S100A4 downregulates filopodia formation through increased dynamic instability

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Cell migration requires the initial formation of cell protrusions, lamellipodia and/or filopodia, the attachment of the leading lamella to extracellular cues and the formation and efficient recycling of focal contacts at the leading edge. The small calcium binding EF-hand protein S100A4 has been shown to promote cell motility but the direct molecular mechanisms responsible remain to be elucidated. In this work, we provide new evidences indicating that elevated levels of S100A4 affect the stability of filopodia and prevent the maturation of focal complexes. Increasing the levels of S100A4 in a rat mammary benign tumor derived cell line results in acquired cellular migration on the wound healing scratch assay. At the cellular levels, we found that high levels of S100A4 induce the formation of many nascent filopodia, but that only a very small and limited number of those can stably adhere and mature, as opposed to control cells, which generate fewer protrusions but are able to maintain these into more mature projections. This observation was paralleled by the fact that S100A4 overexpressing cells were unable to establish stable focal adhesions. Using different truncated forms of the S100A4 proteins that are unable to bind to myosin IIA, our data suggests that this newly identified functions of S100A4 is myosin-dependent, providing new understanding on the regulatory functions of S100A4 on cellular migration.

Introduction

The formation of secondary tumor at distant metastatic sites from the original site of growth is a multi-step progression, which leads to poor prognosis for cancer patients. To acquire invasive properties, tumor cells undergo major changes in shape and motility. Significant changes in localized actin structures at the leading edges of tumor cells with polymerization and depolymerization under dynamic control allow them to protrude thin sheet-like lamellipodia or needle-like membrane extensions called filopodia. Filopodia are highly dynamic structures that extend and retract over very short time frames which act as sensory organelles for the extracellular matrix or other cells. Their protrusion is powered by actin polymerization at their tips while their overall structure is the result of tightly packed bundles of actin fibers cross linked by fascin.4 Such finger-like extensions play essential regulatory roles in cell spreading and adhesion during migration through the formation of nascent focal complexes (FX).6-9 Proper organization of these FX, and more importantly, tight adhesion to external cues through interactions of transmembrane receptors such as integrins or cadherin,5 result in the maturation of the complex into canonical focal adhesions (FA). Maturation of FA is accompanied by a large increase in their size, during a timely regulated process where markers like Paxillin and vinculin, among numerous others,9 interact at these focal contacts with each other and with actin stress fibers and integrins to provide a link to the extracellular matrix. Importantly, kinetic analyses have recently demonstrated the involvement of myosin II in the timely maturation of FX and FA.7,10 The biological mechanisms deciphering how myosin II functions in FA maturation remain to be fully unraveled, but could be through either the generation of tension, which directly affects the conformation of proteins in the adhesion complex or its cross-linking activity.11,13 Consequently, factors that can regulate both myosin II’s cross-linking activities and contraction are good candidates to govern FA formation.13,15

One factor known to influence myosin activity is the small calcium binding EF-hand protein S100A4. S100A4 can bind to the heavy chain of myosin IIA both in vitro and in vivo16-19 where it promotes the disassembly of pre-existing filaments or their actual assembly.21 It is thought that such recruitment to the actin/myosin fibers may be directly linked to S100A4’s ability to promote cell motility and metastasis.22,23 Consistently with this idea, elevated levels of S100A4 in the primary tumor have been correlated with its progression to a metastatic stage and to a poor prognosis for patient survival in breast cancer.26,27 S100A4 also induces a metastatic phenotype when transfected into benign rat mammary tumor cells in a transgene model of breast cancer.28,29 Both, the C-terminal region of S100A4 and its EF-hand motifs are required for interaction of S100A4 with myosin IIA and abrogation of its Ca2+ binding properties or truncation of its C-terminus lead to reduced metastasis promotion.24,25,30

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to determine whether increased levels of S100A4 proteins in cells could also affect their ability to promote wound closure using the established scratch assay (Fig. 1). Rama 37 cells stably expressing full-length S100A4 or their control counterparts were seeded in 12-well plates, scratched and monitored by time-lapse microscopy. Expression of wild-type S100A4 led to dramatic changes in cell migration and wound healing repair, with differences in gap closure observed as soon as 2 h post-scratching (data not shown). Eight hours following injury, the scratch was nearly completely filled in Rama 37 cells expressing high levels of S100A4, whereas control cells had only migrated about 50% across the wound (Fig. 1A). Given the short time of the experiment, these differences are unlikely to be due to increased proliferation by the S100A4 overexpressing cells. Real time dynamics of migration and wound repair were also measured using the CellIQ system and demonstrated that the Rama 37 cells expressing full length S100A4 were much more efficient in closing the wound, requiring nearly half the time than the control counterparts (Fig. 1B).

To establish whether such properties of S100A4 were linked to its ability to interact with myosin IIA, we tested if expression of nested truncated forms of S100A4 also affected wound closure. Deletion of the last two or six C-terminal amino acids, referred to as S100A4Δ2 and S100A4Δ6 transfected cell lines respectively, have been shown to contain similar levels to wild-type S100A4 in the Rama 37 transfected cell system. These truncated forms of S100A4 dramatically reduce the ability of S100A4 to bind to myosin IIA in vitro.24 Expression of either truncated versions of S100A4 did not accelerate the wound healing process and led to migration/gap closure dynamics similar to that for control cells (Fig. 1), demonstrating that the C-terminal portion of S100A4 that is required for myosin IIA binding is equally essential for its ability to promote wound repair.

High level of S100A4 leads to a reduction in focal adhesion formation. Having determined that overexpression of S100A4 leads to an increase in wound repair and cellular motility, we were interested to comprehend what happens at both the cellular and molecular levels to promote such changes. Cellular migration is driven by the ability of cells to interact with the extracellular matrix, through the formation of focal adhesion (FA) where transmembrane proteins of the integrin type interact with the actin cytoskeleton via adaptor proteins like paxillin and vinculin.

### Results

**Overexpression of S100A4 increases wound healing migration and overall cell motility.** The effects of S100A4 overexpression on promoting cell invasion are well documented.23-25 We sought to determine whether increased levels of S100A4 proteins in cells could also affect their ability to promote wound closure using the established scratch assay (Fig. 1). Rama 37 cells stably expressing full-length S100A4 or their control counterparts were seeded in 12-well plates, scratched and monitored by time-lapse microscopy. Expression of wild-type S100A4 led to dramatic changes in cell migration and wound healing repair, with differences in gap closure observed as soon as 2 h post-scratching (data not shown). Eight hours following injury, the scratch was nearly completely filled in Rama 37 cells expressing high levels of S100A4, whereas control cells had only migrated about 50% across the wound (Fig. 1A). Given the short time of the experiment, these differences are unlikely to be due to increased proliferation by the S100A4 overexpressing cells. Real time dynamics of migration and wound repair were also measured using the CellIQ system and demonstrated that the Rama 37 cells expressing full length S100A4 were much more efficient in closing the wound, requiring nearly half the time than the control counterparts (Fig. 1B). To establish whether such properties of S100A4 were linked to its ability to interact with myosin IIA, we tested if expression of nested truncated forms of S100A4 also affected wound closure. Deletion of the last two or six C-terminal amino acids, referred to as S100A4Δ2 and S100A4Δ6 transfected cell lines respectively, have been shown to contain similar levels to wild-type S100A4 in the Rama 37 transfected cell system. These truncated forms of S100A4 dramatically reduce the ability of S100A4 to bind to myosin IIA in vitro.24 Expression of either truncated versions of S100A4 did not accelerate the wound healing process and led to migration/gap closure dynamics similar to that for control cells (Fig. 1), demonstrating that the C-terminal portion of S100A4 that is required for myosin IIA binding is equally essential for its ability to promote wound repair.
that aggregate early on. We therefore sought to determine whether high level of S100A4 proteins would lead to changes in the localization of such foci in the Rama 37 cells. In immunocytochemical experiments clear localization of paxillin in clusters at the periphery of the cells was observed in the control cells (Fig. 2A-A'). Expression of high level of the full length S100A4 resulted in the total loss of foci formation around the cells (Fig. 2B-B'). The expression of either S100A4Δ2 or S100A4Δ6 in Rama 37 cells led to a total reversion of the S100A4 phenotype (Fig. 2C and D, respectively), with a clear recovery of paxillin containing FA and actin organization similar to that of the control cells. Analysis of another marker for FA formation, vinculin, generated comparable results (data not shown).

Overall actin organization was also dramatically affected by the expression of S100A4. While a high number of actin projections could be seen at the cell periphery in the control cells, there was a less organized actin network in the S100A4 overexpressing cells, with the distinct formation of large leading edges and a fibroblastic like morphology (Fig. 2B). The expression of either S100A4Δ2 or S100A4Δ6 in Rama 37 cells led to a total reversion of the S100A4 phenotype (Fig. 2C and D, respectively).

Taken together, these data demonstrate that expression of S100A4 induces critical changes in both FA formation and actin organization, including a significant reduction in actin projection and a change in cellular morphology from a filopodia to lamellipodia-dependent process. The ability of S100A4 to induce such changes is coincidental with its ability to interact with myosin IIa in vitro.

S100A4 reduces filopodia formation. The fact that induction of S100A4 expression resulted in significant reduction in actin projections away from the cell stroma prompted us to determine the exact nature of these extensions. Because protrusion of filopodia is powered by actin polymerization at their tips and their overall structure is the result of tightly packed bundles of actin fibers cross-linked by fascin, we analyzed the localization of the fascin protein in the filamentous structures. Immunofluorescent staining for fascin in the four different cell lines (Fig. 3) and quantitative analysis of the number of those stably attached projections (Table 1) were performed. An average number of 41.2 ± 8.6 protrusions from the cell cortex could be seen in the control cells. Staining for fascin was seen throughout the length of the extension with an accumulation at the tip (Fig. 3). Expression of the full length S100A4 led to a dramatic reduction to 3.5 ± 1.5 projections per cell, with a very small amount of fascin present at the cell periphery.

Figure 2. Overexpression of wild-type S100A4 but not the C-terminal truncated forms Δ2 or Δ6 prevents formation of mature focal adhesions. Rama 37 Control cells (A-A'), or cells expressing wild-type S100A4 cells (B-B'), S100A4Δ2 (C-C') and S100A4Δ6 (D-D') were grown for 48 h on glass coverslips coated with fibronectin prior to fixing and staining by immunofluorescence for paxillin and actin. Cells were mounted and viewed using confocal microscopy. (A'–D') correspond to focused regions of the cell. Scale bar = 50 μm.
shows that Rama 37 control cells, S100A4Δ2 and S100A4Δ6 expressing cells displayed similarities in overall number of FA present (89.4 ± 13.4, 93.6 ± 6.3 and 83.6 ± 12.1, respectively). Overexpression of full length S100A4, on the other hand, led to a significant reduction in the number of GFP-vinculin incorporated into FA (50.2 ± 11.5). These results confirmed our data from immunostaining analyses given above.

To assess more specifically the rates of FA assembly, the number of FA formed over 20 min was quantified (Table 2). The number of FA formed during this time course, in wild-type S100A4 protein expressing cells (11.4 ± 3.5), was significantly lower than in cell lines expressing either the S100A4Δ2 or S100A4Δ6 proteins (28.2 ± 6.9 and 23.6 ± 6.6 respectively). Surprisingly, Rama 37 control cells did not show significant difference in FA formation (14.4 ± 2.1) when compared with wild-type S100A4 expressing cells (p = 0.0704) although the mean value was slightly higher in the former cell line. Except for the Rama 37 control cells, the formation of focal contacts for the other cell lines correlates with the total number of FA present during the 20 min time period. However, one needs to keep in mind that for this representative experiment the number of analyzed cells is rather low, making it difficult to draw strong conclusions at this point.

Quantification of filopodia on live cells was performed by randomly halting images at one time point and by monitoring dynamics changes in filopodia and retraction fibers over subsequent times (Fig. 5). This analysis indicated that wild-type S100A4 expressing cells formed a significantly higher number of filopodia compared with S100A4Δ6 protein expressing cells and Rama 37 control cells (Table 2) in the course of our time analysis. This suggests that while overall number of stabilized filopodia are reduced in the S100A4 overexpressing cells, this is due to a reduction in their formation, but more possibly due to their fragility and high dynamics. Although the filopodia quantified in S100A4Δ2 shows no significant difference, the mean value is still lower than those of wild-type S100A4 protein expressing cells (Table 2). Since recent work in keratinocytes indicated that FA formation depend on stably adhered filopodia, we followed stably adhered filopodia containing GFP-vinculin by fluorescence

in the S100A4Δ6 expressing cells (19.7 ± 8.7). All together, these data demonstrate that S100A4 downregulates the number of filopodia and that this ability may be dependent on its myosin IIA binding properties.

Elevation of wild-type S100A4 protein expression causes overall reduction in focal adhesion dynamics. Our initial observations using immunofluorescence staining (Fig. 2) indicated that the number of FA is greatly reduced when the wild-type S100A4 protein is overexpressed. We were interested to determine whether such reduction in their formation was due to changes in their dynamicity. To validate this, all cell lines were transfected with GFP-vinculin expressing plasmid and analyzed over time by confocal microscopy. A representative image of the control Rama 37 cell is shown in Figure 4. From the collected images, focal contacts which were newly formed within 20 min (black arrows) were distinguished from those that were already present and both sets of information were recorded and analyzed (Table 2). The quantification analysis

Figure 3. High levels of full-length S100A4 reduce the number of adhered filopodia. Rama 37 control cells (A–A’), or cells expressing wild-type S100A4 (B–B’), S100A4Δ2 (C–C’) and S100A4Δ6 (D–D’) were grown for 48 h on glass coverslips coated with fibronectin prior to fixing and staining by immunofluorescence for fascin and actin. Cells were mounted and viewed using confocal microscopy. (A–D’) correspond to focused regions of the cell. Scale bar = 50 μm.
and phase contrast over time (Fig. 4, black arrow and Fig. 5). These data clearly show that FA are formed along filopodial actin bundles upon contact with the lamellipodium. Furthermore filopodia dependent formation of FAs was detected in all four cell lines (see Movies S1–S4 for all cell lines).

**Discussion**

The S100A4 protein has become a prominent player during cancer progression and the metastasis stage since it is overexpressed at high concentration in a vast repertoire of malignancies ranging from melanoma to non-small cell lung carcinoma and cancers of the breast and stomach.\(^ {32-35} \) Whereas S100A4 has not been directly implicated with the tumorigenesis process as its overexpression does not result in uncontrolled cellular growth,\(^ {36} \) it is thought that S100A4 is a key player regulating the migration or invasion steps required in the metastatic cascade. Thus high expression of S100A4 increases the migratory properties of transformed fibroblasts and non-metastatic adenocarcinoma cell line.\(^ {22,23,37} \) Conversely, ablation of S100A4 expression in tumor cells correlates with decreased cellular motility.\(^ {38,39} \) There is little doubt that S100A4 can promote cell migration in both tumor and normal cells; however, the underlying biological mechanism for such changes is still poorly understood. To shed more lights on some of the mechanisms that could explain the gain in migratory properties, we have utilized a non-metastatic rat cell lines expressing truncated versions of the S100A4 protein. We show that forcing high levels of expression of wild-type S100A4 reduces the number of projections per cell

| Cell lines          | Number of projections per cell ± s.e. (n = 5) | p value* | p valueb | p valuec |
|---------------------|-----------------------------------------------|----------|----------|----------|
| Rama 37 control     | 41.20 ± 8.58                                  |          |          |          |
| Rama 37 S100A4 WT   | 3.50 ± 1.52                                   | 0.0001   |          |          |
| Rama 37 S100A4Δ2    | 3.17 ± 1.11                                   | 0.0008   | 0.0004   | 0.0062   |
| Rama 37 S100A4Δ6    | 19.71 ± 8.69                                  |          |          |          |

Rama 37 control cells, cells expressing wild-type S100A4, S100A4Δ2 and S100A4Δ6 were fixed and stained for fascin and actin after seeding on fibronectin coated coverslips and growth for 48 h. Data shown are means ± s.e. corresponds to the average number of projection containing fascin observed per cells. *p value obtain from Student t-test where total number of projections present in Rama 37 Control were compared with Rama 37 S100A4WT, Rama 37 S100A4Δ2 and Rama 37 S100A4Δ6. **p value obtain from Student t-test where total number of projections present in Rama 37 S100A4Δ6 were compared with Rama 37 S100A4Δ2. "p value obtain from Student t-test where total number of projections present in Rama 37 S100A4Δ2 were compared with Rama 37 S100A4Δ6.

As cells migrate, they organize their leading edge in three chronological steps; these involve the initial formation of cell protrusions (lamellipodia and/or filopodia), attachment of the leading lamella to extracellular cues and formation of focal contacts at the leading edge that mature through the production of tensile forces. Our initial observations have demonstrated the formation of abundant actin and fascin containing protrusions at the cell’s leading edge (Figs. 2B and 3B) in the control Rama37 cells when compared to full length S100A4 overexpressing cells, which only show very little of such extensions. When two truncated forms of S100A4 which are unable to bind to myosin IIA (mutants S100A4Δ2 and S100A4Δ6) are overexpressed, they do not induce such changes, suggesting that this induction is probably dependent on myosin IIA. Such findings are consistent with those of Bresnick’s works, which have shown that S100A4 can
Table 2. Expression of wild-type S100A4 protein causes overall reduction in focal adhesion

| Cell lines (protein encoded in expression vector) | Number of focal adhesion present (over 20 min) ± s.e. (n = 5) | p value<sup>a</sup> | Number of focal adhesion formed (within 20 min) ± s.e. (n = 5) | p value<sup>b</sup> | Number of filopodia present ± s.e. (n = 9) | p value<sup>c</sup> |
|-----------------------------------------------|-------------------------------------------------|-----------------|-------------------------------------------------|-----------------|----------------------------------|-----------------|
| Rama 37 Control                              | 89.4 ± 13.4                                     | 0.0028          | 14.4 ± 2.1                                      | 0.0705          | 10.9 ± 2.71                      | 0.0271          |
| Rama 37 S100A4 WT                            | 50.2 ± 11.5                                     | 0.0001          | 11.4 ± 3.5                                      | 0.0326          | 17.3 ± 7.2                       | 0.0540          |
| Rama 37 S100A4Δ2                             | 93.6 ± 6.3                                      | 0.0100          | 28.2 ± 6.9                                      | 0.00410         | 14.6 ± 6.9                       | 0.0540          |
| Rama 37 S100A4Δ6                             | 83.6 ± 12.1                                     | 0.0001          | 23.6 ± 6.6                                      | 0.00410         | 10.9 ± 4.5                       | 0.0307          |

Rama 37 control cells, cells expressing wild-type S100A4, S100A4Δ2 and S100A4Δ6 were transfected with pGZ21 GFP-vinculin expressing plasmid and then seeded on fibronectin coated coverslips for 24 h before analysis. Cells were analyzed over a 20 min time period in phase contrast and fluorescence. Quantification of the total numbers of FA present, newly formed FA within the 20 min time frame, as well as the number of forming filopodia was performed using the Image J software. <sup>a</sup>p value obtain from Student t-test where total number of focal adhesions present during 20 min live cell imaging of Rama 37 Control, Rama 37 S100A4Δ2 and Rama 37 S100A4Δ6 were compared with those of Rama 37 S100A4 WT. <sup>b</sup>p value obtain from Student t-test where total number of focal adhesions formed during 20 min live cell imaging of Rama 37 Control, Rama 37 S100A4Δ2 and Rama 37 S100A4Δ6 were compared with those formed during imaging of Rama 37 S100A4 WT. <sup>c</sup>p value obtain from Student t-test where total number of filopodia present on Rama 37 Control, Rama 37 S100A4Δ2 and Rama 37 S100A4Δ6 were compared with those on Rama 37 S100A4 WT.

Figure 5. Establishment of stably adhered filopodia, retraction fibers and unstable filopodia in Rama 37 cells Rama 37 S100A4Δ6 cells transfected with pGZ21 GFP-vinculin expressing plasmid were seeded on fibronectin coated coverslips for 24 h before analysis. Cells were analyzed over a 20 min time period in phase contrast and fluorescence to quantify stably adhered filopodia (white arrow, (A), retraction fibers (B) and unstable filopodia yellow arrow, (C). Area (A) was enlarged to show the formation of stable filopodia (top part), the subsequent maturation of FA (middle part) and the overlay of both time lapse images (bottom part) over time (A). Area (B) was enlarged with a white line drawn above to show the retracting FA right behind the retraction fibers over time (B). Area (C) was enlarged to show the unstable formation of filopodia over time (C). For (A–C), the top part is shown in phase contrast, the middle part in fluorescence and the bottom part as overlays of both images. Time points are given in seconds. Note that the time lapse has shorter intervals for (C) whereas (A and B) have a similar time lapse. First image of all analysis corresponds to the main figure (time zero) and then subsequent time lapse images are shown. For better visualization, phase contrast images were sharpened and GFP-images were background-subtracted and enhanced in contrast before overlaying the images. Scale bar = 20 μm.
induce forward protrusions during chemotaxis and that enrichment of the myosin IIA-S100A4 complex occurs at the leading edge of polarized cells. When high levels of wild-type S100A4 are induced, significant reorganization of actin structures also occurs, with the distinct formation of large leading edges and a fibroblastic like morphology (Fig. 2B). Interestingly, the lamella and stress fibers were again clearly visible in cells containing high levels of the truncated forms of S100A4 (mutants S100A4Δ2 and S100A4Δ6) indicating that S100A4 may regulate their formation through its interaction with myosin IIA. This finding is in agreement with previous work which has shown that myosin IIA has been implicated in regulating the reorganization of actin network in the lamellipodia of spreading cells. Our data suggest that S100A4 may be one of the cellular regulator of such process.

Our data also shows that formation of cellular protrusion is modified upon expression of wild-type S100A4 when analyzed in fixed cells. Control cells demonstrated a large number of filopodia formation while high levels of S100A4 resulted in significant reduction (Fig. 3 and Table 1). However, fixed cells only allow the characterization of stably adhered filopodia while unstable ones are lost due to the analysis procedure. By contrast, live cell imaging quantification of filopodia numbers indicated, that in fact, the number of newly formed filopodia in all cells was not greatly affected. In fact, filopodia formation was even accelerated in the presence of high levels of S100A4 (Table 2). These data therefore suggest that S100A4 inhibits in some way the adhesion of stably adhered filopodia, although their actual initial formation rate is higher. During formation of filopodia, myosin-X acts as a catalyst by bringing filaments together to facilitate bundling at the actin-membrane interface. S100A4 has so far been shown to interact directly with myosin IIA and, with much less efficiency to IIB but not at all with myosin V. It is unclear, at this time, whether S100A4 can influence myosin X activity but it is tempting to speculate that such interactions, if true, could induce significant changes in stabilization and promotion of filopodia.

Previous experiments by Schäfer et al. using migrating keratinocytes have shown that there is a clear dependence of stable FAs on stably adhered filopodia. Since we could also observe a filopodia-dependent formation of FAs and a reduced number of FAs upon expression of S100A4 one is tempted to argue that a similar dependence between filopodia and FAs also exists in our cell types. However, at the current state we cannot finally answer this hypothesis. This is mainly because of the different migration type of our cells compared with the stably polarized migrating keratinocytes used in previous work. Cancer cells as used here permanently form lamellipodial extensions in all directions but barely move the center of the cell over time. Therefore, filopodia permanently switch into retraction fibers making it very difficult to distinguish whether FAs are formed right behind filopodia or just enlarged because of a new outgrowth over former retraction fibers. Here, time lapse analyses over very long time frames might solve this question which is out of the scope of this work.

Our analysis therefore demonstrates that high levels of S100A4 induce the formation of many nascent filopodia, but that only a very small and limited number of these can stably adhere. We suggest that this phenomenon is the result of inhibited myosin function leading to filopodia and filopodial complexes that cannot be stabilized. Such possibility is supported by recent findings demonstrating the importance of myosin II in the timely maturation of focal complexes (FX) and FA. Such lack of maturation would, therefore, lead to filopodial retraction and a low number of FAs resulting in the observed effects on morphology and migration. The reasons for S100A4 to induce such effects through myosin IIA remain to be explicitly demonstrated. It is thought that S100A4 can function as a myosin-IIA inhibitor in vivo, since it has been reported to inhibit the actin-activated ATPase of myosin-IIA. Inhibiting the molecular motor properties of myosin IIA would lead to a loss of tension on actin stress fibers, resulting in significant changes in the conformation of proteins in the adhesion complex. Alternatively S100A4 has been shown to impair myosin polymerization and its organization into highly defined filaments. The aggregation of myosin IIA into fibers is thought to induce cross-linking activities on adjacent actin filaments, a process that is also required for FX maturation. Consequently, high levels of S100A4 could similarly affect FX formation through the disruption of myosin IIA fibers.

Although our data argues that S100A4 may regulate filopodia extension as well as FX and FA formation through its potential interaction with myosin, given that none of the truncated forms of S100A4 which have impaired myosin binding could reciprocate, there could be other potential candidates that we should factor in for such regulation. Other prospective targets for S100A4 are the cytoskeletal proteins, tropomyosin and F-actin. The consequences of their interactions are not known, especially in vivo, but their prominent roles in both filopodia formation and FA maturation may yet prove to be influential in delivering the enhanced migratory activities, as well as filopodial organization induced by S100A4.

Material and Methods

Cell culture and lines. The rat mammary (Rama 37) non-metastatic benign tumor-derived cell line expresses barely detectable levels of S100A4 mRNA and protein. The four cell lines used during this work were: (a) the control cells transfected with the empty pBKCMV vector, (b) the cell line transfected with the full length S100A4 open reading frame inserted in the pBKCMV expression plasmid (S100A4WT), (c) and (d) cell lines S100A4Δ2 and S100A4Δ6, which were obtained by transfection of the expression vectors for S100A4 protein with C-terminal truncations lacking amino acids 100–101 or 96–101 respectively. Both truncations have been shown to affect the ability of S100A4 to bind and interact with myosin IIA heavy chain using Biosensor analysis.

The cells were routinely cultured in a humidified atmosphere at 37°C, 5% (v/v) CO2, 95% (v/v) air in Dulbecco minimal Eagle’s medium (DMEM) supplemented with 2 mM L-glutamine (50 U per ml) penicillin/streptomycin (50 mg per ml), 10% (v/v) fetal bovine serum, 5 ng/ml insulin and hydrocortisone and 0.8 mg/ml genicin (G418).

Scratch assay and cell migration measurement. Cell migration of the various cell clones was assessed using scratch assays.
10^5 cells were seeded into each well of 12-well tissue culture plates and left to grow for 48 h when they reached 90% confluency. Confluent monolayers were scored with a sterile pipette tip to leave a scratch of approximately 0.4–0.5 mm in width. Culture media was removed and replaced with fresh media. Wound closure was monitored by collecting digitalized images at various time intervals after the scratch. Digitized images were captured in phase contrast with inverted epifluorescence microscope (DMI14000B, Leica) using a N-Plan 10x/0.25 PH1 objective. Digitized images were also obtained using the Cell IQ automated image capture system, (Chip-Man Technologies) in which pre-selected fields were imaged using phase contrast microscopy on a continuous loop until wound closure was complete. The Cell-IQ Analyzer Software, using Machine Vision Technology, was able to detect and measure remaining areas deprived of cells/migrating sheets. Data from this equipment has been presented as the velocity of wound closure, i.e., the time that was required for full closure to be completed.

**Immunofluorescence staining of cultured cells.** Rama 37 cells stably expressing either wild-type S100A4, S100A4Δ2, S100A4Δ6 or cells transfected with empty pBKCMV vector were plated at a concentration of 1.5 x 10^4 cells/well onto fibronectin-coated (2.5 μg/cm^2) glass coverslips in 24-well plates. Cells were grown for 48 h prior to being washed once in cytoskeleton buffer [CB: 150 mM NaCl, 5 mM MgCl_2, 5 mM EGTA, 5 mM glucose, 1 g/l streptomycin, 10 mM 2-(N-morpholino) ethanesulfonic acid, pH 6.1] and being fixed with 3.7% (w/v) paraformaldehyde in CB at 37°C for 20 min. Cells were then further washed in glycine-CB (30 mM) before being permeabilized with 5% (v/v) Triton X-100 in CB for 2 min and blocked with blocking solution [5% (v/v) goat serum in CB] for 60 min at room temperature. Samples were incubated with primary antibodies vinculin (Sigma), paxillin (Invitrogen) or fascin (Chemicon, Millipore) (dilution between 1/60, 1/100 and 1/500, respectively) in blocking solution [1% (v/v) goat serum in CB] for 45 min at 37°C. After washing three times with the blocking solution, cells were incubated with the appropriate secondary antibodies, anti-rabbit and anti-mouse antibodies labeled with FITC (Dako) in blocking solution for 45 min at 37°C with a dilution of 1:100. For actin staining, rhodamine phalloidin (Invitrogen) and left to grow for 48 h prior to pGZ21 GFP-vinculin expressing plasmid (a kind gift from Benjamin Geiger, Weizmann Institute of Science) transfection with jetPei™. The transfectants were trypsinated and reseeded at a density of 5 x 10^4 cells into a customized 170 μm-thickness glass bottom Petri dish, pre-coated with 2.5 μm/cm^2 of fibronectin (BD Biosciences) for 30 min at 37°C. The analyses were performed 24 h post-seeding with a confocal microscope (LSM710, Zeiss) using EC Plan-Neofluar 40x/1.3 OIL Ph3 objective (Zeiss) at 37°C and 5% CO_2. The duration for single cell analysis was set to 20 min per cell with 10 s intervals. Phase contrast and GFP imaging was taken with argon ion laser line with a wavelength of 488 nm and 2.4–2.8% of laser intensity. All the parameters were kept constant throughout the analyses. Images obtained were optimized (background subtraction and contrast adjustment) with ImageJ for better visualization.

**Statistical analysis.** Data presented in this study are listed as mean ± standard error (SE). These data were tested for normal distribution by Kolmogorov-Smirnov test using Analyse-it software. As all data were shown to be normally distributed the Student t-test was used to determine significant differences between samples.

**Disclosure of Potential Conflict of Interest**

No potential conflicts of interest were disclosed.

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**Note**

Supplemental material can be found at: www.landesbioscience.com/journals/celladhesion/article/17773

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