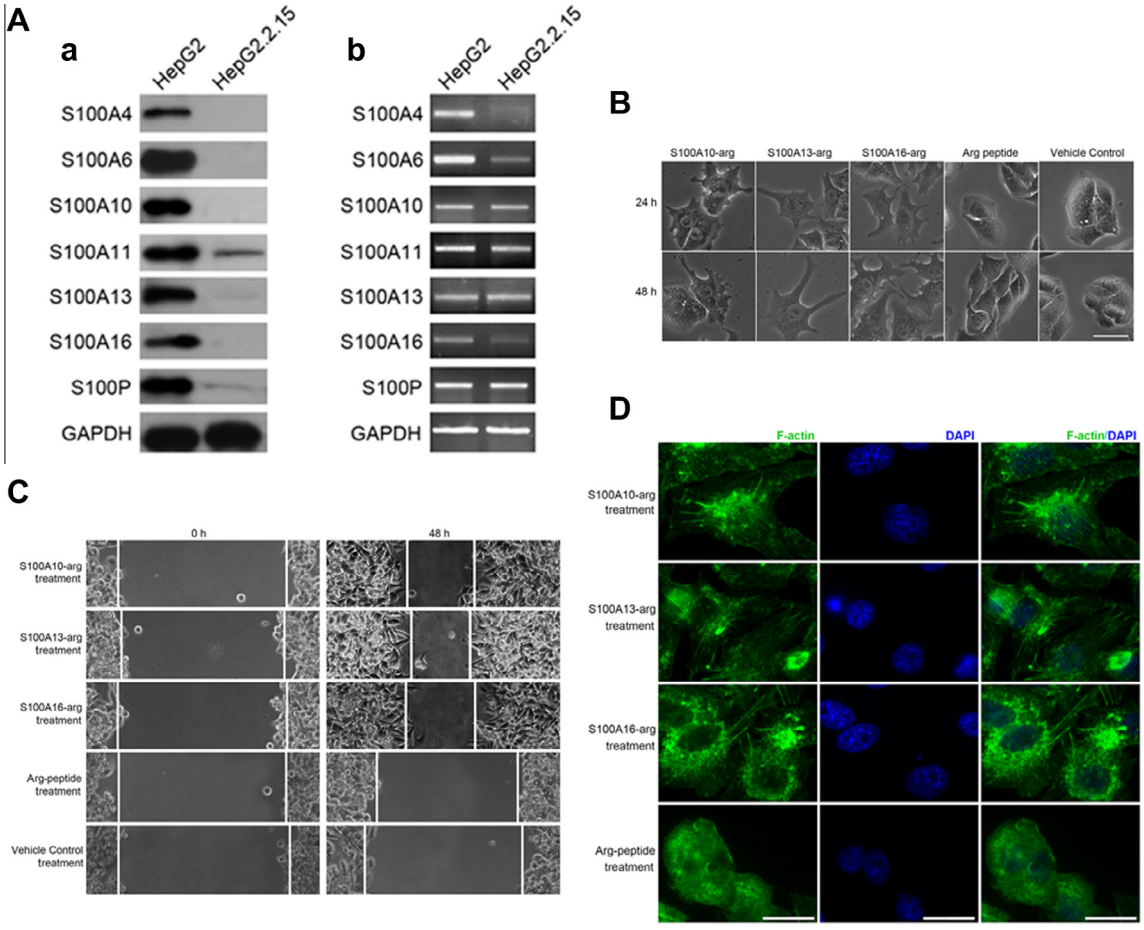

Fig. 1. Re-expression of S100A10, S100A13 or S100A16 promoted membrane protrusion formation, cell migration and F-actin polymerization in HepG2.215 cells. (A) Down-regulation of seven members of the S100 family in HepG2.2.15 cells compared with its parental HepG2 cells. (a) Western blot analysis of the expression level of seven S100 members in HepG2.2.15 cells and HepG2 cells. (b) RT-PCR analysis. Total RNA extraction were performed using Trizol reagent. GAPDH were used as internal control. (B) Cells were incubated with serum-free media containing the indicated S100 proteins (10 μg/ml), or equal molar poly-arginine peptide (Control), or PBS control for 48 h. Bar, 20 μm. (C) Wound healing analysis of HepG2.2.15 cells following the same treatment for 48 h. S100-arg means S100 proteins tagged with polyarginine peptide; Arg peptide means polyarginine peptide; Vehicle control means PBS buffer. (D) Cells were incubated with serum-free media containing the indicated S100 proteins (10 μg/ml) or equal molar poly-arginine peptide (Control) for 48 h. F-actin were visualized by FITC-conjugated phalloidin. Bar, 20 μm.

3.2. Interaction of S100A10 with β-tubulin, β-actin and ANXA2

To further elucidate the experimental observation, we performed pull-down and Co-IP assays to analyze the potential interacting proteins of S100A10. As shown in Fig. 2A, though polyarginine-tag-based pull down and mass spectrometry analysis, four candidate proteins (hnRNP U, β-tubulin, β-actin and ANXA2) were identified. Because three of them (β-tubulin, β-actin and ANXA2) are important cytoskeleton components or regulators, they were further verified by Co-IP experiments. As a result, endogenous β-actin, β-tubulin and ANXA2 can be coimmunoprecipitated with S100A10-EGFP together (Fig. 2B).

3.3. Both S100A10 and ANXA2 promoted membrane protrusion

As shown in Fig. 3A, it was interesting that ANXA2 was almost undetectable in HepG2.2.15 cells by Western blot analysis although it can be visualized in above pull-down and IP assays. Both S100A10 and ANXA2 were negative in HepG2.2.15 cells in immunofluorescent staining assay, and re-expression of any one of them obviously promoted cell protrusion compared with GFP-transfected control cells (Fig. 3B–D). Fig. 3E showed that S100A10 was positive and co-located with ANXA2 in HepG2 cells.

3.4. Cdc42 activation contributed to S100A10-promoted membrane protrusion

Rho GTPases are known principally for their pivotal role in regulating the actin cytoskeleton and cell motility. So firstly, the total levels and activities of Cdc42, Rac1 and ROCK1 were analyzed by Western blot. As shown in Fig. 4A and B, the total level of Rac1 did-not change after stimulated with S100A10-arg, while its active form increased significantly. And both the total level and the active form of Cdc42 were up-regulated apparently after treatment with S100A10-arg. On the other hand, the total level of ROCK1 decreased significantly following treatment. By adding inhibitors of the three Rho GTPases, it was shown that ML141 treatment totally abolished the membrane protrusions induced by S100A10-arg, while NSC23766 had little effects. Consistent with the decrease of ROCK1 following S100A10-arg treatment, Y27632 not only failed to abolish the protrusions in S100A10-treated group but were prone to promote membrane protrusion in control group (Fig. 4C).

4. Discussion

Most cell types express several S100 proteins which are often present at very high concentrations. It is difficult to find the precise

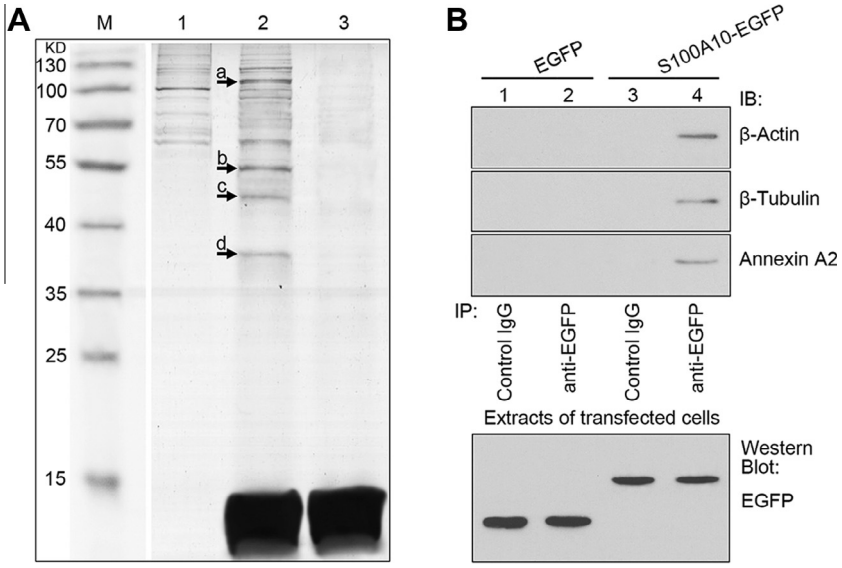

Fig. 2. Interaction of S100A10 with β-actin, β-tubulin and ANXA2.(A) Lysates from HepG2.2.15 cells were subjected polyarginine-tag-based pull down assays with purified S100A10-arg or polyarginine peptide as a control. Extracts of HepG2.2.15 cells were mixed with SP Sepharose coated with S100A10-arg protein (Lane 2) or polyarginine-peptide control (Lane 1). Bound proteins were co-eluted with polyarginine-tag fusion proteins in high salt concentration buffer (600 mM). The eluates were subjected to SDS–PAGE and Coomassie staining with purified S100A10-arg as control (Lane 3). Potential interacting protein bands were subjected to mass spectrometry analysis (a, hnRNP U; b, β-tubulin; c, β-actin; d, ANXA2). (B) S100A10-EGFP and EGFP control plasmids were transfected into HepG2.2.15 cells. Endogenous β-actin, β-tubulin and ANXA2 was coimmunoprecipitated with S100A10-EGFP. Extracts of transfected cells were used as input. Experiments were repeated at least three independent times.

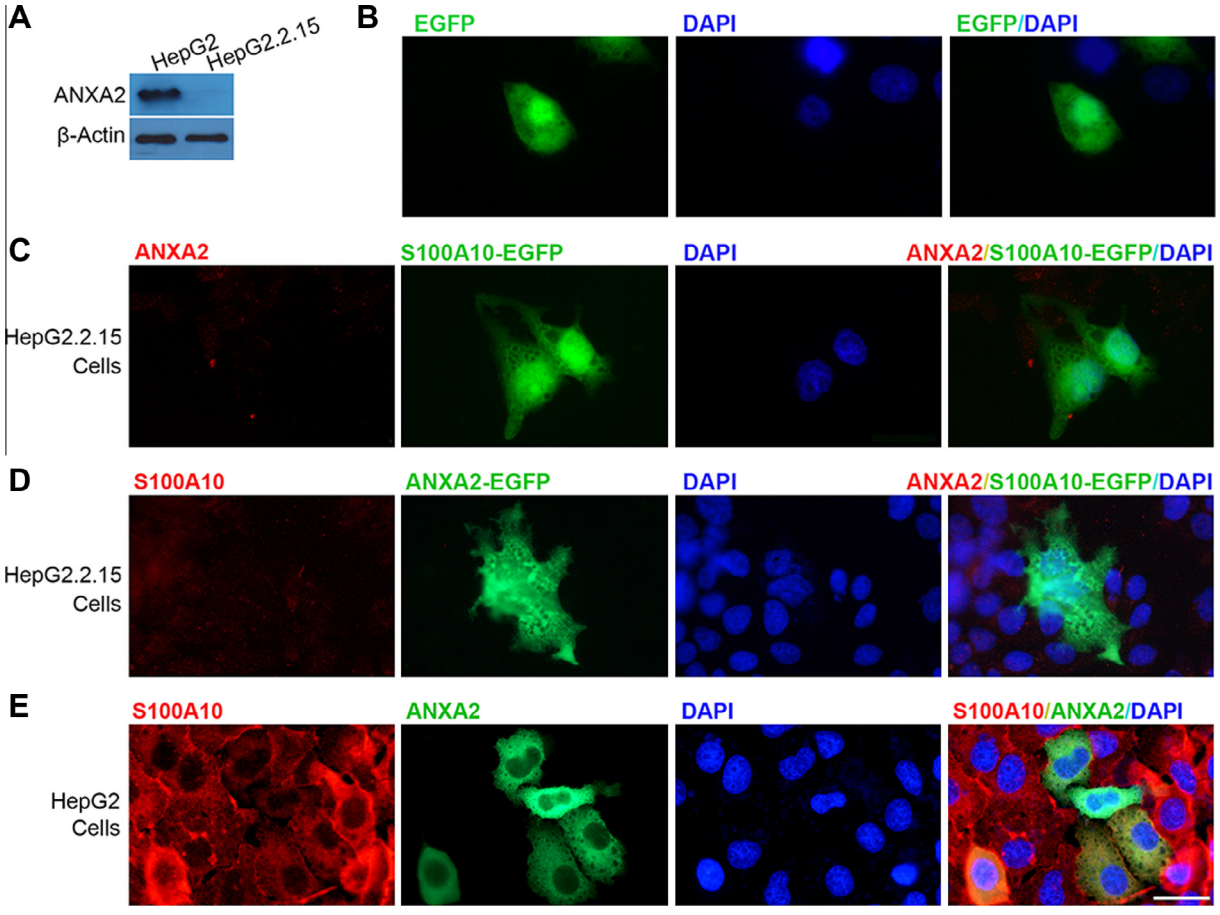

Fig. 3. Both S100A10 and ANXA2 promoted membrane protrusion. (A) Expression level of ANXA2 in HepG2.2.15 and its parental HepG2 cells. As shown, it was interesting that ANXA2 was almost undetectable in HepG2.2.15 cells by Western blot analysis although it can be visualized in the experiments of pull-down and IP assays. (B) HepG2.2.15 cells 48 h post-transfected with EGFP empty vector. (C) Immunofluorescent staining of ANXA2 in HepG2.2.15 cells transfected with S100A10-EGFP. (D) Immunofluorescent staining of S100A10 in HepG2.2.15 cells transfected with ANXA2-EGFP. (E) Immunofluorescent staining of endogenous S100A10 in HepG2 cells transfected with ANXA2-EGFP. Bar, 20 μm.

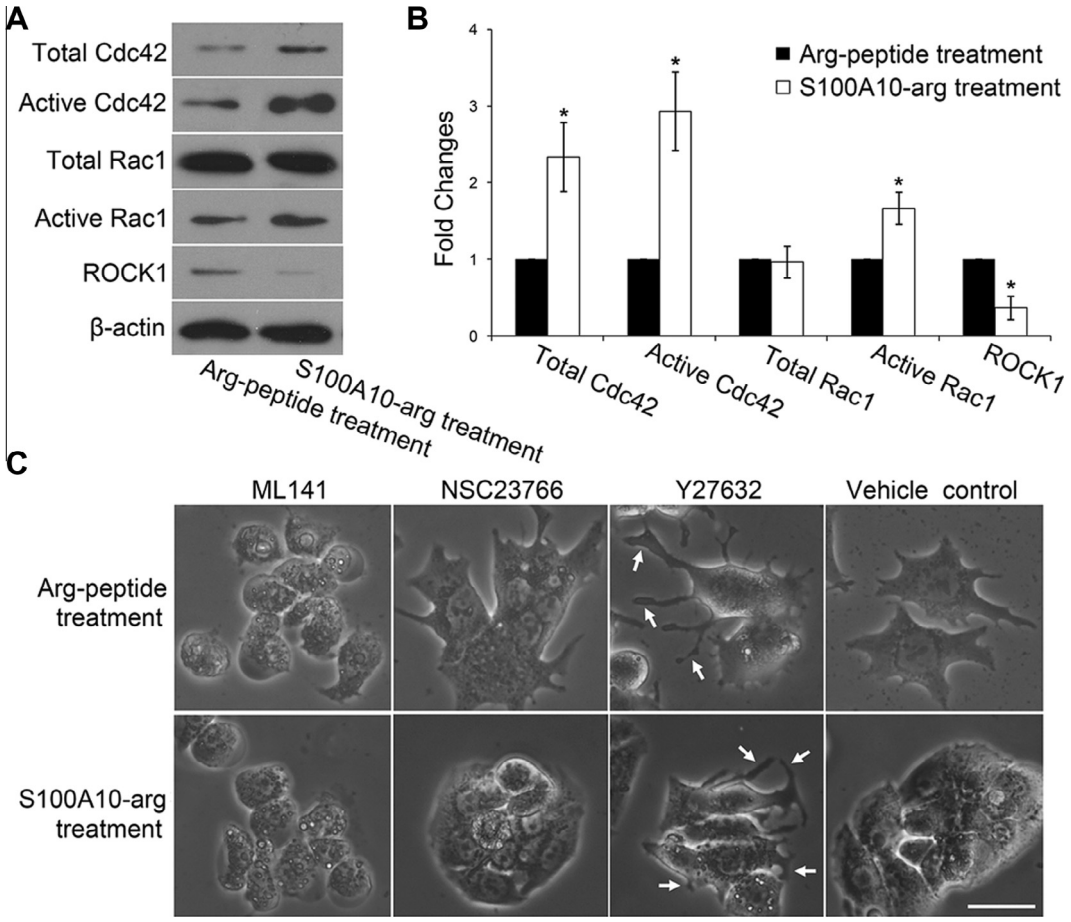

Fig. 4. Cdc42 activation contributed to S100A10-promoted membrane protrusion. (A) Representative Western blot analysis of total levels and activities of Cdc42, Rac1 and ROCK1 24 h post-stimulated with 10 μg/ml S100A10-arg or equal molar quantity poly-arginine peptide in HepG2.2.15 cells. (B) Densitometry analysis from three independent experiments. Expression alterations of Rho GTPase following treatment with S100-arg proteins were expressed as fold changes over polyarginine treated control. Data are presented as means ± SD. $^{*}P < 0.05$. (C) 24 h post-stimulated with S100A10-arg or poly-arginine peptide, cells were treatment with ML141 (10 μM, inhibitor of Cdc42), NSC23766 (10 μM, inhibitor of Rac1), Y27632 (10 μM, inhibitor of ROCK1) and DMSO vehicle control. As shown, ML141 treatment totally abolished the membrane protrusions induced by S100A10-arg while NSC23766 had little effects on the cell protrusion. Consistent with the decrease of ROCK1 following S100A10-arg treatment (A & B), Y27632 treatment not only failed to abolish the protrusions in S100A10-treated group but were prone to promote membrane protrusion in control group (arrows). All experiments were repeated at least three independent times.

functions of each S100 protein through over-expression or knock-down only one member due to functional redundancy and compensation [1–3]. Several S100 proteins have been reported to be implicated in cell cytoskeleton regulation and migration [12–16]. HepG2.2.15 cells exhibit a rounded morphology, and are defect in cell cilia, protrusion, spreading and migration, and interestingly the expression of seven S100 proteins in it are almost totally missing compared with its parental HepG2. Thus, as a cellular model, HepG2.2.15 will definitely facilitate the study of cell protrusion, migration and other cell motility related processes regulated by, but not limited to, S100 proteins. And through re-expressing some of the S100 proteins, in the present study we exhibit the convenience of this cell model for studying cell protrusion and migration.

Within cells, the majority of S100A10 resides in a heterotetrameric form containing 2 light chains of S100A10 and 2 heavy chains of ANXA2 [20]. ANXA2 is a member of the larger annexin family of Ca^{2+} and phospholipid binding proteins. ANXA2 has been proposed to play a key role in many processes including exocytosis, endocytosis, membrane organization, and also cell proliferation and migration [21,22]. S100A10 have been reported to be associated with several human diseases, such as depression, virus infection, inflammation and cancer [5,17–19]. Recently, it was shown that S100A10 was required for the organization of actin stress fibers, and protein interactions between surface ANXA2 and S100A10 mediate adhesion of breast cancer cells to microvascular endothelial cells [23,24]. Consistently, in the present study, we found S100A10 significantly promoted cell protrusion and migration. In particular, interestingly, although ANXA2 almost cannot be detected in HepG2.2.15 cells by western blot and immunofluorescences, it did appear in the pull down assays. And over-expression of ANXA2 promoted protrusion and spreading in HepG2.2.15 cells. So the expression of ANXA2 in a very low level may also mediate the partial effects of S100A10 on cell protrusion and migration.

Spatially controlled polymerization of actin is at the origin of cell motility and is responsible for the formation of cellular protrusions like lamellipodia. At the leading edge of the cell, actin is organized in parallel bundles which form filopodia and in a dense meshwork that forms ruffling lamellipodia and promotes forward movement [25]. In this study, we found re-expression of S100A10, S100A13, or S100A16 significantly promoted polymerization of F-actin. Through pull-down and IP assays, we shown that S100A10 may interact with ANXA2, β-actin and β-tubulin. ANXA2, both as a monomer and in the heterotetrameric complex with S100A10, has been shown to be capable of binding to and also bundling actin filaments [26]. It is known that actin, tubulin, intermediate filaments and their binding partners interact extensively and compose the cytoskeleton network. So the complex molecular

mechanisms underlying the regulation of cytoskeleton by S100 family members need further investigation.

Rho GTPases are molecular switches that control a wide variety of signal transduction pathways in all eukaryotic cells [27]. They are known principally for their pivotal role in regulating the actin cytoskeleton, cell polarity, microtubule dynamics, membrane transport pathways, transcription factor activity and cell proliferation [28,29]. At least 20 Rho family proteins have so far been identified in humans, and of these Rho and Rho kinase/ROCK, Rac and Cdc42 have been the most widely studied for their effects on cell cytoskeleton organization and migration. In this study, we found that the activities of Cdc42 and Rac1were up-regulated by S100A10-arg treatment, and ML141 treatment totally abolished the membrane protrusions induced by S100A10-arg while NSC23766 had little effect on the cell protrusion. On the other hand, consistent with the decrease of ROCK1 following S100A10-arg treatment, Y27632 treatment not only failed to abolish the protrusions in S100A10-treated group but was prone to promote membrane protrusion in control group. It has been reported that depending on the cell type and conditions, ROCK inhibitors can either inhibit or enhance cell migration [30]. It has also been reported that ROCK can limit membrane protrusions and the inhibition of ROCK with Y28632 induced multiple membrane protrusions in THP-1 monocytes [31]. These results suggested that Cdc42 activation may play a major role in S100A10-mediated cell protrusion in HepG2.2.15 cells.

S100A13 has been known to be a component of the pathway for the release of FGF1 [32]. Recent studies showed that S100A13 play a role in angiogenesis and tumor metastasis [33]. It was also reported that IL1α is exported through a non-classical release pathway involving the formation of a specific multiprotein complex, which includes IL1α and S100A13 [34]. S100A16 protein is a newly identified member of the EF-hand Ca^{2+}-binding proteins, and its function is largely unknown [35]. It has been reported that S100A16 was up-regulated in human tumors of bladder, lung, thyroid gland, pancreas, and ovary [36]. Recent researches showed that S100A16 may play a role in adipogenesis [37]. In the present study, we shown that, just like other S100 family members, S100A13 and S100A16 also have the functions in cytoskeleton regulation.

In conclusion, the present study showed that HepG2.2.15 can serve as a useful cellular model for studying cell protrusion, migration and other cell motility related processes regulated by, but not limited to, S100 proteins. Through this model, we proved the involvement of S100A10 in cytoskeleton regulation and cell migration, analyzed its potential interacting proteins, showed its effects on membrane protrusion may be partially dependent on Cdc42 activation, and found for the first time, to our knowledge, the implication of S100A13 and S100A16 in F-actin polymerization, cell protrusion and migration.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.05.010.

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