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Critical role of the Ror-family of receptor tyrosine kinases in invasion and proliferation of malignant pleural mesothelioma cells

Takeshi Saji[1], Michiru Nishita[1, *], Hiroyuki Ogawa[2], Takefumi Doi[2], Yasuhiro Sakai[3], Yoshimasa Maniwa[2], and Yasuhiro Minami[1, *]

[1]Division of Cell Physiology, Department of Physiology and Cell Biology, Graduate School of Medicine, Kobe University, 7-5-1, Kusunoki-cho, Chuo-ku, Kobe, Hyogo 650-0017, Japan
[2]Division of Thoracic Surgery, Graduate School of Medicine, Kobe University, 7-5-2, Kusunoki-cho, Chuo-ku, Kobe, Hyogo 650-0017, Japan
[3]Department of Pathology, Hyogo Cancer Center, 13-70, Kitaoji-cho, Akashi, Hyogo 673-8558, Japan

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[*]corresponding authors:
Michiru Nishita and Yasuhiro Minami, Division of Cell Physiology, Department of Physiology and Cell Biology, Graduate School of Medicine, Kobe University, 7-5-1, Kusunoki-cho, Chuo-ku, Kobe, Hyogo 650-0017, Japan; Tel: +81-78-382-5560, 5561; Fax: +81-78-382-5579; E-mail: nishita@med.kobe-u.ac.jp and minami@kobe-u.ac.jp

Abstract

Malignant pleural mesothelioma (MPM) is a highly aggressive tumor with poor prognosis and closely related to exposure to asbestos. MPM is a heterogeneous tumor with three main histological subtypes, epithelioid, sarcomatoid and biphasic types, among which sarcomatoid type shows the poorest prognosis. The Ror-family of receptor tyrosine kinases, Ror1 and Ror2, are expressed in various types of tumor cells at higher levels and affect their aggressiveness. However, it is currently unknown whether they are expressed in and involved in aggressiveness of MPM. Here, we show that Ror1 and Ror2 are expressed in clinical specimens and cell lines of MPM with different histological features. Studies using MPM cell lines indicate that expression of Ror2 is associated tightly with high invasiveness of MPM cells, while Ror1 can contributes to their invasion in the absence of Ror2. On the other hand, both Ror1 and Ror2 promote proliferation of MPM cells. We also show that promoted invasion and proliferation of MPM cells by Ror signaling can be mediated by the Rho-family of small GTPases, Rac1 and Cdc42. These findings elucidate the critical role of Ror signaling in promoting invasion and proliferation of MPM cells.

Introduction

Malignant mesothelioma is a highly aggressive tumor originated from mesothelial cells of the pleura, pericardium or peritoneum (Bueno 2005; Robinson & Lake 2005). Among them, malignant pleural mesothelioma (MPM) is the most common one with increasing frequency throughout the world and closely related to exposure to asbestos (Kanazawa *et al.,* 2006; Carbone *et al.,* 2012; Frank & Joshi 2014). Asbestos causes chronic inflammation that has been linked to the initiation and progression of MPM (Carbone *et al.,* 2012). The prognosis of patients with MPM is poor in general, with a median overall survival of 9-10 months from the time of diagnosis (Nojiri *et al.,* 2011). MPM is a highly heterogeneous tumor with three main histological subtypes, epithelioid, sarcomatoid and biphasic types, among which sarcomatoid type shows the poorest prognosis and in most cases resistant to chemo- and radiation therapy (Nutt *et al.,* 2010; Kao *et al.,* 2013). Recent studies have revealed that MPM is associated with loss-of-function mutations within the genes involved in the Hippo pathway, such as *neurofibromatosis type 2* (*NF2*) and *large tumor suppressor kinase 2* (*LATS2*), resulting in an aberrant activation of the transcriptional coactivator YAP (Chen *et al.,* 2012; Felley-Bosco & Stahel 2014; Moroishi *et al.,* 2015).

The Ror-family of receptor tyrosine kinases, consisting of Ror1 and Ror2, act as receptors for Wnt5a to mediate the non-canonical Wnt signaling, which plays essential roles during developmental morphogenesis (Minami *et al.,* 2010; Nishita *et al.,* 2010; Endo *et al.,* 2015). Ror1 and Ror2 are expressed highly in various types of tumor cells, resulting in aberrant activation of Ror-mediated signaling (Ror signaling) that affects their proliferation and/or invasion, depending on the cell types (Nishita *et al.,* 2010; Endo *et al.,* 2015). Our previous studies using osteosarcoma cells have shown that aberrantly activated Ror2 signaling promotes their invasiveness by inducing expression of matrix metalloproteinase-13 (MMP-13) and intraflagellar transport 20 (Enomoto *et al.,* 2009; Nishita *et al.,* 2017). It is currently unknown whether Ror1 and Ror2 are expressed in and involved in aggressiveness of MPM.

Here, we show for the first time that Ror1 and Ror2 are expressed in clinical specimens and cell lines of MPM with different histological and pathological features and uncover their roles in regulating proliferation and invasion of MPM cells. These findings shed novel insight into how Ror signaling acts in conferring aggressiveness to MPM cells.

Results and discussion

Expression of Ror1 and Ror2 in clinical specimens and cell lines of MPM

We first examined expression of Ror1 and Ror2 in resected specimens from MPM patients by immunohistochemical staining. All the specimens examined (N=5) contained tumor cells that are positive for Ror1 and Ror2, irrespective of their histological and pathological features, i.e. epithelioid and/or sarcomatoid types (Figure 1a). Neither Ror1 nor Ror2 was detected in normal lung tissues (Figure S1a) and desmoplastic stroma (Figure 1a) adjacent to the epithelioid and sarcomatoid tumors, respectively. Importantly, however, expression of Ror2 and Ror1 was also detectable at apparent and marginal levels, respectively, in non-invasive, dysplastic pleural mesothelial cells within the MPM specimens (Figure S1b), probably associated with asbestos-induced chronic inflammation. In fact, expression of Ror1 and Ror2 has been shown to be induced during tissue damage-induced inflammatory responses in adult tissues (Li *et al.,* 2013; Endo *et al.,* 2017; Kamizaki *et al.,* 2017; Takahashi *et al.,* 2017). These findings suggest that Ror1 and Ror2 might be involved in initiation and progression of MPM associated with chronic inflammation and could be novel molecular markers for MPM. Further studies with a larger number of MPM specimens will be required to clarify further correlation of expression of Ror1 and/or Ror2 with clinical and histo-pathological features of MPM.

We next examined expression of Ror1 and Ror2 in human MPM cell lines, H28 (epithelioid), H2452 (biphasic), and H2052 (sarcomatoid), by Western blotting. Notably, expression of Ror2 was the most prominent in H2052 cells (Fig. 1b), and its level was much higher than that in osteosarcoma SaOS2 cells (data not shown), which exhibit highly invasive properties due to, at least partly, their sustained expression of Ror2 (Enomoto *et al.,* 2009; Nishita *et al.,* 2017). Ror2 was also detected in H2452 cells, while it was almost undetectable in H28 cells (Figure 1b). Unlike Ror2, expression of Ror1 was more prominent in H2452 cells than that in H28 and H2453 cells (Figure 1b).

Roles of Ror1 and Ror2 in invasive and proliferative properties of MPM cells

Among the three MPM cell lines, H2052 cells, expressing Ror2 at the highest level, exhibited the highest activity of invasiveness, compared to H28 and H2452 cells (Figure 1c), raising a possibility that expression levels of Ror2 might be associated with invasive activities of MPM cells. To this end, we examined the effect of siRNAs against *Ror2* on MPM cell invasion by Matrigel invasion assay. In agreement with undetectable expression of Ror2 in H28 cells, siRNAs against *Ror2* failed to affect their invasiveness (Figure 2a). In contrast, knockdown of *Ror2* inhibited significantly invasion of both H2452 and H2052 cells (Figure 2b, c). These results indicate that Ror2 plays an important role in promoting invasion of MPM cells, as observed in other types of cancer cells, such as osteosarcoma and melanoma cells (Enomoto *et al.,* 2009; O'Connell *et al.,* 2010). We also found that knockdown of *Ror1* inhibited significantly invasion of H28 cells, but not H2452 and H2052 cells (Figure 2d-f), suggesting that the role of Ror1 in promoting invasion would become evident in the absence of Ror2.

We next studied the role of Ror1 and Ror2 in proliferation of MPM cells. Like invasive activity, proliferative activity was the most prominent in H2052 cells among the three MPM cell lines (Figure 2g-i), and knockdown of *Ror2* reduced significantly proliferative rate of H2452 and H2052 cells (Figure 2h, i). Significant reduction in proliferative rate was also observed by *Ror1* knockdown in all three MPM cell lines (Figure 2g-i), suggesting that both Ror1 and Ror2 play a role in promoting proliferation of MPM cells.

Ror signaling is mediated by Rac and Cdc42 to promote invasion and proliferation of MPM cells

It has been established that Ror1 and Ror2 can mediate the non-canonical Wnt signaling, which involves various signaling molecules, such as Dishevelled, Rho-family of small GTPases, JNK, and Src (Oishi *et al.,* 2003; Akbarzadeh *et al.,* 2008; Enomoto *et al.,* 2009; Sato *et al.,* 2010; Endo *et al.,* 2012; Nishita *et al.,* 2014). Among these

molecules, activities of Rac and Cdc42, members of the Rho-family of small GTPases, were inhibited substantially by siRNAs against *Ror1*, but not *Ror2*, in H28 cells (Figure 3a). On the other hand, knockdown of *Ror2*, but not *Ror1*, inhibited the activities of Rac and Cdc42 in H2452 cells (Figure 3b). Activity of another member of the Rho-family of small GTPase, RhoA, was unaffected by knockdown of either *Ror1* or *Ror2* in both H28 and H2452 cells (data not shown). We then examined whether Rac and Cdc42 have pro-invasive and/or pro-proliferative roles in H28 and H2452 cells. As shown in Figure 3c-f, knockdown of either *Rac1* or *Cdc42* inhibited significantly invasive and proliferative rates of both H28 and H2452 cells. Taken together, these results indicate that Ror1 and Ror2 signaling, respectively, in H28 and H2452 cells, is mediated by both Rac1 and Cdc42, to promote their invasion and proliferation. Since knockdown of *Ror1* inhibited proliferation of H2452 cells without affecting significantly the activities of Rac and Cdc42, Rac/Cdc42-independent pathway might also be involved in Ror1-induced proliferation of H2452 cells. At present, it is unclear how Ror-Rac1/Cdc42 signaling promotes invasion and proliferation of H28 and H2452 cells. It can be envisaged that this signaling can activate transcription factors like AP-1, which play an important role in transcriptional activation of various genes required for cell invasion and proliferation, such as *MMPs* and *Cyclin D1*, respectively (Westermarck & Kahari 1999; Shaulian & Karin 2001; Yan & Boyd 2007). Further study will be required to clarify the mechanism by which Ror-Rac1/Cdc42 signaling promotes invasion and proliferation of MPM cells.

In contrast to H28 and H2452 cells, we failed to detect any apparent changes in the activities of the Rho-family of small GTPases as well as the other above mentioned signaling molecules in H2052 cells (data not shown). With this respect, it is note-worthy that, unlike H28 and H2452 cells, H2052 cells have mutations within the genes involved in Hippo signaling, including *NF2*, resulting in aberrant activation of YAP that confers highly proliferative and invasive properties to MPM cells (Miyanaga *et al.,* 2015; Zhang *et al.,* 2017). In fact, knockdown of *YAP* inhibited invasion and

proliferation of H2052 cells (Figure S2 and data not shown). Thus, it would be of interest to examine a possible functional relationship between Ror signaling and YAP in regulating invasion and proliferation of H2052 cells.

In summary, we have shown for the first time that Ror1 and/or Ror2 are expressed highly in human MPM tissues and cell lines with different histological features. In MPM cell lines, expression of Ror2 is associated with high invasive and proliferative properties. Although Ror1 can also contribute to proliferation of MPM cells, its pro-invasive role becomes evident in MPM cells lacking expression of Ror2. Our results also reveal that Ror signaling is mediated at least partly byRac1 and Cdc42 to promote invasion and proliferation of MPM cells. These findings shed light on our understanding of the role of Ror-mediated signaling in promoting invasion and proliferation of MPM cells. Further study will be required to clarify the molecular mechanisms how expression of Ror1 and Ror2 are induced in MPM cells and how Ror-Rac1/Cdc42 signaling can be mediated to promote initiation and/or progression of MPM. Our findings also indicate an interesting possibility that Ror1, Ror2, and possibly molecules involved in Ror signaling might be novel diagnostic and/or therapeutic targets for MPM.

Experimental procedures

Cell culture and Transfection

Human MPM cell lines, NCI-H28, NCI-H2052 and NCI-H2452, were cultured in RPMI-1640 medium (Nacalai tesque, Kyoto, Japan) containing 10 % (v/v) fetal bovine serum (Bio-West, Nuaille, France) at 37 ℃. For plasmid and siRNA transfection, Viafect (Promega, Madison, WI, USA) and RNAiMAX (Thermo Fisher Scientific, Waltham, MA, USA) were used, respectively.

Antibodies

The following antibodies were used: anti-Ror1 (4102, 1:25 for immunohistrochemistry, 1:500 for Western blotting, Cell Signaling Technology, Danvers, MA, USA), anti-Ror2 (sc-80329, 1:250 for Western blotting, Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-Ror2 (AF2064, 1:50 for immunohistrochemistry, R & D systems, Minneapolis, MN, USA), anti-Rac1 (23A8, 1:500, Millipore, Burlington, MA, USA), anti-Cdc42 (2462, 1:500, Cell Signaling Technology), and anti-α-tubulin conjugated with horse radish peroxidase (PM054-7, 10,000, MBL, Nagoya, Japan).

Small interfering RNA (siRNA)

The following target sequences were used for the respective siRNAs: si-*Ror1*#1, CCCAGAAGCTGCGAACTGT; si-*Ror1*#2, CAGCAATGGATGGAATTTCAA; si-*Ror2*#2, GCAACCTTTCCAACTACAA; si-*Rac*1, GAGGAAGAGAAAATGCCTG; si-*Cdc42*, GACTCCTTTCTTGCTTGTT. The sequences of si-*Ror2*#1 and negative control siRNA (si-Ctrl) were described previously (Enomoto *et al.,* 2009).

Immunoprecipitation and Western blotting

Cells were solubilized with ice-cold lysis buffer [50 mM Tris-HCl (pH7.5), 150 mM NaCl, 1% (v/v) Nonidet P-40, 1 mM EDTA, 10 mM NaF, 1 mM Na_3VO_4, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM *p*-amidinophenylmethanesulfonyl fluorid]. The

resultant lysates were subjected to immunoprecipitation, SDS-PAGE, and Western blotting analyses were performed as described previously (Nishita *et al.,* 2006).

GST pull-down Assay

To detect active GTP-bound forms of Rac and Cdc42, respectively, cells were solubilized and mixed with Glutathione S-transferase (GST)-PBD (p21-binding domain of PAK1) coupled with glutathione-Sepharose beads, as described previously (Nishita *et al.,* 2002). Proteins contained in the pelleted beads and total cell lysates were separated by SDS-PAGE and subjected to Western blotting using antibodies to Rac and Cdc42, respectively.

Immunohistochemical analysis

Primary MPM tissue specimens were resected from 5 patients at Kobe University Hospital, fixed, and embedded in paraffin for sectioning. The resultant tissue sections were incubated with antibodies against Ror1 and Ror2 overnight at 4℃ and then with anti-rabbit (EnVision+ System-HRP; DakoCytomation, Carpinteria, CA, USA) and anti-goat (ImmPRESS Reagent kit Peroxidase; Vector Laboratories, Burlingame, CA, USA) IgG conjugated with HRP-labeled polymer, respectively, for 30 min at room temperature. Secondary antibodies were visualized with DAB Chromogen (DakoCytomation), and nuclei were counterstained with hematoxylin. Clinical tissue specimens were obtained and analyzed in accordance with procedures approved by the institutional review board of Kobe University Hospital (No. 170196).

Matrigel invasion assay

Matrigel invasion assay was performed as described previously (Enomoto *et al.,* 2009). In brief, 2.5×10^4 cells in serum-free RPMI-1640 were loaded onto the upper well of the Transwell chamber (8 μm ϕ pore size; Corning, NY, USA), precoated with Matrigel (1:40 in RPMI-1640) on an upper side of the chamber. The lower well was filled with

600 μl of RPMI-1640 containing 10% FBS. After incubation for 24 hr, cells invaded to lower surface of the membrane were counted.

WST-8 assay

WST-8 assay was performed using Cell Counting Kit-8 (Dojindo, Kumamoto, Japan), according to the manufacturer's instructions. In brief, 5×10^3 cells were seeded onto a 96-well plate in triplicate and transfected with the respective siRNAs. After cells were cultured for various periods of time, WST-8 reagent was added to the culture media. After incubation for 150 min, the absorbance at 450 nm was measured.

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Figure legends

Figure 1. Expression of Ror1 and Ror2 in MPM specimens and cell lines. (a) Immunohistochemical analyses of Ror1 and Ror2 with surgical specimens from patients with MPM. Representative images of hematoxylin and eosin (H&E) staining and anti-Ror1 and -Ror2 immunohistochemistry of MPM tissue sections are shown. The asterisks indicate desmoplastic stroma. Scale bar, 100 μm. (b) Western blotting analysis of Ror1 and Ror2 in MPM cell lines, H28, H2452 and H2052 cells. The asterisk denotes a nonspecific band. (c) Invasive activity of MPM cells was measured by Matrigel invasion assay. Data represent means ± SD of three independent experiments.

Figure 2. Effect of *Ror1* or *Ror2* knockdown on invasiveness and proliferation of MPM cells. (a-f) The respective cells were transfected with the indicated siRNAs and cultured for 3 days. (Upper panels) The respective cell lysates were subjected to SDS-PAGE, followed by Western blotting analysis with antibodies to the indicated proteins. In (a) and (b), Ror2 protein was concentrated by anti-Ror2 immunoprecipitation prior to Western blotting. In (c)-(f), the asterisks indicate nonspecific bands. (Lower panels) Cells were analyzed by Matrigel invasion assay. Data represent means ± SD of three independent experiments. *P<0.05; **P<0.01, *t* test. (g-i) Cells were transfected with the respective siRNAs. On day 1 and day 7 after siRNA transfection, viable cell number was measured by WST-8 assay. Data represent means ± SD of three independent experiments. *P<0.05; **P<0.01, *t* test.

Figure 3. Rac1 and Cdc42 are activated downstream of Ror signaling and are required for high invasive and proliferative properties of H28 and H2452 cells. (a, b) H28 (a) and H2452 (b) cells were transfected with the indicated siRNAs. After 3 days in culture, cells were analyzed by GST-PBD pull-down assay to detect active GTP-bound forms of Rac and Cdc42, respectively. Representative results of three independent experiments

are shown, respectively. (c, d) Effect of *Rac1* or *Cdc42* knockdown on invasiveness of H28 (c) and H2452 (d) cells. Cells transfected with the indicated siRNA were analyzed by Western blotting (upper panels) and Matrigel invasion assay (lower panels). Data represent means ± SD of three independent experiments. **P<0.01, *t* test. (e, f) Effect of *Rac1* or *Cdc42* knockdown on proliferation of H28 (e) and H2452 (f) cells. Cells were transfected with the respective siRNAs. On day 1 and day 7 after siRNA transfection, viable cell number was measured by WST-8 assay. Data represent means ± SD of three independent experiments. *P<0.05; **P<0.01, *t* test.

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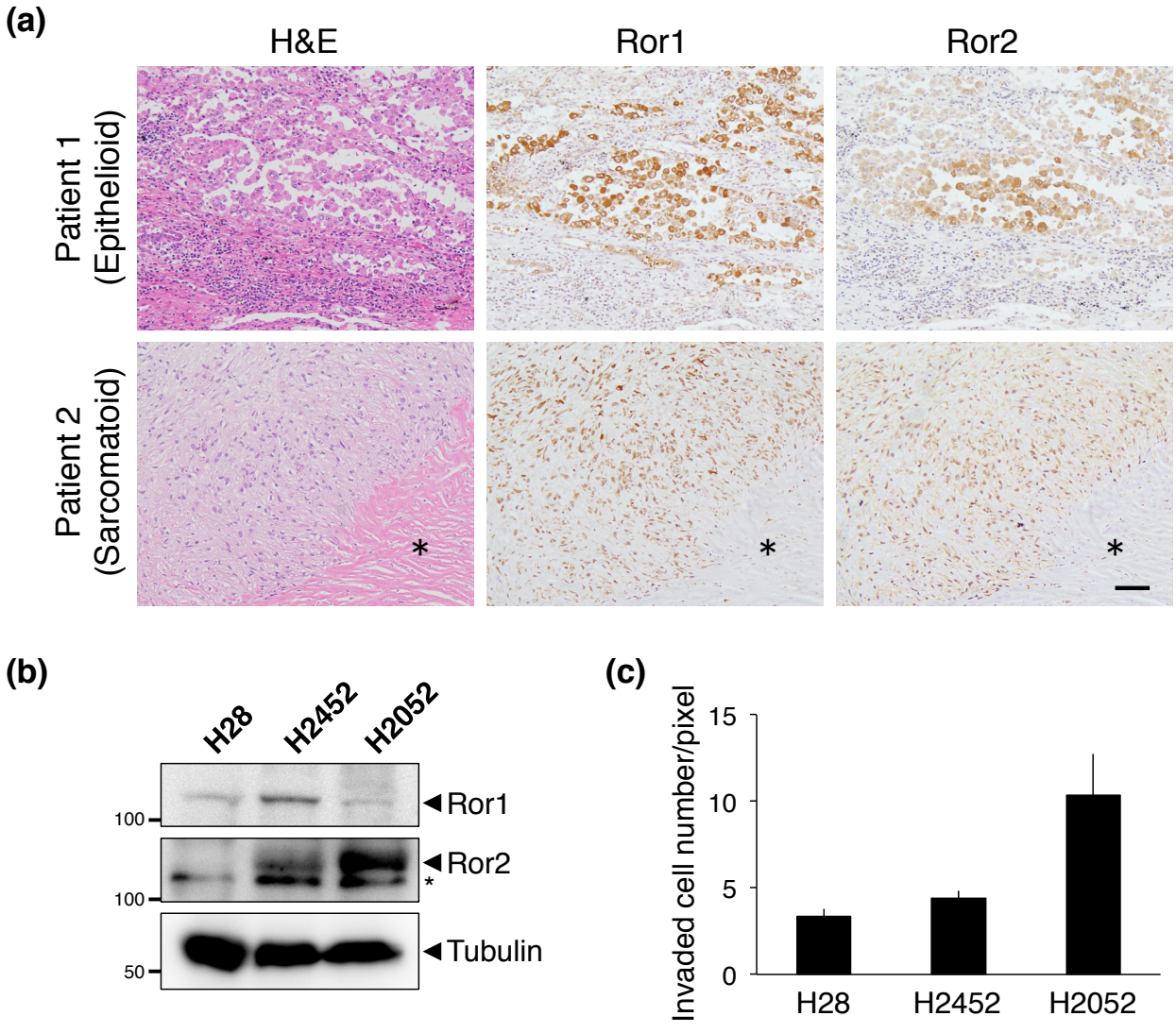

Figure 2 Saji *et al*.

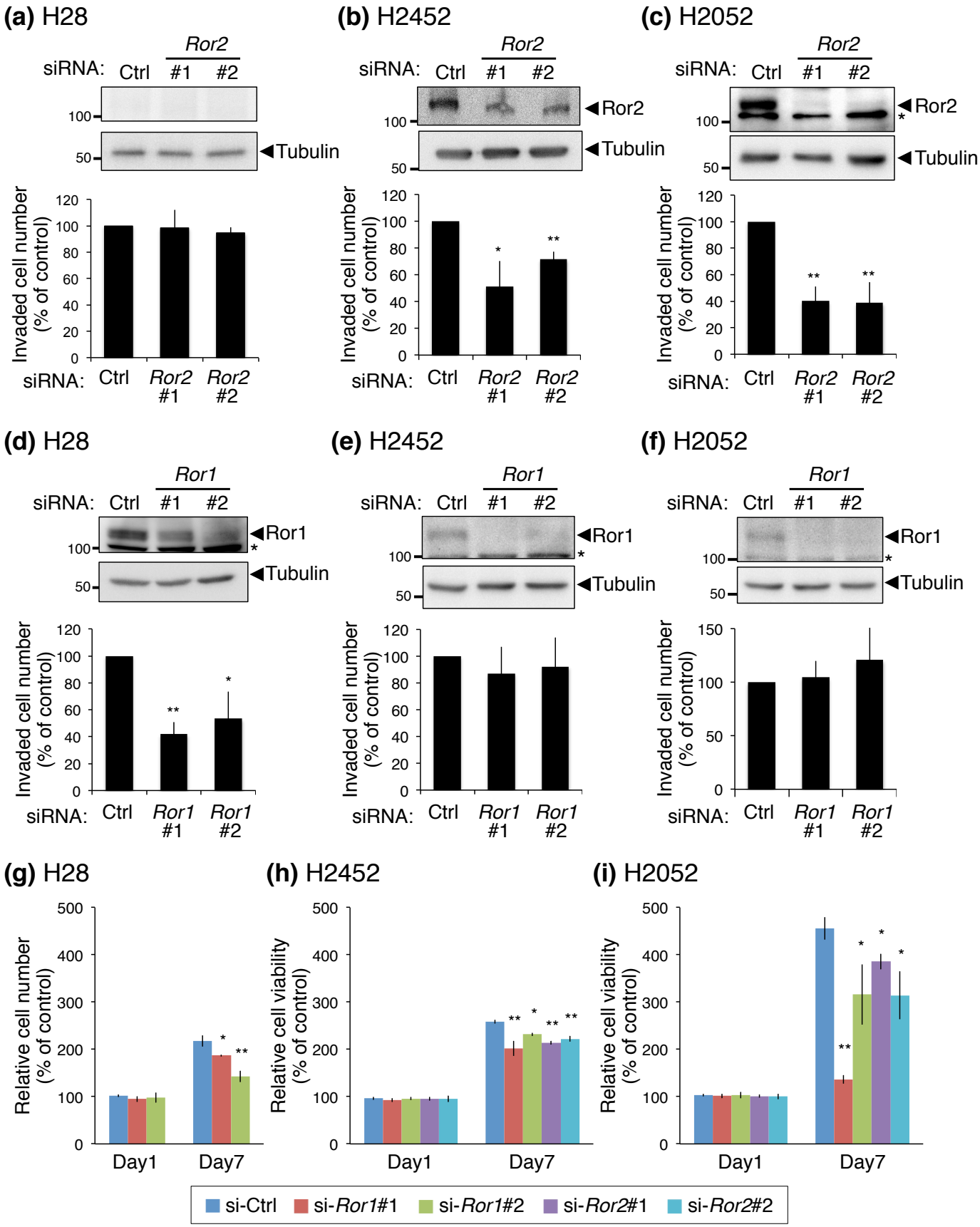

Figure 3 Saji *et al*.

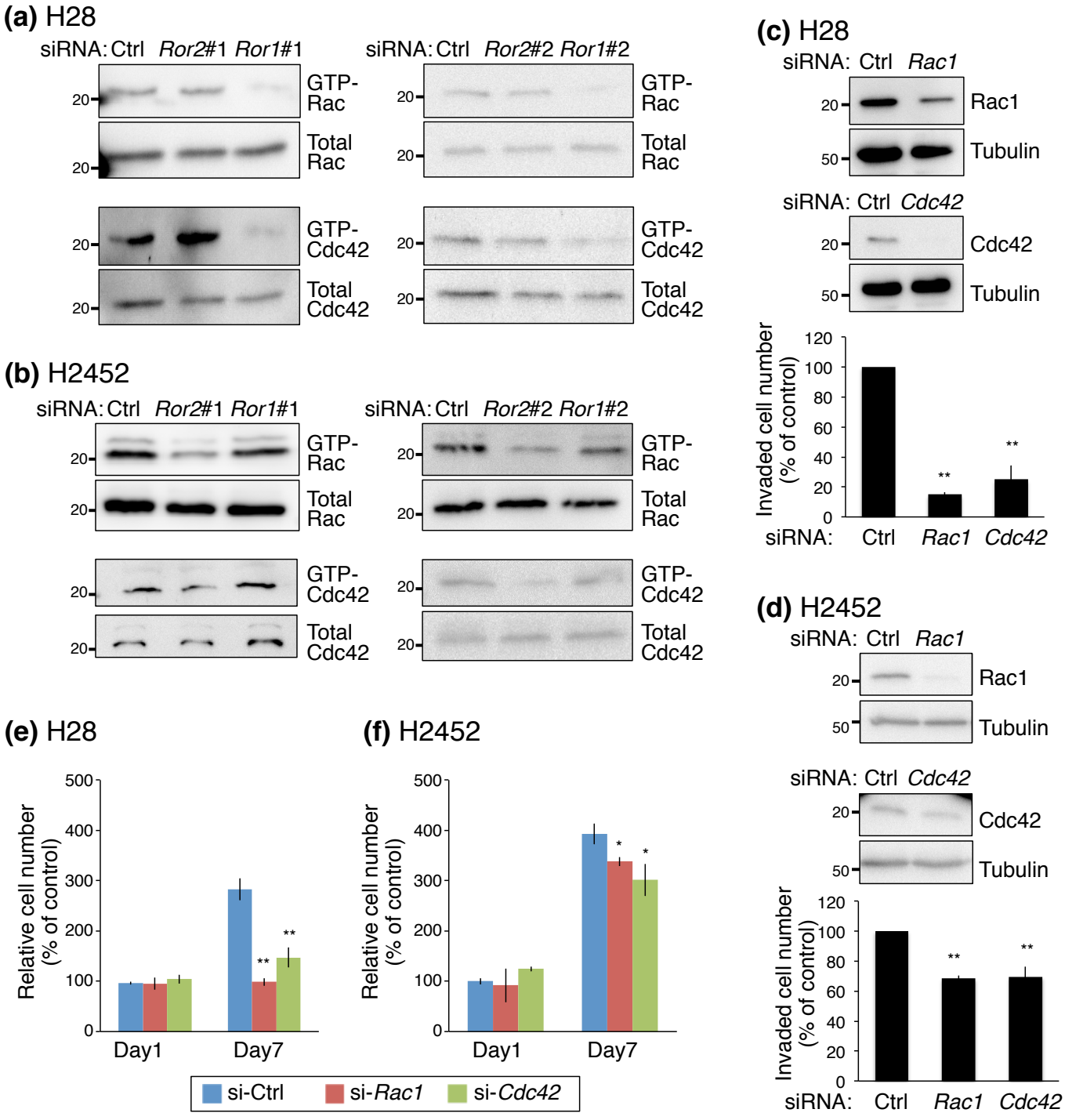

Figure S1 Saji *et al*.

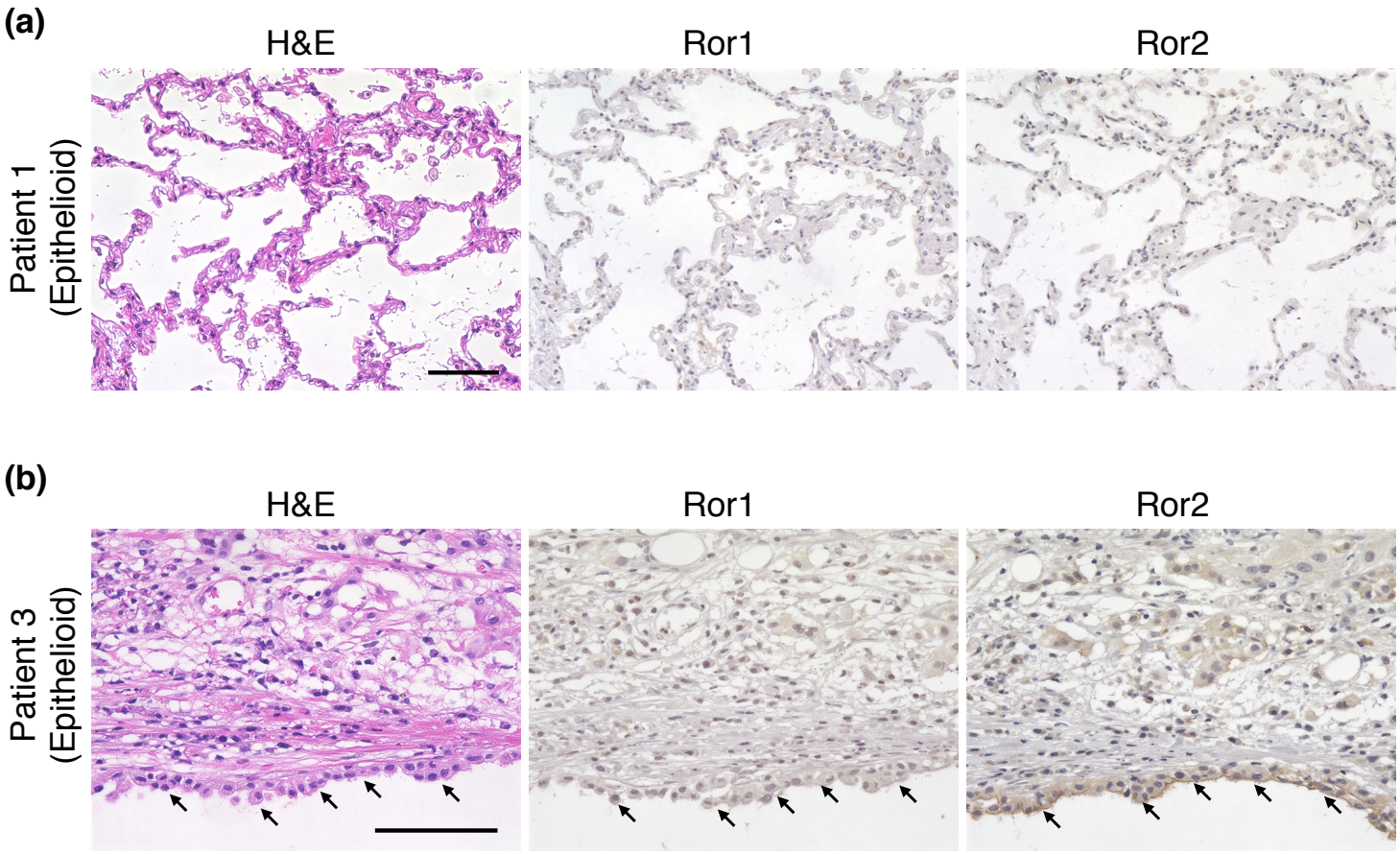

Figure S1. Immunohistochemical analysis of Ror1 and Ror2 in normal lung tissues (a) and non-invasive, dysplastic pleural mesothelial cells (b) within MPM specimens. MPM tissue sections were stained with hematoxylin and eosin (H&E) and anti-Ror1 and -Ror2 antibodies, respectively. Representative images were shown. Arrows in (b) indicate mesothelial cells. Scale bars, 100 μm.

Figure S2 Saji *et al*.

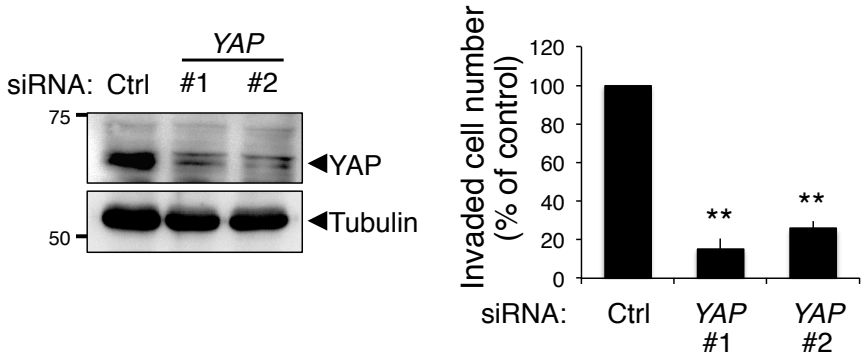

Figure S2. Effects of siRNA against *YAP* on invasiveness of H2052 cells. H2052 cells were transfected with the indicated siRNAs. After 3 days in culture, the respective cell lysates were subjected to Western blotting analysis with anti-YAP antibody to confirm knockdown efficiency (left panel) and Matrigel invasion assay (right panel). Data represent means ± SD of three independent experiments. **P<0.01, *t* test.