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ORIGINAL ARTICLE

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Metastasis-inducing protein S100A4 interacts with p53 in the nuclei of living cells

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Abstract

Objective: S100A4 can bind to p53 *in vitro*. However, it is unclear whether the proteins interact in the nuclei of living cells.

Methods: Initially, using an optical biosensor, the ability of CFP-S100A4 to bind with p53-YFP was examined *in vitro*. The fluorescence resonance energy transfer (FRET) technique using laser confocal microscopy was applied to detect the interaction of S100A4 with p53 in living cells.

Results: Imaging FRET in HeLa cells expressing S100A4 and p53 showed an increase in cyan fluorescent protein fluorescence intensity, after photobleaching yellow fluorescent protein in cell nuclei. In comparison, the imaging of cell nuclei expressing mutant S100A4-C and p53 or S100A4 and yellow fluorescent protein as the negative control showed the occurrence of FRET, and demonstrated that the interaction between S100A4 and p53 occurred in the nuclei of living HeLa cells. However, FRET did not occur in the cytoplasm of cells, showing that S100A4 did not interact with p53 in the cytoplasm.

Conclusion: S100A4 can interact directly with p53 in the nuclei of living cells. This might be a molecular basis for metastasis-inducing S100A4 *in vivo*. The results also suggest that the inhibition of the calcium-binding site of S100A4 might be a possible anticancer therapy.

KEYWORDS

fluorescence resonance energy transfer, interaction, metastasis, p53, S100A4

1 | INTRODUCTION

S100A4 is a member of the S100 family of Ca^{2+}-binding proteins, a polypeptide of 101 amino acids, and has a molecular mass of ~11.5 kd.[1] The expression of this protein is significantly associated with a reduced survival rate for patients with cancers.[2–5] Experiments with different transgenic mice or rats models have provided evidence of the effect of S100A4-induced tumor metastasis.[6-9] S100A4 is therefore a potential target for antimetastatic therapy.[10–12]

S100A4 protein has been reported to interact in a calcium-dependent manner with multiple molecular target proteins, such as the heavy chain of non-muscle myosin, liprin1, and p53 *in vitro*, all of which might be involved in its metastasis-inducing abilities.[13] Although we have found that the C-terminal end of S100A4 plays a key role in the interaction with the heavy chain of non-muscle myosin and the metastasis-inducing tumor *in vitro* and *in vivo*, the mechanism of metastasis induction by S100A4 *in vivo* remains unknown.[12,14] S100A4 has been reported to show a Ca^{2+}-dependent interaction with p53 *in vitro*.[15] Although S100A4 was shown to be colocalized with p53 in individual cancer cell nuclei, there is no direct evidence to confirm the interaction of S100A4 with p53 in living cell nuclei.[16]

The interaction of S100A4 with p53 in living HeLa cells has now been investigated using confocal microscopy and the fluorescence resonance energy transfer (FRET) technique. FRET, the non-radiative transfer of photon energy from an excited fluorophore (donor) to a neighboring fluorophore (acceptor), occurs only when the donor and acceptor are in close proximity (<100 A). This approach can be used to measure nanometer scale distances, and has made it possible to use

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this technique to detect protein–protein interactions in the nuclei of living cells.[2,17]

The aim of the present study was to find out whether S100A4 interacts with p53 in the nuclei of living cells, and thereby acts as a potential therapeutic strategy.

2 | METHODS

2.1 | Plasmids

Living Colors plasmid vectors pEYFP and pECFP were obtained from Clontech (Palo Alto, CA, USA). The amplified, recombinant, full-length human *p53* mRNA was cloned between the HindIII and BamHI sites of a Living Color EYFP vector, such that a fusion protein, consisting of enhanced yellow fluorescent protein (EYFP) attached to p53 (p53-YFP), was encoded. The construction of pCFP-S100A4 plasmids, containing S100A4 cDNA, was as previously described.[14]

2.2 | Production of recombinant fusion proteins in *Escherichia coli*

The coding sequences of CFP-S100A4, CFP-S100A4-C, p53-YFP were excised from the Living Color expression vectors and were inserted into an engineered pET16 vector between an NheI site (inserted adjacent to the existing NdeI site) and a BamHI site, as previously described.[14] The open reading frames of the fusion proteins were confirmed through automated DNA sequencing. Recombinant fusion proteins were expressed in the *E. coli* strain, BL21-DE3, and purified using HisBind Resin (Novagen, Madison, WI, USA).

2.3 | Target binding by CFP-S100A4 and p53-YFP

To assess either S100A4 or mutant S100A4-C and p53 interaction *in vitro*, the interactions between the CFP-S100A4s fusion proteins and the p53-YFP fusion protein were determined using a dual-channel IAsys resonant mirror biosensor (Thermo Electron, Basingstoke, UK), as described previously.[14]

2.4 | Cell culture and transfection

HeLa cervical carcinoma cells were grown and transfected, as described previously.[14]

2.5 | Fluorescence microscopy and FRET analysis

HeLa cells (3×10^5) were transiently transfected with plasmids encoding CFP-S100A4, CFP-S100A4-C, and p53-YFP using FuGene (Roche, Basel, Switzerland). Fluorescence was visualized using a confocal laser-scanning microscope (Zeiss-LSM-510 META; Carl Zeiss, Oberkochen, Germany). Cyan fluorescent protein (CFP) fluorescence was excited at 458 nm and signals were detected, with the META detector set at 490–520 nm. YFP fluorescence was achieved at an excitation of 514 nm and detected at 559–615 nm. For the acquisition of FRET signals, CFP was excited with a laser, set to 458 nm, and FRET signals were detected in the YFP channel of the META detector set to 559–615 nm. Protein interactions were further confirmed by acceptor photobleaching.[18] Cells were treated by scanning a region of interest 100 times, using a laser set to 514 nm (YFP settings) at 100% intensity. Before and after treatment, CFP images were collected to monitor changes in donor fluorescence. Based on this procedure, FRET efficiency was calculated by photobleaching of the acceptor (YFP), resulting in an increase in donor fluorescence (CFP). The average intensity of CFP fluorescence, for five points' time after and before photobleaching YFP, was analyzed.

3 | RESULTS

3.1 | Identification of S100A4 interacting with p53 *in vitro* using an optical biosensor

To examine the effect of wild-type S100A4 binding to p53, an optical biosensor was used to detect their interaction *in vitro*. The recombinant p53-YFP was immobilized onto the amino silane surface of the biosensor cuvette. As the mS100A4-C, a variant S100A4 protein from another study,[12] has lost the functions of wild-type S100A4 described previously, it was used as a negative control. CFP-S100A4 and CFP-mS100A4-C, at different concentrations, were added individually to the p53-YFP surface in the presence of 0.5 mM calcium iron. The results show that the extent of wild-type S100A4 binding to p53 is significantly higher than that of variant mS100A4-C (Figure 1a,b), indicating that wild-type S100A4 can interact with p53 *in vitro*. However, mS100A4-C showed a virtually undetectable interaction with p53 *in vitro* ($P < 0.0001$, Student's test; Figure 1a,b).

3.2 | Imaging FRET by photobleaching YFP fluorescence in cells transfected with CFP-S100A4 and p53-YFP

To test whether S100A4 directly associates with p53 in living cells, cells (Figure 2) co-transfected with CFP-S100A4 and p53-YFP cDNA were detected by measuring the intensity of CFP fluorescence before and after photobleaching YFP fluorescence, using FRET imaging through laser confocal microscopy. The results showed that the CFP fluorescence intensity in the cell nuclei (red cycle) increased after photobleaching YFP fluorescence (top, post-), when compared with that before photobleaching YFP fluorescence (top, pre-; Figure 2a). This increase indicated the occurrence of FRET, and showed that the interaction between S100A4 and p53 occurred in living HeLa cells. However, no increase in CFP fluorescence intensity in the cell cytoplasm (yellow cycle) was observed after photobleaching of YFP fluorescence (Figure 2b), suggesting that FRET did not occur in the cytoplasm. Therefore, S100A4 did not interact with p53 in the cytoplasm of cells co-transfected with CFP-S100A4 and p53-YFP. These observations were further verified using six HeLa cells (Figure 3a,b).

Due to the loss in the ability of mS100A4-C to bind its target proteins, such as NMMHCIIA, *in vitro* and *in vivo*,[12,14] and p53, *in vitro* (Figure 1), transfected cells (Figure 4), expressing both CFP-mS100A4-C and p53-YFP proteins, were also examined by measuring CFP

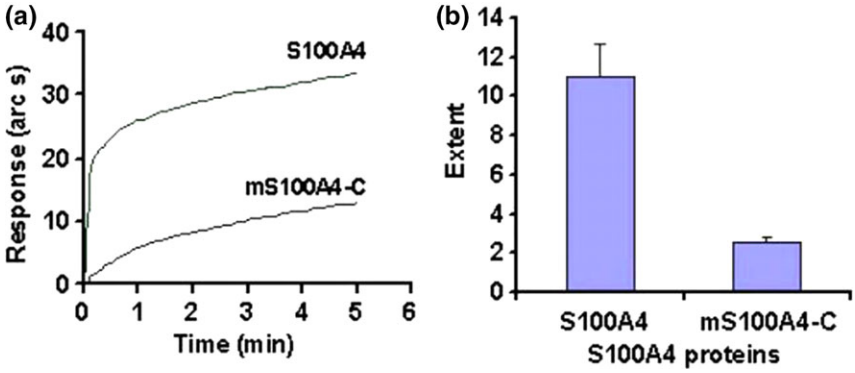

FIGURE 1 Interaction of wild-type S100A4 with immobilized p53 using an optical biosensor. The extents of binding of wild-type S100A4 (S100A4) and mutant S100A4-C (mS100A4-C) to immobilized p53 were measured using an optical biosensor. Purified p53-YFP was immobilized on a planar amino silane surface. After the addition of CFP-S100A4 and S100A4-C-YFP in two biosensor cuvettes, respectively, the extents of binding of the S100A4s were followed in real-time for at least, 5 min. Data were collected every 0.3 s during the course of the experiment and analyzed using FastFit software ($n = 3$ for each bar)

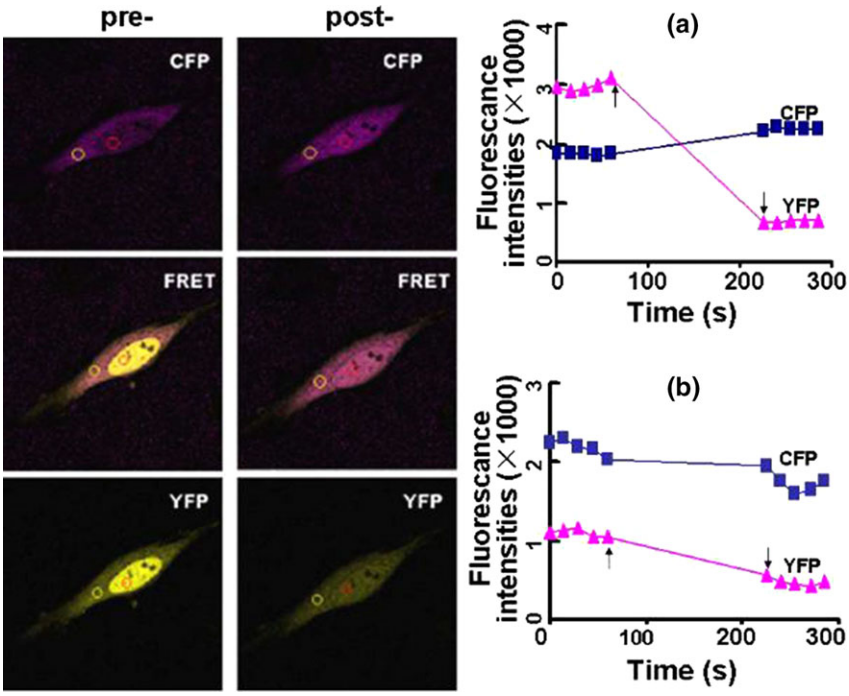

FIGURE 2 Cyan fluorescent protein (CFP), fluorescence resonance energy transfer (FRET), and yellow fluorescent protein (YFP) images taken before and after photobleaching in HeLa cells co-transfected with CFP-S100A4 and p53-YFP. A HeLa cell transiently co-transfected with the CFP-S100A4 and p53-YFP cDNA construct was illuminated with a Zeiss two-photon laser and examined 18–24 h after transfection by Zeiss confocal microscopy. Two regions of interest (red and yellow cycles) were analyzed for CFP fluorescence intensity after (post-) and before (pre-) photobleaching of YFP. The photobleaching image of YFP fluorescence in the cell is shown in the lower panels (YFP), CFP fluorescence in the upper panels (CFP), and total fluorescence intensity distributions in the middle panels (FRET). (a,b) Changes in CFP and YFP fluorescence intensities of two regions of interest are shown. Changes in CFP and YFP fluorescence intensities before and after photobleaching YFP in the (a) cell nucleus and (b) cytoplasm. The locations of upward and downward arrows indicate the beginning and end of photobleaching of YFP, respectively

fluorescence intensity after photobleaching YFP fluorescence. As a negative control, the results, as expected, showed a decrease in CFP fluorescence intensity in the cell nuclei (red cycle, pre/post-, Figure 4a) and cytoplasm (yellow cycle, pre/post, Figure 4B) after photobleaching YFP fluorescence, compared with that before photobleaching YFP fluorescence.

Data from nine HeLa cells (Figure 5) showed no significant changes in the mean CFP fluorescence intensities between pre-photobleaching YFP and post-photobleaching YFP in the nuclei (Figure 5a,b) or cytoplasms (Figure 5c,d) of cells co-transfected with CFP-S100A4-C and p53-YFP cDNA. This suggests a lack of occurrence of both FRET and the interaction between the mutant S100A4-C and p53 molecules in the nuclei and cytoplasms of living HeLa cells co-expressing the CFP-S100A4-C and p53-YFP proteins.

Using the same approach for FRET signal acquisition and analysis, the interactions between CFP-S100A4 and YFP, CFP and p53-YFP, and the paralleling negative controls were examined separately. Cells co-transfected with CFP-S100A4 and YFP, and CFP and p53-YFP, like the paralleling negative control cells co-transfected with CFP-mS100A4-C and p53-YFP, showed decreases in CFP fluorescence intensities after photobleaching YFP fluorescence in the nuclei and cytoplasm (data not shown), indicating the absence of FRET, and lack of interactions between CFP-S100A4 and YFP, and CFP and p53-YFP in transfected living cells.

4 | DISCUSSION

FRET-based techniques provide a way of studying the interaction between proteins *in vivo*, and are potentially useful for studying protein interaction dynamics as well.[14,19] In the present study, we confirmed that neither S100A4 nor CFP-S100A4 interacted with YFP *in vitro* and *in vivo*, as described previously,[14] and verified that the fusion of the fluorescent proteins did not affect the ability of either S100A4 or its target proteins to interact. Simultaneously, the negative control, variant S100A4-C, was used alongside the experimental samples so as to monitor false positives.

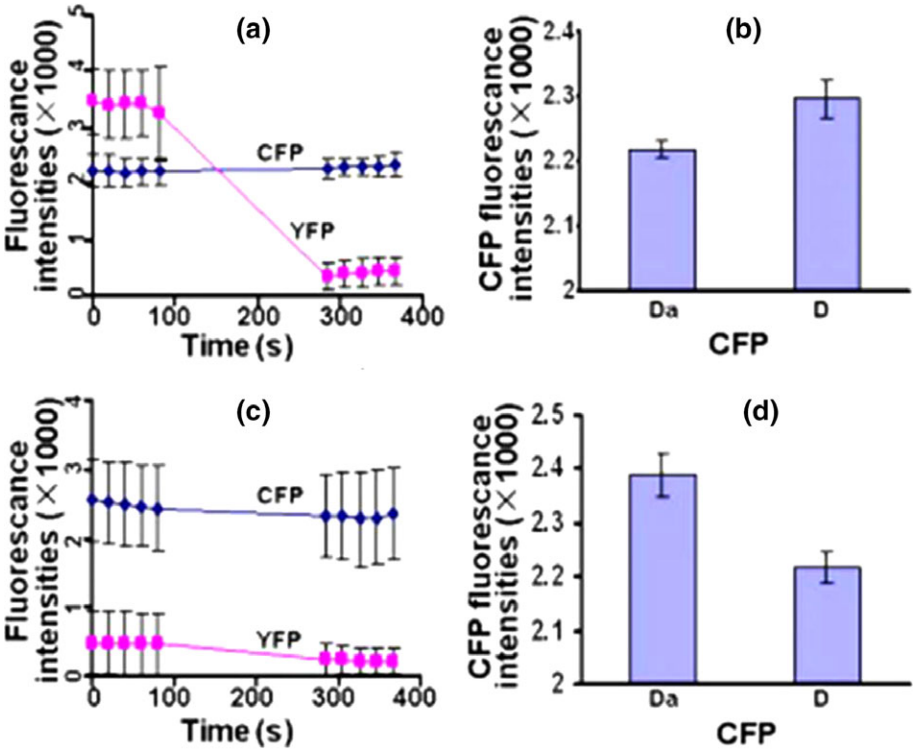

FIGURE 3 Changes in cyan fluorescent protein (CFP) fluorescence intensities after photobleaching yellow fluorescent protein (YFP) fluorescence in the nuclei and cytoplasm of HeLa cells co-transfected with CFP-S100A4 and p53-YFP. FRET in six HeLa cells expressing CFP-S100A4 and p53-YFP was examined by calculating the mean intensity of CFP fluorescence before/after photobleaching YFP fluorescence. Donor (CFP) fluorescence in the presence of acceptor (YFP) fluorescence (Da); that is, before the photobleaching of YFP fluorescence. Donor (CFP) fluorescence in the absence of acceptor (YFP) fluorescence (D); that is, after the photobleaching of YFP fluorescence. (a,b) The mean intensity (pixels) of CFP fluorescence after photobleaching YFP fluorescence (D) in the nuclei of six HeLa cells co-transfected with CFP-S100A4 and p53-YFP cDNA increased compared with that before photobleaching YFP fluorescence (Da), indicating FRET occurrence (in the nucleus). (c,d) The mean intensity (pixels) of CFP fluorescence after photobleaching YFP fluorescence (D) in the cytoplasm of the HeLa cells decreased compared with that before photobleaching YFP fluorescence (Da), indicating the absence of FRET (in the cytoplasm). Total $n = 6$ cells for each bar, three experiments

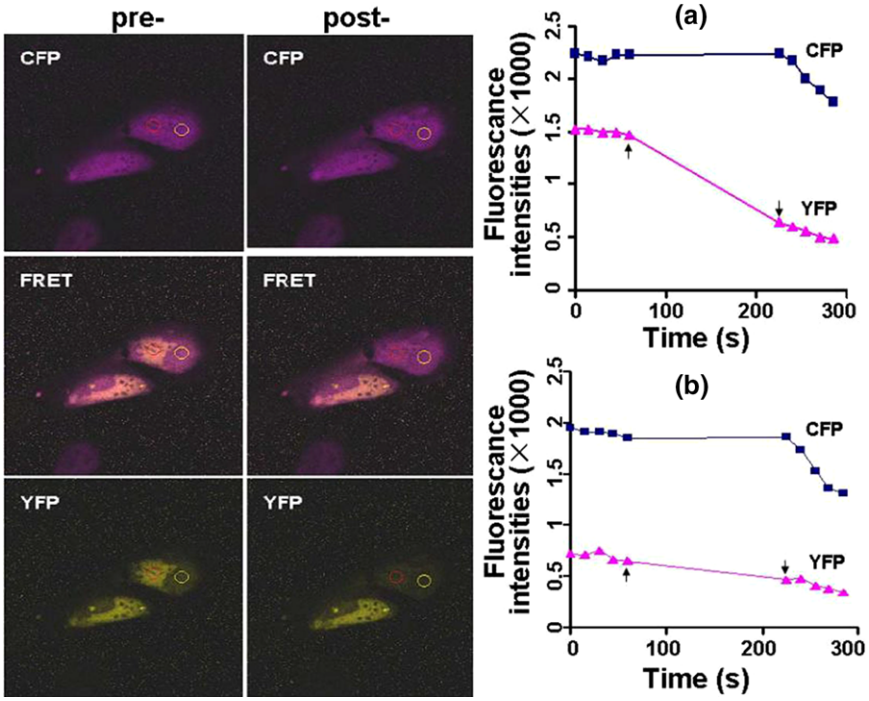

FIGURE 4 Cyan fluorescent protein (CFP), fluorescence resonance energy transfer (FRET), and yellow fluorescent protein (YFP) images taken before and after photobleaching YFP in HeLa cells co-transfected with CFP-mS100A4-C and p53-YFP. Two regions of interest (red cycle for nucleus and yellow cycle for cytoplasm) of HeLa cells transiently co-transfected with CFP-mS100A4-C and p53-YFP cDNA constructs were analyzed for CFP fluorescence intensity after (post-) and before (pre-) photobleaching YFP fluorescence. The photobleaching image of YFP fluorescence in one cell is shown in the lower panels (YFP), CFP fluorescence in the upper panels (CFP), and the total fluorescence intensity distributions in the middle panels (FRET). Changes in CFP and YFP fluorescence intensities before and after photobleaching YFP in the (a) cell nucleus and (b) cytoplasm. The locations of upward and downward arrows indicate the beginning and end of photobleaching, respectively

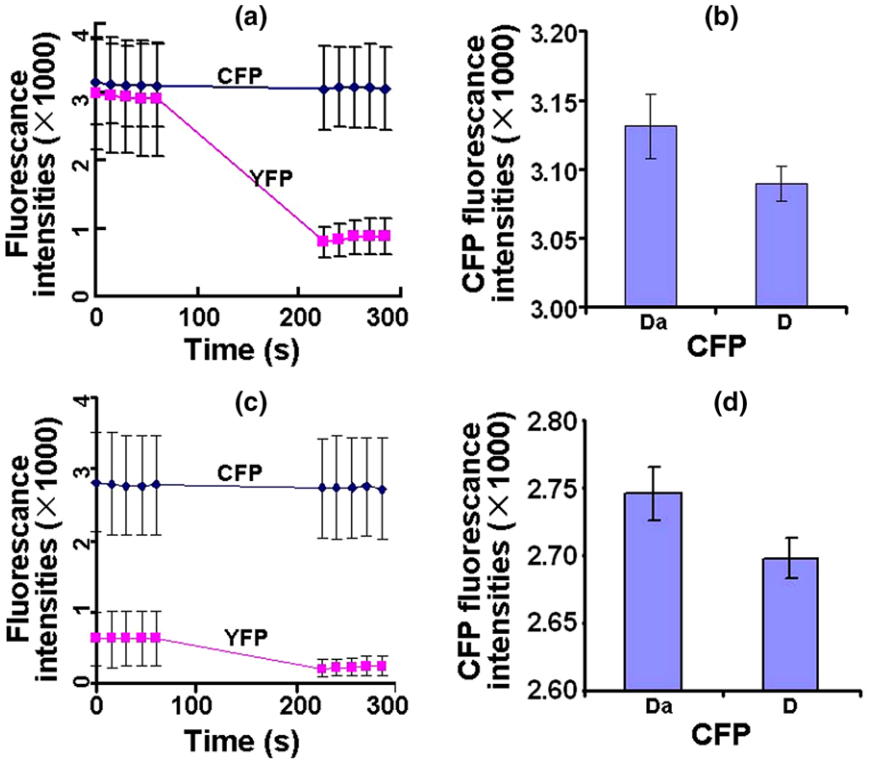

FIGURE 5 Fluorescence resonance energy transfer (FRET) in the nucleus and cytoplasm of HeLa cells co-transfected with CFP-S100A4-C and p53-YFP cDNA. FRET in nine HeLa cells expressing CFP-S100A4-C and p53-YFP was analyzed by calculating the mean intensity of cyan fluorescent protein (CFP) fluorescence before/after photobleaching yellow fluorescent protein (YFP) fluorescence. Donor (CFP) fluorescence in the presence of acceptor (YFP) fluorescence (Da); that is, before the photobleaching of YFP fluorescence. Donor (CFP) fluorescence in the absence of acceptor (YFP) fluorescence (D); that is, after the photobleaching of YFP fluorescence. (a,b) The mean intensity of (b) CFP fluorescence decreased after photobleaching YFP fluorescence (D) in the nucleus of nine HeLa cells co-transfected with CFP-S100A4-C and p53-YFP cDNA, indicating the absence of FRET in the nucleus. (c,d) Similarly, the mean intensity (pixels) of (d) CFP fluorescence decreased after photobleaching YFP fluorescence (D) compared with that before photobleaching YFP fluorescence (Da) in the cytoplasm of HeLa cells; that is, no FRET occurred in the cytoplasm. Total $n = 9$ cells for each bar, three experiments

FRET data from HeLa cells co-transfected with CFP-S100A4s and p53-YFP were first collected and analyzed. These showed an active functional interaction between the S100A4 and p53 proteins (Figures 2a, 3a,b), and the co-localization of this interaction in the nuclei (Figure 2a, 3a,b), but not in the cytoplasm (Figure 2b, 3c/d) of living HeLa cells. However, no FRET occurred between the mS100A4-C (inactivated calcium binding site) and p53 (Figure 5a–d), confirming that the mutant S100A4-C certainly lost the ability to interact with p53 *in vivo*.

Indeed, these might be expected, because the results are completely consistent with *in vitro* studies. In detecting the ability of wild-type and mutant S100A4 to bind to p53 *in vitro*, using an optical biosensor (Figure 1), wild-type S100A4 proteins showed strong abilities to bind to p53. However, interaction between mutant S100A4-C and p53 was undetectable. Interestingly, the effects of S100A4 interaction with p53 *in vitro* and *in vivo* show similar effects to that of S100A4 with rNMMHCIIA *in vitro* and *in vivo*,[14] respectively.[20–22] Conversely, the co-localizations of their interactions in living cells are different.[16] Immunofluorescent staining of cultured mammary tumor S100A4 cells localized much of the S100A4 protein in the perinuclear cytoskeleton. The same trend of co-localization of interactions of S100A4 with rNMMHCIIA was observed in the FLIM study.[14] It was reported that p53 can physiologically rapidly import into the nucleus and also export,[23] and can be excluded from the nucleus during DNA synthesis.[24] However, the present data showed that the co-localization of interactions of S100A4 with p53 is inside the nuclei of Hela cells. We observed that p53-YFP fused proteins begin to migrate from the cytoplasm to the nucleus 1.5 h after co-transfection of p53-YFP and CFP-S100A4, and then accumulate in the nucleus, and do not move out of the nucleus until observation time, 4 h later. This finding is coincidentally consistent with a report that showed that S100A4 can regulate the subcellular localization of p53 by disrupting the tetramerization of p53 when S100A4 binds to its tetramerization domain.[25] Therefore, we propose that S100A4 might interact with the C-terminus of the p53 molecule in the nucleus, induce conformational changes in p53, and then modulate functional activities of p53. This is because the C-terminus of the p53 molecule is responsible for activities, such as oligomerization, nuclear translocation, binding to damaged DNA, regulating specific DNA binding activity in the core domain, and modulating p53 transcriptional activity, which likely take place in the nucleus.[15] This further strongly indicates that nuclear co-localization is most likely important for the interaction of S100A4 with p53 in living cells.

Elevated S100A4 levels in tumor metastasis suggest a role in tumor progression.[2,26] As S100A4 itself is unable to initiate tumors, it was proposed that it might act in cooperation with other oncogenes.[27] p53 prevents tumorigenic transformation through the induction of cell cycle arrest or apoptosis.[25] S100A4 expression was inversely associated with elevated levels of wild-type p53.[2,27] and implied that the physical interaction between wild-type p53 and S100A4 might result in a stimulation of cell entry into the S phase.[15]

In conclusion, the S100A4 protein, but not the variant S100A4-C protein with a mutation in the C EF-hand domain (control), can interact directly with p53 in the cell nucleus. The pattern of interactions between S100A4 and p53, as indicated by FRET images, coincides with the pattern of S100A4 binding to immobilized p53 *in vitro* in optical biosensor studies. This suggests that the interaction of S100A4 and p53 occurs in the nuclei of living HeLa cells, which might be a molecular basis for metastasis induction by S100A4. The present results also suggest that the inhibition of the calcium-binding site in the C EF-hand of S100A4 might be a possible anticancer therapy.

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CONFLICT OF INTEREST

The authors declare that they had read the article and there are no competing interests.

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