Accumulation of the myosin-II-spectrin complex plays a positive role in apical extrusion of Src-transformed epithelial cells

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ABSTRACT

At the initial stage of carcinogenesis, transformation occurs in single cells within the epithelium. Recent studies have revealed that the newly emerging transformed cells are often apically eliminated from epithelial tissues. However, the underlying molecular mechanisms of this cancer preventive phenomenon still remain elusive. In this study, we first demonstrate that myosin-II accumulates in Src-transformed cells when they are surrounded by normal epithelial cells. Knockdown of the heavy chains of myosin-II substantially diminishes apical extrusion of Src cells, suggesting that accumulated myosin-II positively regulates the apical elimination of transformed cells. Furthermore, we have identified β-spectrin as a myosin-II-binding protein under the co-culture of normal and Src-transformed epithelial cells. β-Spectrin is also accumulated in Src cells that are surrounded by normal cells, and the β-spectrin accumulation is regulated by myosin-II. Moreover, knockdown of β-spectrin significantly suppresses apical extrusion of Src cells. Collectively, these results indicate that accumulation of the myosin-II-spectrin complex plays a positive role in apical extrusion of Src-transformed epithelial cells. Further elucidation of the molecular mechanisms of apical extrusion would lead to establishment of a novel type of cancer preventive medicine.

1 | INTRODUCTION

In epithelial tissues, abnormal or functionally suboptimal cells are often eliminated from epithelia. This homeostatic mechanism is, at least partly, mediated by cell competition. Cell competition is a process by which cells with different properties compete with each other for survival and space (Amoyel and Bach, 2014; Baker, 2017; Claveria and Torres, 2016; Di Gregorio et al., 2016; Johnston, 2009; Maruyama and Fujita, 2017; Merino et al., 2016; Morata and Ballesteros-Arias, 2015; Ohsawa et al., 2018; Vincent et al., 2013; Wagstaff et al., 2013). For example, when oncogenic Ras- or Src-transformed cells are surrounded by normal epithelial cells, transformed cells are apically extruded from the epithelial layer. The apical eradication of transformed cells has been observed in cell culture systems (Hogan et al., 2009; Kajita et al., 2010; Leung and Brugge, 2012; Wu et al., 2014) as well as in *Drosophila* (Vidal et al., 2006), zebrafish (Kajita et al., 2010), and mouse (Kon et al., 2017; Sasaki et al., 2018) *in vivo*. In this process, normal epithelial cells recognize the presence of the neighboring transformed cells and actively eliminate them from epithelia, implying that normal epithelia have anti-tumor activity that does not involve immune cells, which is termed EDAC (epithelial defense against cancer) (Kajita et al., 2014). However, the underlying molecular mechanisms still remain largely obscure.

Non-muscle myosin-II binds to actin and generates mechanical forces, thereby regulating various cellular processes such as cell migration, cytokinesis, and cell shape changes (Heissler and Manstein, 2013; Pecci et al., 2018; Vicente-Manzanares et al., 2009). Myosin-II consists of a homodimer of heavy chains, two regulatory light chains, and two essential light chains, and activity of myosin-II is regulated by phosphorylation of regulatory light chains. Mammalian cells express three isoforms of non-muscle myosin-II, NM IIA, IIB, and IIC, which differ in their heavy chains;

among them NM IIA is most widely distributed in tissues. To investigate the competitive interaction between normal and transformed cells, we have established Madin-Darby canine kidney (MDCK) epithelial cells stably expressing oncogenic cSrcY527F or RasV12 in a tetracycline-inducible manner (Hogan et al., 2009; Ohoka et al., 2015). In Src- or Ras-transformed cells that are surrounded by normal cells, phosphorylation of myosin light chain (MLC) of myosin-II is elevated (Hogan et al., 2009; Kajita et al., 2010). The increased MLC phosphorylation is not observed when transformed cells alone are cultured, indicating that activation of myosin-II is induced by the presence of the neighboring normal cells in a non-cell-autonomous manner. Addition of the myosin-II inhibitor blebbistatin or expression of the dominant-negative form of MLC (MLC-AA) in transformed cells suppresses apical extrusion (Hogan et al., 2009; Kajita et al., 2010), indicating that activity of myosin-II plays a crucial role in the elimination of transformed cells.

In this study, we demonstrate that not only activity of myosin-II but also its accumulation is involved in apical extrusion of transformed cells.

2 | RESULTS

2.1 | Accumulation of myosin-IIA plays a positive role in apical extrusion of Src-transformed cells

To further understand the role of myosin-II in apical extrusion of Src-transformed cells, we examined the localization of heavy chains of myosin-II (NMHC-IIA: non-muscle myosin heavy chain IIA) by immunofluorescence. When Src-transformed cells were surrounded by normal epithelial cells, NMHC-IIA was profoundly accumulated at the apical side of Src cells (Figure 1a, b). When Src-transformed cells alone were present, the accumulation of NMHC-IIA was not observed, and the immunofluorescence level of NMHC-IIA was comparable to that in normal cells (Figure 1a, b), suggesting that the presence of the neighboring normal cells induces accumulation of NMHC-IIA in Src cells in a non-cell-autonomous fashion. These results imply that not only activity of myosin-II (Hogan et al., 2009; Kajita et al., 2010) but also accumulation of myosin-II is regulated during apical extrusion. Accumulation of NMHC-IIA also occurred in RasV12-transformed cells that were surrounded by normal cells (Figure S1a, b), but less prominently than that in Src-transformed cells; therefore, in this study we focused on the functional role of myosin-II in apical extrusion of Src-transformed cells.

Previous studies have shown that F-actin is not accumulated in Src- or RasV12-transformed cells that are surrounded by normal cells (Hogan et al., 2009; Kajita et al., 2010). Accordingly, addition of cytochalasin D, the inhibitor of actin polymerization, did not significantly affect the accumulation of NMHC-IIA in Src cells (Figure S3a, b). In contrast, the myosin-II inhibitor blebbistatin significantly suppressed the accumulation of NMHC-IIA (Figure S3e, f), suggesting the presence of certain molecular machinery that recruits active myosin-II to the apical side of Src cells.

Collectively, these results suggest that NMHC-IIA is accumulated in Src-transformed cells surrounded by normal cells in an F-actin-independent, but myosin-activity-dependent fashion.

To examine the functional role of the myosin-II accumulation in apical extrusion of Src-transformed cells, we established cSrcY527F-transformed cells stably expressing NMHC-IIA-shRNA (Figure 1c). When NMHC-IIA-knockdown Src cells were surrounded by normal cells, apical extrusion was substantially diminished (Figure 1d), demonstrating that myosin-II plays a crucial role in apical elimination of Src-transformed cells.

2.2 | Accumulation of the myosin-II-spectrin complex plays a positive role in apical extrusion of Src-transformed epithelial cells

Previous studies have reported that myosin IIA interacts with the spectrin-actin membrane skeleton (Puszkin et al., 1978; Smith et al., 2018). Spectrins are large skeletal molecules that exist mainly as heterotetramers consisting of α-spectrin and β-spectrin. They form a filamentous network on the cytoplasmic face of the membrane, thereby providing a scaffold for various proteins (Machnicka et al., 2014; Machnicka et al., 2012). By immunoprecipitation, we showed that β-spectrin interacted with NMHC-IIA under the co-culture condition (Figure 2a). Next, subcellular localization of β-spectrin was examined by immunofluorescence. We found that β-spectrin was accumulated at the apical side of Src-transformed cells that were surrounded by normal cells (Figure 2b, c). In contrast, accumulation of β-spectrin was not observed when Src cells alone were cultured (Figure 2b, c). The non-cell-autonomous accumulation of β-spectrin was diminished by blebbistatin (Figure S3e, g), but not by

cytochalasin D (Figure S3c, d). Thus, the mode of accumulation of β-spectrin was akin to that of myosin-II.

To examine the functional role of spectrin in apical extrusion, we established cSrcY527F-transformed cells stably expressing β-spectrin-shRNA (Figure S2a). The amount of co-immunoprecipitated β-spectrin was profoundly diminished when β-spectrin was knocked down in Src-transformed cells (Figure S2b), suggesting that the interaction between NMHC-IIA and β-spectrin predominantly occurs in Src-transformed cells under the mix culture condition. We found that knockdown of β-spectrin did not significantly affect accumulation of myosin-II (Figure 3a, b). In contrast, knockdown of NMHC-IIA substantially diminished accumulation of β-spectrin (Figure 3c, d), indicating that myosin-II regulates localization of β-spectrin in Src cells surrounded by normal cells. Finally, we investigated the effect of knockdown of β-spectrin on the behavior of Src-transformed cells. When β-spectrin-knockdown Src cells were surrounded by normal cells, apical extrusion was significantly suppressed (Figure 3e). Collectively, these results suggest that accumulation of the myosin-II-spectrin complex plays a positive role in apical extrusion of Src-transformed epithelial cells.

3 | DISCUSSION

In this study, we demonstrate that myosin-II and β-spectrin accumulate at the apical region of transformed cells surrounded by normal cells, which positively regulates apical extrusion. As shown in Figure 2a, the higher amount of β-spectrin binds to myosin-II in a single culture of Src-transformed cells than that of normal cells, but is further elevated under the co-culture condition, suggesting that the interaction between normal and transformed cells somehow promotes the complex formation between β-spectrin and myosin-II. Both myosin-II and spectrin have actin-binding property. However, we have not observed accumulation of F-actin at the apical region of Src-transformed cells that are surrounded by normal cells (Kajita et al., 2010). Consistently, the actin-polymerization inhibitor cytochalasin D does not significantly affect the accumulation of myosin-II or spectrin, implying that the interaction between myosin-II and spectrin is regulated by an F-actin-independent mechanism.

Previous studies have shown that the paxillin-plectin-EPLIN complex is accumulated in RasV12- or Src-transformed cells when they are surrounded by normal epithelial cells, which promotes apical elimination of the transformed cells (Kadeer et al., 2017; Kasai et al., 2018; Ohoka et al., 2015). But, knockdown of EPLIN does not significantly influence accumulation of NMHC-IIA, and knockdown of NMHC-IIA does not affect accumulation of EPLIN (Figure S4a-e). Thus, it is likely that the myosin-II-spectrin complex regulates apical extrusion of Src-transformed cells independently of the paxillin-plectin-EPLIN complex. It has been revealed that other than the accumulation of the paxillin-plectin-EPLIN complex, a variety of non-cell-autonomous changes can occur in transformed cells during apical extrusion such as induction of the Warburg effect-like metabolic changes and elevated

endocytosis (Kon et al., 2017; Saitoh et al., 2017). In future studies, the upstream and downstream regulators of the myosin-II-spectrin complex need to be identified.

Apical extrusion of transformed cells has been also observed in various epithelial tissues in mouse *in vivo* systems (Kon et al., 2017; Sasaki et al., 2018). In addition, environmental factors such as obesity and inflammation profoundly influence the frequency of elimination of transformed cells (Sasaki et al., 2018). It should be determined whether and how the myosin-II-spectrin complex is involved in these processes *in vivo*. Further investigation of apical extrusion of transformed cells would lead to a novel approach for cancer prevention.

4 | EXPERIMENTAL PROCEDURES

4.1 | Antibodies and Materials

Rabbit anti-NMHC-IIA (LS-B2802) antibody was purchased from LifeSpan BioSciences, Inc. Rabbit anti-NMHC-IIA (M 8064) antibody was from Sigma-Aldrich. The former was used for immunofluorescence, and the latter was for western blotting and immunoprecipitation. Mouse anti-EPLIN (sc-136399) and mouse anti-β-spectrin (sc-136074) antibodies were from Santa Cruz Biotechnology. Mouse anti-actin (Clone C4) antibody was from Millipore. Alexa-Fluor-568- and -647-conjugated secondary antibodies were from ThermoFisher Scientific. Hoechst 33342 (Life Technologies) was used at a dilution of 1:5,000. For immunofluorescence, the primary antibodies described above were diluted in phosphate-buffered saline (PBS) containing 1% BSA at 1:100. All secondary antibodies were used at 1:200. Alexa-Fluor-647-conjugated phalloidin (Life Technologies) was used at 1.0 U ml^{-1}. For western blotting, all primary antibodies were used at 1:1,000, and secondary antibodies were at 1:1,000. The following inhibitors were used where indicated: cytochalasin D (Sigma-Aldrich, 100 nM) and blebbistatin (Millipore, 30 μM). DMSO (Sigma-Aldrich) was added as a control.

4.2 | Cell Culture

MDCK, MDCK-pTR GFP-cSrcY527F, and MDCK-pTR GFP-RasV12 cells were cultured as previously described (Hogan et al., 2009; Ohoka et al., 2015). MDCK-pTR GFP-cSrcY527F cells stably expressing EPLIN-shRNA were established as previously described (Ohoka et al., 2015). MDCK-pTR GFP-cSrcY527F cells stably expressing NMHC-IIA-shRNA or β-spectrin-shRNA were established as follows:

Double-stranded DNA fragments coding NMHC-IIA-shRNA sequences (NMHC-IIA -shRNA1: 5′- GATCCCCGCTGCTAAGAAGCTGGTATTTCAAGAGA ATACCAGCTTCTTAGCAGCTTTTTC -3′ and 5′- TCGAGAAAAAGCTGCTAAGAAGCTGGTATTCTCTTGAAATACCAGCTTCT TAGCAGCGGG -3′ or NMHC-IIA-shRNA2: 5′- GATCCCC GCAACTGACAGCCATGAAATTCAAGAGATTTCATGGCTGTCAGTTGCTTTT TC -3′ and 5′- TCGAGAAAAAGCAACTGACAGCCATGAAATCTCTTGAATTTCATGGCTGT CAGTTGCGGG -3′) or β-spectrin-shRNA sequences (β-spectrin-shRNA1: 5′- GATCCCCGCACAGGTTTGAGAGCCTTTTCAAGAGAAAGGCTCTCAAACCT GTGCTTTTTC -3′ and 5′- TCGAGAAAAAGCACAGGTTTGAGAGCCTTTCTCTTGAAAAGGCTCTCAAA CCTGTGCGGG -3′ or β-spectrin-shRNA2: 5′- GATCCCCGCACACTACATTTGAGCATTTCAAGAGAATGCTCAAATGTAGT GTGCTTTTTC -3′ and 5′- TCGAGAAAAAGCACACTACATTTGAGCATTCTCTTGAAATGCTCAAATGT AGTGTGCGGG -3′) were inserted into the *Bgl*II and *Xho*I site of pSUPER.neo + gfp (Oligoengine). MDCK-pTR GFP-cSrcY527F cells were transfected with pSUPER.neo + gfp NMHC-IIA-shRNA or β-spectrin-shRNA using Lipofectamine 2000 (Invitrogen), followed by antibiotic selection in the medium containing 5 μg ml^{-1} blasticidin (InvivoGen), 400 μg ml^{-1} zeocin (InvivoGen), and 800 μg ml^{-1} G418 (Life Technologies). To induce the expression of GFP-cSrcY527F, the tetracycline-inducible MDCK-pTR GFP-cSrcY527F cells were treated with 2 μg ml^{-1} tetracycline (Sigma-Aldrich). For inhibitor treatment, the indicated

inhibitors were added together with tetracycline, and cells were further cultured for 16 h or 24 h. For analyses of immunofluorescence or apical extrusion, cells were seeded onto Type-I collagen-mounted coverslips. Type-I collagen (Cellmatrix Type I-A) was obtained from Nitta Geratin and was neutralized on ice to a final concentration of 2 mg ml^{-1} according to the manufacturer's instructions.

4.3 | Immunofluorescence

MDCK-pTR GFP-cSrcY527F or MDCK-pTR GFP-RasV12 cells were mixed with MDCK cells at a ratio of 1:50 and cultured on the collagen matrix as previously described (Hogan et al., 2009). For immunofluorescence analyses, the mixture of cells was incubated for 8–12 h until they formed a monolayer, followed by tetracycline treatment for 16 h. Cells were fixed with 4% paraformaldehyde in PBS and permeabilized with 0.5% Triton X-100 in PBS, followed by blocking with 1% BSA in PBS. Primary or secondary antibodies were incubated for 2 h or 1 h, respectively at ambient temperature. Immunofluorescence images were acquired by the Olympus FV1200 system and the Olympus FV10-ASW software. For quantification of immunofluorescence intensity, more than 30 transformed cells were analyzed for each experiment using the MetaMorph software (Molecular Devices). For analyses of apical extrusions, the samples were prepared as described above, except that cells were treated with tetracycline for 24 h. More than 30 cells were analyzed for each experiment, and apically extruded cells were quantified.

4.4 | Immunoprecipitation and western blotting

For immunoprecipitation, 0.9×10^7 MDCK cells and 0.9×10^7 MDCK-pTR GFP-cSrcY527F cells for mix culture or 1.8×10^7 MDCK or MDCK-pTR GFP-cSrcY527F

cells for single culture were seeded in 14.5-cm dishes (one or two dishes for each experimental condition) (Greiner-Bio-One) and cultured for 9 h until a monolayer was formed. Cells were then incubated with tetracycline to induce c-SrcY527F expression. After 16 h culture in the presence of tetracycline, cells were washed with ice-cold PBS containing 1 mM Na_3VO_4 and lysed for 30 min in Triton X-100 lysis buffer (20 mM Tris-HCl [pH 7.5], 150 mM NaCl, and 1% Triton X-100) containing the following inhibitors: 1 mM Na_3VO_4, 10 mM NaF, 5 μg ml^{-1} leupeptin, 1 mM phenylmethylsulfonyl fluoride, and 7.2 trypsin inhibitor units of aprotinin. After centrifugation of the cell lysates at 21,500 g for 10 min, the supernatant was first pre-cleared with Protein G sepharose (Sigma-Aldrich) at 4°C for 30 min. The pre-cleared cell lysates were then incubated with Dynabeads protein G (Life Technologies) conjugated to control rabbit IgG (5 or 10 μg) at 4°C for 30 min and finally subjected to immunoprecipitation with Dynabeads Protein G conjugated to rabbit anti-NMHC-IIA antibody (5 or 10 μg) at 4°C for 1 h. Immunoprecipitated proteins were subjected to SDS-PAGE, followed by western blotting with anti-β-spectrin antibody. Western blotting was performed as described previously (Hogan et al., 2004). Primary antibodies were used at 1:1,000. Western blotting data were analyzed using ImageQuant™ LAS4010 (GE healthcare). To examine the knockdown efficiency of NMHC-IIA, β-spectrin, or EPLIN, MDCK-pTR GFP-cSrcY527F cells stably expressing NMHC-IIA-shRNA, β-spectrin-shRNA, or EPLIN-shRNA were seeded onto 6-well dishes (Corning). After 12-24 h, the incubated cells were lysed with Triton X-100 lysis buffer containing protease inhibitors (5 μg ml^{-1} leupeptin, 1 mM phenylmethylsulfonyl fluoride, and 7.2 trypsin inhibitor units of aprotinin) and directly boiled with SDS-PAGE sample buffer.

4.5 | Data Analyses

Two-tailed Student's *t*-tests were used to determine *P*-values for statistical analyses.

4.6 | Data Availability

The datasets generated and/or analyzed during the current study are available from the corresponding author on reasonable request.

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ACKNOWLEDGEMENTS

This study was supported by Japan Society for the Promotion of Science (JSPS) Grant-in-Aid for Scientific Research on Innovative Areas 26114001, Grant-in-Aid for Scientific Research (A) 18H03994, Strategic Japanese-Swiss Science and Technology Program, AMED under Grant Number JP17ck0106361 and JP18cm0106234, SAN-ESU GIKEN CO. LTD, the Naito Foundation, and the Takeda Science Foundation.

FIGURE LEGENDS

FIGURE 1 Accumulation of myosin-II positively regulates apical extrusion of Src-transformed cells that are surrounded by normal epithelial cells. (a) Immunofluorescence images of MDCK-pTR GFP-cSrcY527F cells mixed with normal MDCK cells or cultured alone. Cells were incubated with tetracycline for 16 h and stained with anti-NMHC-IIA antibody (red) and Hoechst (blue). Scale bar, 10 μm. (b) Quantification of the immunofluorescence intensity of NMHC-IIA. Data are mean ± SD from three independent experiments. *P<0.05 (Student t-test). More than 30 cells were analyzed in each experimental condition. (c) Establishment of MDCK-pTR GFP-cSrcY527F cells stably expressing NMHC-IIA-shRNA1 or -shRNA2. Cell lysates of each NMHC-IIA-knockdown cell line were analyzed by western blotting with the indicated antibodies. (d) Effect of knockdown of NMHC-IIA on apical extrusion. MDCK-pTR GFP-cSrcY527F, MDCK-pTR GFP-cSrcY527F NMHC-IIA-shRNA1 or -shRNA2 cells were co-cultured with normal MDCK cells, and at 24 h after tetracycline addition, apical extrusion was analyzed. Data are mean ± SD from three independent experiments. *P<0.05; **P<0.005 (Student t-test). More than 30 cells were analyzed in each experimental condition.

FIGURE 2 β-Spectrin binds to myosin-II and accumulates in Src-transformed cells surrounded by normal cells. (a) Co-immunoprecipitation of β-spectrin with anti-NMHC-IIA antibody. Cells were cultured under three different conditions: i) normal MDCK cells alone, ii) 1:1 mix culture of normal MDCK and MDCK-pTR GFP-cSrcY527F cells, and iii) MDCK-pTR GFP-cSrcY527F cells alone. At 16 h after induction of cSrcY527F expression, the cell lysates were subjected to

immunoprecipitation with control IgG or anti-NMHC-IIA antibody, followed by western blotting with anti-β-spectrin antibody. Note that the doublet bands of β-spectrin were not observed in a reproducible manner (e.g. Figure S2b), and the significance and identity of these bands are not known at present. (b) Immunofluorescence images of MDCK-pTR GFP-cSrcY527F cells mixed with normal MDCK cells or cultured alone. Cells were incubated with tetracycline for 16 h and stained with anti-β-spectrin antibody (red) and Hoechst (blue). Scale bar, 10 μm. (c) Quantification of the immunofluorescence intensity of β-spectrin. Data are mean ± SD from three independent experiments. *P<0.05 (Student t-test). More than 30 cells were analyzed in each experimental condition.

FIGURE 3 β-Spectrin also plays a positive role in apical extrusion of Src-transformed cells. (a and c) Immunofluorescence images of MDCK-pTR GFP-cSrcY527F or MDCK-pTR GFP-cSrcY527F β-spectrin-shRNA1 (a) or NMHC-IIA-shRNA1 (c) cells mixed with normal MDCK cells. Cells were incubated with tetracycline for 16 h and stained with anti-β-spectrin (red) and anti-NMHC-IIA (gray) antibodies and Hoechst (blue). (b and d) Quantification of the immunofluorescence intensity of NMHC-IIA (b) or β-spectrin (d). Data are mean ± SD from three independent experiments. n.s.: not significant. *P<0.05 (Student t-test). More than 30 cells were analyzed in each experimental condition. (e) Effect of knockdown of β-spectrin on apical extrusion. MDCK-pTR GFP-cSrcY527F, MDCK-pTR GFP-cSrcY527F β-spectrin-shRNA1 or -shRNA2 cells were co-cultured with normal cells, and at 24 h after tetracycline addition, apical extrusion was analyzed. Data are mean ± SD from three independent experiments. *P<0.05 (Student t-test). More than 30 cells were analyzed in each experimental condition.

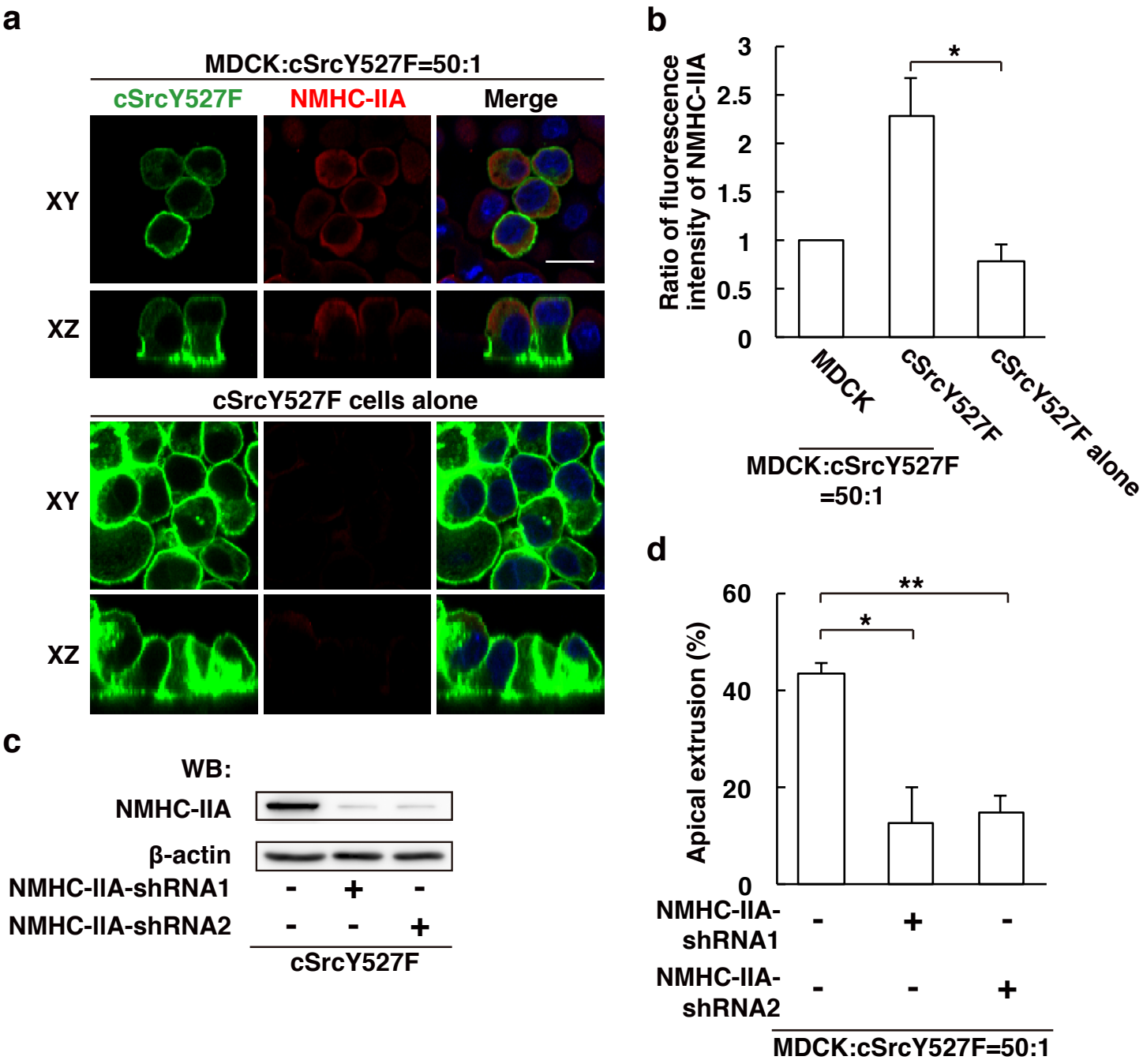

Figure 1 Takagi et al.

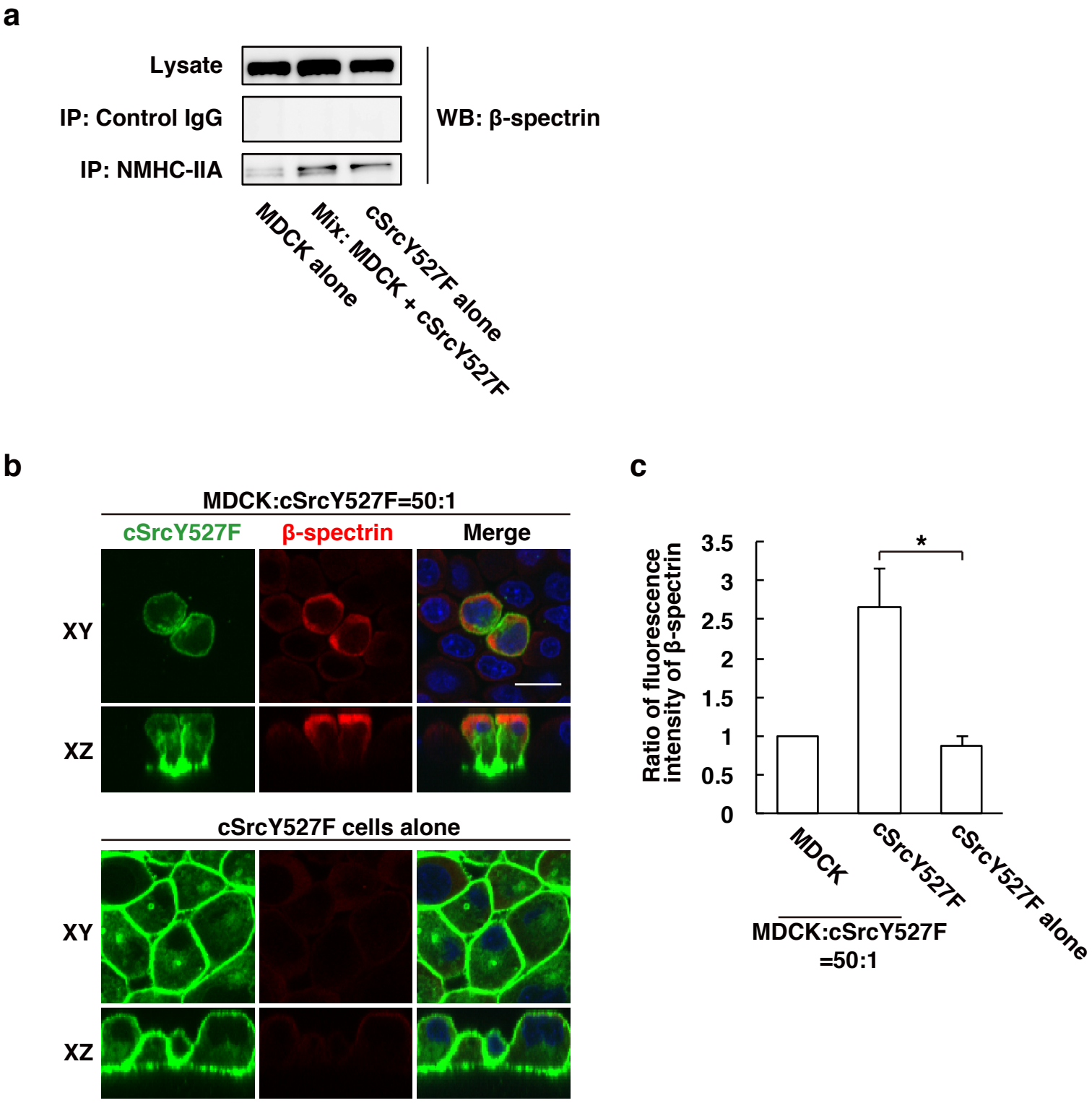

Figure 2 Takagi et al.

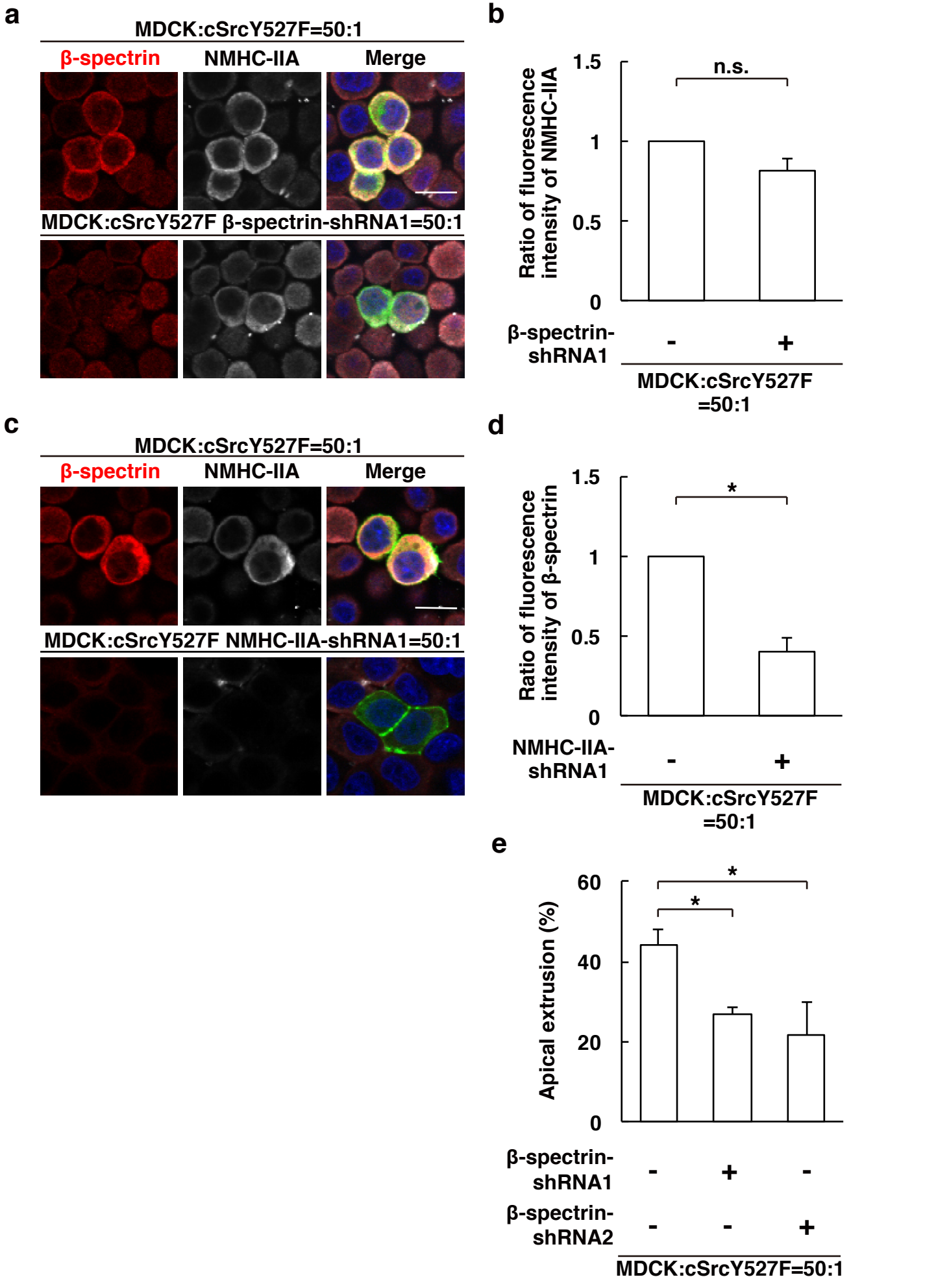

Figure 3 Takagi et al.