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Novel Cellular Activities of The Cell Cycle Checkpoint Protein Rad1 Revealed by a New High-quality anti-Rad1 Antibody*

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Abstract *Rad9*, *Rad1* and *Hus1* are critical for the cell cycle checkpoint and can form a heterotrimer complex called 9-1-1 complex which was supposed to play important roles in the cell cycle checkpoint and other activities required for the maintenance of genome integrity. However, lack of high quality anti-Rad1 antibodies has seriously hindered the research on Rad1 as well as working mechanisms of the 9-1-1 complex at molecular level. In this study, a mouse anti-Rad1 monoclonal antibody (mAb) was successfully generated. The mAb possesses high affinity and specificity, and recognizes both endogenous mouse Rad1 (mRad1) and human Rad1 (hRad1) proteins and was successfully used in ELISA, Western blot analysis, immunoprecipitation and immunofluorescence assays. Using this mAb, we found that mRad1 protein expression was increased in $Rad9^{+/+}$ mouse embryonic stem (MES) cells after hydroxyurea (HU, a genotoxic agent) treatment while not in $Rad9^{-/-}$ MES cells, suggesting that mRad1 expression is under Rad9 regulation. Furthermore, endogenous mRad1 was distributed mainly in the cytoplasm and did not migrate to the nucleus after HU treatment, contradicting the generally accepted hypothesis that Rad9, Rad1 and Hus1 form the 9-1-1 complex in the nucleus in response to genotoxic stresses. In summary, the exact molecular roles of Rad1 and the 9-1-1 complex are likely more complicated than previously expected and this anti-Rad1 mAb is a powerful tool for the future investigation on Rad1 as well as the 9-1-1 complex.

Key words Rad9-Hus1-Rad1 (9-1-1) complex, Rad1, anti-Rad1 monoclonal antibody, hydroxyurea (HU)
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Rad9, *Rad1* and *Hus1* genes are conserved from yeast to humans and their coded proteins can form a heterotrimer complex called the 9-1-1 complex [1–4] which is critical for the cell cycle checkpoint control and plays important roles in the maintenance of genomic integrity[5–18]. Aside from cell cycle checkpoint functions, there is mounting evidence that Rad9 has many other functions such as DNA repair, 3′ -5′ exonuclease activity [19], radioresistance [6], telomere maintenance [20] and transactivation of downstream genes [21]. The majority of knowledge on the 9-1-1 complex was derived from the experimental data of Rad9 largely because high quality anti-Rad9 antibodies were available [22–27]. Rad1 is highly conserved in evolution, the immunogenicity of Rad1 is very low, and high quality anti-Rad1 antibodies have not been generated so far, thus few articles on Rad1 have been published and the knowledge on the functions of Rad1 is very limited. Therefore the prediction on Rad1 functions and the conclusions on the functions of the 9-1-1 complex need to be further substantiated by

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studying Rad1 directly with good quality anti-Rad1 antibodies.

Some research groups investigated the functions of Rad1 by overexpression of various tagged Rad1-fusion proteins and the usage of the corresponding anti-Tag antibodies [12, 26, 28–30]. However, these might not reflect the actual functions of endogenous Rad1. Several groups studied the functions of Rad1 using self-made anti-Rad1 polyclonal antibodies, and reported that the endogenous hRad1 protein was located mainly in the nucleus and only a small proportion of hRad1 was outside the nucleus [12], but the specificities of the antibodies were not rigorously confirmed. In the past we also generated anti-Rad1 antibodies, but all the antibodies generated fell short in affinity and/or specificity and not suitable for studying functions of endogenous Rad1. Thus, it is of great value to produce high quality antibodies for Rad1 protein detection in the study of Rad1 and 9-1-1 complex.

In this study, we raised a mouse anti-Rad1 mAb, named F10, with high affinity and specificity. F10 was able to recognize both endogenous mRad1 and hRad1 protein and was successfully used in ELISA, Western blot, immunoprecipitation and immunofluorescence assays. We also investigated the subcellular distribution of endogenous Rad1 protein and its response to replication inhibitor hydroxyurea (HU) in MES cells. To our surprise, Rad1 did not moved into the nucleus after the treatment of the genotoxic agent HU, which contradicts the long-standing hypothesis that Rad1 and Hus1 would migrate into nuclei and forms the 9-1-1 complex with Rad9 in response to genotoxic stresses including HU.

1 Materials and methods

Ethics Statement Maintenance of mice and experimental procedures were approved by the Animal Welfare and Research Ethics Committee of the Institute of Biophysics, Chinese Academy of Sciences.

1.1 Cell lines

Myeloma cells Sp2/0 were cultured in IMDM (HyClone) supplemented with 10% fetal bovine serum (FBS, HyClone) and 1% (*v*/*v*) penicillin/streptomycin (Gibco), Human HeLa and HEK 293T cells were cultured in DMEM (Invitrogen, CA) supplemented with 10% fetal bovine serum (FBS, HyClone) and 1% (*v*/*v*) penicillin/streptomycin (Gibco), *Rad9*$^{+/+}$ MES cells, *Rad9*$^{-/-}$ MES cells and *Rad1*$^{-/-}$ MES cells, obtained from Lieberman's laboratory[8, 31] were cultured in standard MES cell medium in the presence of leukemia inhibitory factor (LIF) without a feeder layer [32]. All the cells were maintained at 37 ℃ in a humidified CO_2-controlled (5%) incubator.

1.2 Purification of antigen mRad1 protein

Mouse *Rad1* gene were cloned into a pGEX-6p-1 vector (GE Healthcare), a bacterial vector for expressing GST fusion proteins with a PreScission protease site. The protein was expressed in *E. coli* strain BL21 (DE3). pGEX-PPase plasmid, encoding PreScission protease was obtained from Bi L's laboratory (Institute of Biophysics, Chinese Academy of Science, China), then expressed in *E. coli* strain BL21 (DE3). All GST-tagged proteins were purified by affinity chromatography using Glutathione Sepharose 4B GST bind resins (GE Healthcare) according to the manufacturer's protocol. The purity of the protein was analyzed by SDS-PAGE technique with Image J software and the concentration of the protein was detected by NanoDrop® ND-1000 (Gene Company, USA).

1.3 Generation of monoclonal antibodies

Female BALB/c (10 weeks old) mice were injected subcutaneously with 100 μg of purified antigen protein emulsified in complete Freund's adjuvant. Two additional injections of 50 μg antigen emulsified in incomplete Freund's adjuvant were followed at bi-weekly intervals starting four weeks after the first immunization. One week after the second boost, the serum antibody titer was tested using ELISA. Two weeks after the second boost, the mice were given a booster injection intraperitoneally with 50 μg protein. The boosted mice were continuously maintained for one month. Afterwards, another round of immunization described above except the first 100 μg injection was performed again. One week after that, the mice were given another final booster injection intraperitoneally with 100 μg protein. Two days after the last injection, spleen cells from the immunized mice were fused with myeloma Sp2/0 cells [33] at 2∶1 ratio using polyethylene glycol (PEG) 1500 (Roche Diagnostics GmbH, Mannheim, Germany). Hybridoma cells were seeded into 96-well plates and selected in Hypoxanthine-aminopterin-thymidine (HAT) supplemented IMDM (Hyclone). After cultivation for 7 days aminopterin was omitted from medium and supernatants were screened for antibody reactivity and specificity by ELISA. Antigen mRad1

protein (2 mg/L) was coated on microtiter plates overnight at 4℃. Cells of positive tested wells were subcloned two times (with coated antigen at a concentration of 0.5 mg/L) by limiting dilution. Subtype identification was performed using a mouse Immunoglobulin Isotyping ELISA Kit (BD Pharmingen™, Heidelberg, Germany)according to the manufacturer's protocol. RNA was extracted from Screened hybridoma cells then cDNA was synthesized by reverse transcription followed by the manufacturer's protocol (Invitrogen). General primers[34] was used to amplify the VH and VL genes of the mAbs and clone into PMD18-T vector, then sent it to the company to confirm the sequences. MAbs were obtained from the ascites of mice injected intraperitoneally with screened hybridoma cells and purified by Protein A-agarose (GE Healthcare) affinity chromatography.

1.4 ELISA

To select anti-Rad1 monoclonal antibodies, the purified antigen mRad1 (0.5 mg/L) was coated on microtiter plates overnight at 4℃. Plates were blocked with 3% PBST (PBS with 0.05% (*v*/*v*) Tween 20) containing 3% (*w*/*v*) bovine serum albumin (BSA) for 1 h at 37℃. 100 μl of cell supernatant was added and incubated for 1 h at 37℃. After washing, a 1∶2 000 dilution of HRP-conjugated anti-mouse IgG antibody (Promega) was added for 1 h at 37℃. Plates were washed 3 times between each step with 0.05% PBST. 100 μl substrate (TMB system) was added and the reaction was stopped by the addition of an equal volume of 0.3% H_2SO_4. The optical density was measured at 450 nm using a spectrophotometer (Cytation 3 Cell Imaging Multi-Mode Reader, Bio Tek, USA).

1.5 SPR assay

Anti-mRad1 monoclonal antibody, purified from ascites mentioned above, antibody affinity was measured by Surface Plasmon Resonance (SPR) using a CM5 sensor chip in a BIACORE3000 at 25℃. Anti-mRad1 antibody F10 was captured in the flow cell. Running buffer and different concentrations of antigen mRad1 protein (0.3125, 0.625, 1.25, and 2.5 μmol/L diluted in running buffer) were applied to the antibody-containing flow cell for 2 min at a flow rate of 30 μl/min.

1.6 Western blot

The plasmid pCDNA3.1 was used to construct plasmids pCDNA-*mRad1* capable of expressing mRad1 in cells. Flag-CMV2-*mRad1*, and the Flag-CMV2-*hRad1* were constructed previously[35] to encode Flag-hRad1 and Flag-mRad1. Human HeLa, HEK 293T and *Rad9*$^{+/+}$ MES cells were transfected with the plasmid of interest using Lipofectamine 2000 (Invitrogen, USA). Cells were grown to 60% ~80% confluence in 60 mm tissue culture dishes and transfected with 1.5 μg Flag-CMV2-*hRad1*, Flag-CMV2-*mRad1*, pCDNA-*mRad1* and pCDNA3.1 (as an empty vector control) following the procedure described by Invitrogen. The whole cell lysate of interest were separated by SDS-PAGE and transferred onto PVDF membrane (GE Healthcare). Membranes were blocked with 5% skim milk in PBS and incubated with MAbs (1 g/L, 1∶1000) and anti-β-actin monoclonal antibody(1∶5 000) for 1h at room temperature, followed by incubation with HRP-conjugated anti-mouse IgG (Promega, 1∶2 000) for 1 h at room temperature. Detection was done using chemiluminescence assay. Membranes were washed 4 times for 10 min with PBS after each incubation step. β-actin was used as an internal control. The mean normalized absorbance (A) of Rad1 bands relative to the A of β-actin band was calculated using the ImageJ software.

1.7 Immunoprecipitation

Flag-CMV2-*hRad1* and Flag-CMV2-*mRad1* plasmids were transfected into 293T cells respectively, after 24 h incubation at 37℃, transfected cells were lysed in 0.5 ml ice-cold lysis buffer (150 mmol/L in NaCl, 0.5% NP-40, 50 mmol/L Tris, 10% Glycerine, 1 mmol/L DTT, pH 7.5) containing protease inhibitor cocktail (Roche, Indianapolis, IN). Lysed cells were spun at 20 000 *g* in a microcentrifuge at 4℃ for 20 min. Protein A beads were washed by wash buffer (150 mmol/L in NaCl, 0.5% NP-40, 50 mmol/L Tris, 10% glycerine, pH 7.5) 1 time, centrifuge at 800 *g* for 2 min. The supernatant was precleared by incubating at 4℃ and continuously mixing on a spinning wheel with 20 μl washed protein A together with 2 μg normal mouse IgG (Santa Cruz) for 1 h. The supernatant was collected after spinning for 2 min at 20 000 *g* and immunoprecipitated with MAbs of interest overnight at 4℃. Add 20 μl washed protein A beads into the supernatant with MAbs incubating at 4℃ and continuously mixing for 2 h. Then the beads were washed five times, 20 μl sample buffer was added to the beads. Then, the beads were boiled for 5 min. Samples (10 μl) were loaded into the SDS-PAGE gel, fractionated, and immunoblotted with anti-Flag tag

rabbit polyclonal antibody, HRP-conjugated anti-Rabbit IgG was used to detect FLAG-recombinant protein.

1.8 Test for hydroxyurea (HU) activity

Rad9$^{+/+}$ MES cells and *Rad9*$^{-/-}$ MES cells were seeded into 6-well plates, when cells were grown to 60%～80% confluence, added 1 mmol/L hydroxyurea (HU) to each well continuously cultured for 1 h, 4 h and 12 h respectively. The whole cell lysates were prepared for Western blot analysis as described before.

1.9 Immunofluorescence

Rad9$^{+/+}$ MES and *Rad1*$^{-/-}$ MES cells (grown on glass cover slips) were seeded into six wells plate, all cells were washed three times by PBS when cells reached a density of 40%, then incubate with 1 ml ice cold methyl alcohol (anhydrous) for 15 min, following by 1 h incubation of 3% BSA in PSB at a 37℃ humidified chamber. After that F10 was added and incubated for 1 h, 37℃. FITC conjugated Rabbit anti-mouse immunoglobulin(Jackson ImmunoResearch, 1∶200) was added to cells and incubated for 1 h at room temperature. Cover slips were washed between each step with 1% PBSA. After mounting cover slips onto a glass slide using fluorescent mounting reagent cells were screened for fluorescent staining.

1.10 Statistical analyses

All data were analyzed with GraphPad 5.0 (GraphPad, Software, La Jolla, CA, USA) using a two-Tailed Student's *t*-test. All data are presented as means ± the standard error of the mean (SEM). $P < 0.05$ was considered statistically significant.

2 Results

2.1 Generation and purification of mRad1 protein

In the past we used at least 10 different anti-Rad1 antibodies purchased commercially, offered as gifts from researchers, or generated by ourselves. However, none of these antibodies gave us clear experimental results, due to very low affinity and/or low specificity to Rad1. In this study, we made effort to generate highly pure Rad1 protein without a tag. Rad1 is highly conserved in evolution and the immunogenicity of Rad1 is very low. The purity of the antigen is a key factor to produce high specificity antibody[36], impure proteins might be much more immunogenic than Rad1. Tags are usually highly immunogenic and may serve as dominant immunogens, which when fused to Rad1 may induce antibodies mainly against tags but not against Rad1. In this study, we purified GST tagged full length mRad1 protein produced in *E. coli*, then removed the GST tag by PPase (PreScission protease) *via* the PreScission protease site, obtained pure mRad1 with further purification. As shown in Figure 1, the molecular mass of the purified mRad1 was between 26 and 34 (the predicted m is 31 ku) and its purity was above 98% (analysed by imageJ software). The concentration of mRad1 protein was 3.86 g/L.

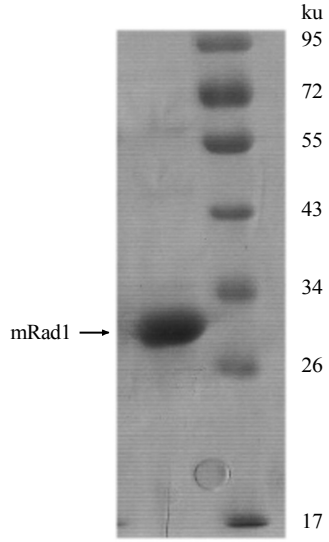

Fig. 1 Purification of mRad1 protein

GST-mRad1 was affinity-purified, GST was removed, and the released mRad1 was further purified. The purified mRad1 protein was at a band of 31 ku with a purity of 98% (analysis by Image J).

2.2 Anti-Rad1 mAb recognizes both human and mouse Rad1 proteins

Anti-Rad1 mAb was generated by immunizing mice with the above purified mRad1 protein. A total of 4 times of immunization, 3 for subcutaneous injection and 1 for intraperitoneal injection were performed, which is a standard procedure for immunizing mice[37]. After the boost injection intraperitoneally, hybridization was performed to generate anti-Rad1 mAbs. The clones of hybridoma were screened using ELISA. Since a properly low concentration of antigen used for screening can contribute to successful selection of high titer clones [38], we coated purified mRad1 protein at a low concentration (0.5 mg/L). A total of 1 296 individual clones of hybridoma cells were screened, and 112 clones were positive in the ELISA test. The strongest positive 20 clones were confirmed by Western blot analysis on purified mRad1 and MES cell lysates. However, all of the 20 clones

demonstrated strong undesirable nonspecific bands and bound very weakly to endogenous mRad1 protein (Data not shown). A remaining mouse after the above immunizing procedure was immunized again one month later for 3 more times, 2 for subcutaneous injection and 1 for intraperitoneal injection. Eighteen strongly positive clones were selected for further characterization. Western blot analysis of 0.1 μg purified mRad1 protein expressed in *E. coli* cells was performed, and two out of the 18 cloned antibodies recognized a single protein band of about 31 ku, consistent with the molecular mass of mRad1. Since the nucleotide sequences of these two antibodies were the same, we considered the two hybridoma clones were derived from a single clone. This clone was named F10. The subtype of F10 mAb was IgG2a heavy chain and mouse kappa light chain. F10 mAb was further purified from ascite by Protein A-agarose and the final concentration of F10 mAb was 3.16 g/L.

To examine whether F10 could detect overexpressed and endogenous Rad1 in cells, Western blot analysis was performed. Lysate of MES cells and human 293T cells transfected with or without the plasmids expressing Flag-tagged mRad1 or hRad1 were probed with F10 mAb. As shown in Figure 2a, F10 reacted with both overexpressed and endogenous mRad1 protein in MES cells. Flag-tagged mRad1 protein migrated slightly slower than the endogenous mRad1 protein as well as the overexpressed mRad1 protein without Flag tag. F10 did not identify an mRad1 protein band in *Rad1*$^{-/-}$ MES cells, confirming the specificity of F10 mAb. Similar results were also shown in Figure 2b indicating that F10 also recognized both overexpressed and endogenous hRad1 protein in human 293T cells. Furthermore, we did not observe undesirable nonspecific bands of other size as shown in a figure covering a large molecular range(Figure 2c). These results indicate that F10 mAb recognizes both mRad1 and hRad1 protein specifically.

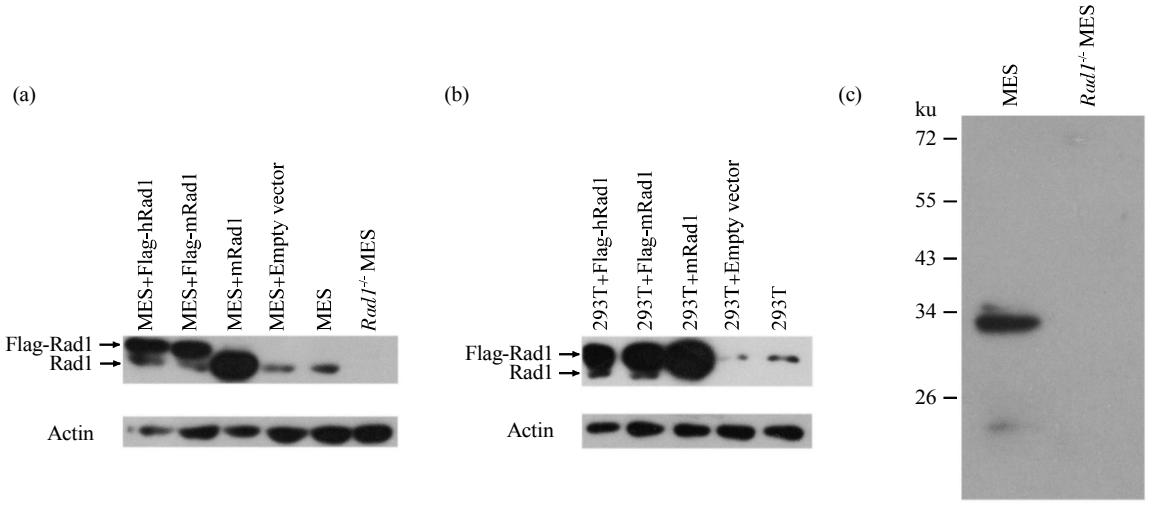

Fig. 2 Anti-Rad1 monoclonal antibody F10 specifically recognizes both human and mouse Rad1 proteins

(a) Plasmids encoding Flag-hRad, Flag-mRad1 and mRad1 proteins (1.5 μg) were transfected into *Rad9*$^{+/+}$ MES cells; MES cells transfected with empty vector and MES and *Rad1*$^{-/-}$ MES cells were used as negative controls. Each lysate equivalent to 5×10^5 cells was subjected to Western blot analyses by anti-mRad1 antibody F10. (b) The same plasmid described above was transefected into Human 293T cells and performed by the same procedure. (c) MES and *Rad1*$^{-/-}$ MES cell lysates were measured by Western blot using F10.

2.3 The affinity of F10 mAb

The affinity of F10 mAb was investigated using Surface Plasmon Resonance (SPR). As detected by SPR, anti-Rad1 monoclonal antibody F10 bound to mRad1 with a high affinity(K_d = 8.12 nmol/L, Figure 3a). As detected by ELISA, F10 bound to mRad1 protein at a low concentration (0.0625 mg/L) with the coated antigen mRad1 protein from 0.1 μg/L to 250 μg/L (Figure 3b). These results indicated that the affinity of F10 mAb was sufficiently high to monitor endogenous Rad1.

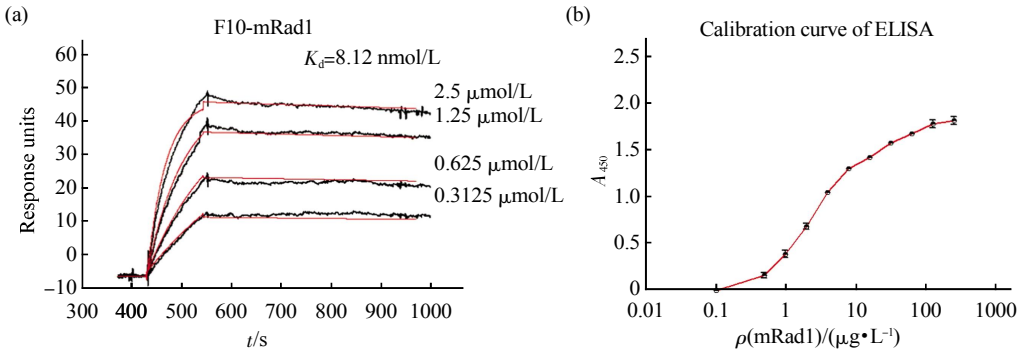

Fig. 3 Binding affinity of anti-Rad1 monoclonal antibody F10

(a) The equilibrium constant of anti-Rad1 antibody F10 was measured by SPR. Black line as the original binding data, red line as the fitting curve. The K_d of F10 was calculated as 8.12 nmol/L. (b) The ELISA was established for anti-mRad1 F10 detection at 0.0625 mg/L and HRP-conjugated anti-mouse IgG at 1∶2 000 dilution for detection. Coated antigen mRad1 protein at 250 μg/L and decreasing concentrations. The optical density was measured at 450 nm using a spectrophotometer (Cytation 3 Cell Imaging Multi-Mode Reader, Bio Tek, USA).

2.4 Comparison of F10 mAb with two commercial anti-Rad1 mAbs

We further compared F10 mAb with two commercial anti-Rad1 mAb, D6 (Santa Cruz) and ab5363[2, 27–28, 39–40] (Abcam) which were used by several groups in Rad1 studies. Flag- *hRad1* plasmid was transfected into human 293T and HeLa cells, F10, D6 and ab5363 (1 mg/L) were used for Western blot analysis. As shown in Figure 4a, both ab5363 and F10 reacted with the overexpressed hRad1 protein, while D6 did not. The whole cell lysates of human 293T, HeLa and wild type MES cells were used for endogenous Rad1 protein detection. D6 and ab5363 failed to mark the Rad1 protein while F10 bound to both hRad1 and mRad1 (Figure 4b). ab5363 instead bound to a few unknown proteins, suggesting that it has low specificity and affinity. F10 was also able to immunoprecipitate hRad1 and mRad1 while the two commercial antibodies could not(Figure 4c and Figure 4d). These results indicate that F10 can be used in Western blot analysis and immunoprecipitation for endogenous Rad1 while the two commercial anti-Rad1 mAb could not.

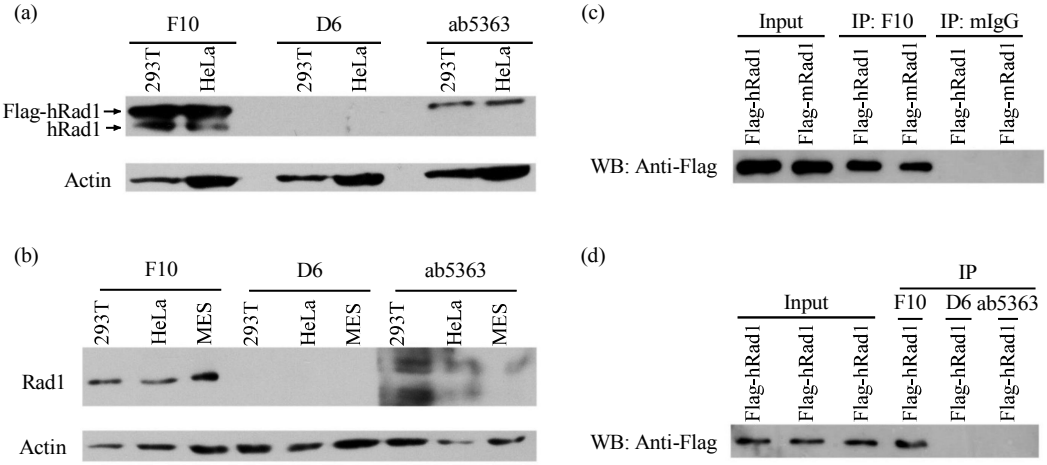

Fig. 4 Comparison of different anti-Rad1 antibodies

(a) Detection of overexpressed hRad1 protein. Plasmid encoding Flag-hRad1 protein (1.5 μg) were transfected into Human 293T and HeLa cells respectively, cell lysates equivalent to 5×10^5 cells was subjected to Western blot analyses by three different anti-Rad1 antibody F10, D6, ab5363. (b) Detection of endogenous Rad1 protein detected using different anti-Rad1 antibodies in different cells. (c) Immunoprecipitation of Flag-hRad1 and Flag-mRad1 by F10 and control mIgG. Overexpressed Flag-hRad1 and Flag-mRad1 in Human 293T cells were immunoprecipitated by anti-mRad1 antibody F10 and control mouse IgG, then detected with anti-Flag tag rabbit polyclonal antibody followed by secondary HRP-conjugated anti-Rabbit IgG. (d) Immunoprecipitation of hRad1 by different anti-Rad1 antibodies. Overexpressed Flag-hRad1 was immunoprecipitated using three different anti-Rad1 antibodies.

2.5 Dependence of mRad1 induction on Rad9

To investigate the response of endogenous mRad1 to the replication inhibitor HU, *Rad9*$^{+/+}$ MES and *Rad9*$^{-/-}$ MES cells were treated with 1 mmol/L HU and expression of mRad1 at each designated time point was detected using Western blot analysis with F10. In *Rad9*$^{+/+}$ MES cells, there was no significant difference in mRad1 expression at 1 or 4 h after HU treatment. At 12 h after HU treatment, mRad1 expression was significantly increased (Figure 5a). Interestingly, there was no significant difference in Rad1 protein expression in *Rad9*$^{-/-}$ MES cells after HU treatment at each indicated time point (Figure 5b), suggesting that Rad9 is involved in the regulation of Rad1 expression in response to genotoxic stresses.

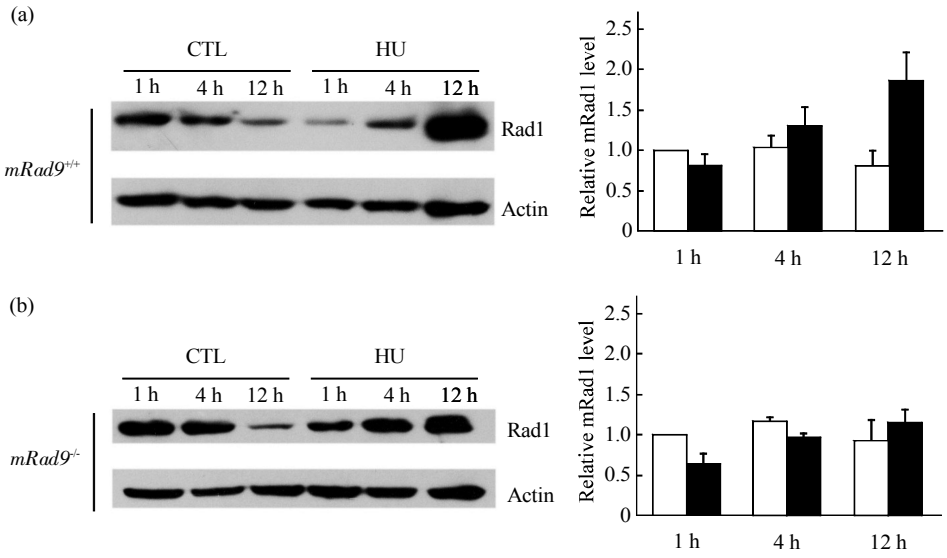

Fig. 5 mRad1 expression level in response to hydroxyurea (HU) treatment

(a) The mRad1 expression level in *Rad9*$^{+/+}$ MES cells was significantly induced by HU. *Rad9*$^{+/+}$ MES cells were treated with 1 mmol/L HU for 1 h, 4 h and 12 h, and the untreated cells used for negative controls. In the right panel, data were derived from three independent experiments as in left panel. The relative mRad1 levels were presented as mean ± the standard error of the mean (SEM). Two-Tailed Student's *t*-test indicated the statistical significance (P=0.02). (b) The mRad1 expression level in *Rad9*$^{-/-}$ MES cells was not induced by HU. Data were derived from three independent experiments. Methods are described as above. □ : Control; ■ : HU.

2.6 Endogenous mRad1 distribution in MES cells

To determine the distribution of endogenous mRad1, immunofluorescence was performed with F10 in *Rad1*$^{+/+}$ MES and *Rad1*$^{-/-}$ MES cells. The nuclear DNA was stained with DAPI. As shown in Figure 6a, in *Rad1*$^{+/+}$ MES cells, the signals of mRad1 mainly located in the cytoplasm. *Rad1*$^{-/-}$ MES cells were used as the negative control and we observed nearly no signals in *Rad1*$^{-/-}$ MES cells which confirmed that the signals detected using immunofluorescence with F10 mAb were indeed from the Rad1 protein. It is reported that hRad9 could help hHus1 transfer into the nucleus after HU treatment and may also have the same effect on the hRad1 [22]. Interestingly, we did not observe the translocation of mRad1 from the cytoplasm to the nucleus at each indicated time after HU treatment in *Rad1*$^{+/+}$ MES cells (Figure 6b).

We also did immunofluorescence assays on *Rad1*$^{+/+}$ MES and *Rad1*$^{-/-}$ MES cells with the two commercial antibodies and found that they were unable to specifically bind endogenous mRad1 on the fixed wild type MES cells (data not shown).

3 Discussion

Lack of high quality anti-Rad1 antibodies has seriously hindered the research on Rad1 itself as well as the 9-1-1 complex. In this study, we produced an anti-Rad1 mouse monoclonal antibody with high specificity and affinity (F10). The following two points likely contributed to the success in obtaining this antibody: the high purity of the antigen without a tag and three more times of immunization with mRad1.

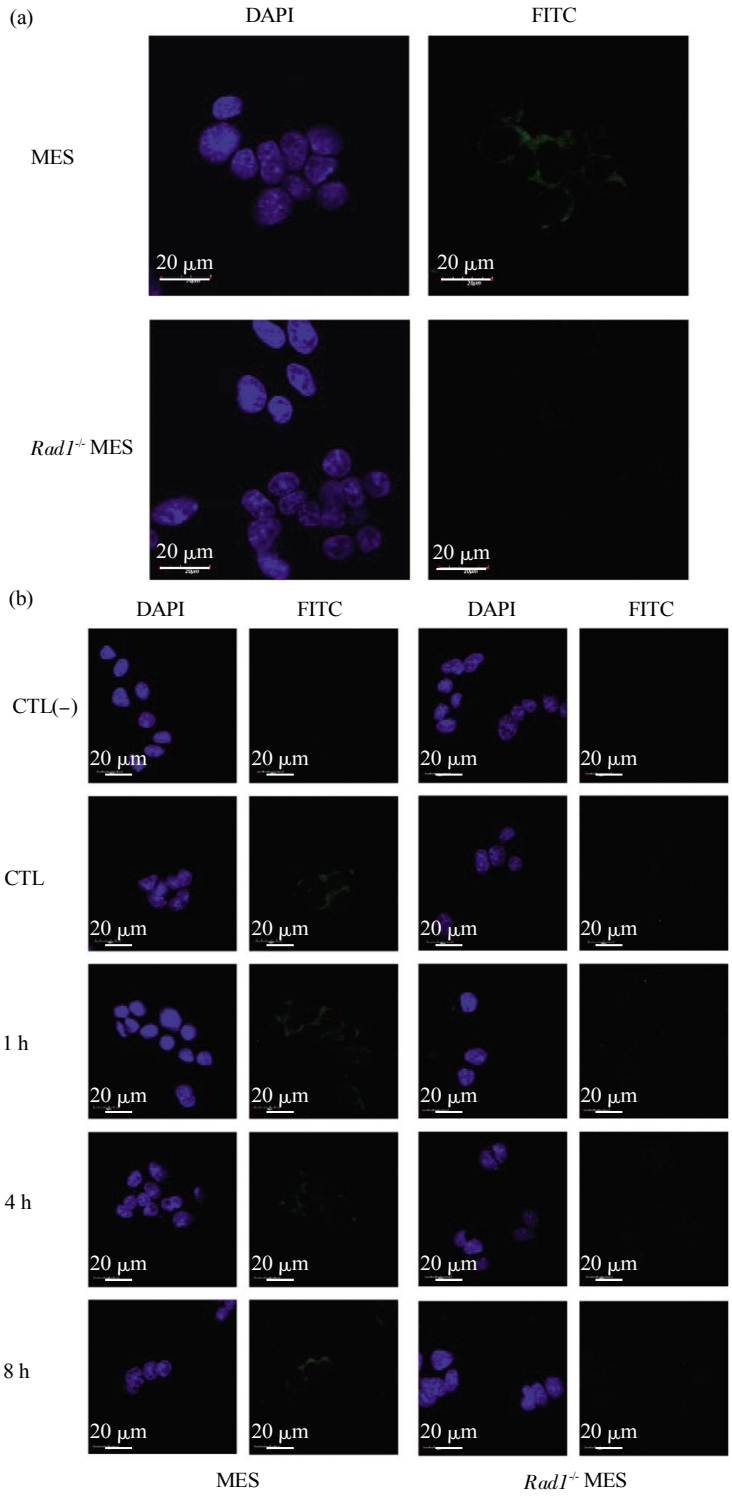

Fig. 6 Confocal images of MES and Rad1$^{-/-}$ MES cells with anti-Rad1 antibody F10

(a) Immunofluorescence microscopy of MES and *Rad1*$^{-/-}$ MES cells with anti-Rad1antibody F10 using a FITC-conjugated anti-mouse secondary antibody (green), and staining of nuclear DNA with DAPI (blue). Immunostaining reveals endogenous mRad1 in MES cells. (b) mRad1 protein was not migrated into the nucleus after HU treatment. MES and *Rad1*$^{-/-}$ MES cells were treated with 1 mmol/L HU for 1 h, 4 h and 8 h, then analysed by immunofluorescence. CTL (-): without F10, only labeled by FITC-conjugated anti-mouse antibody. CTL: without HU treatment.

In comparison with the two commercial anti-Rad1 mAbs, ab5363 (Abcam) and D6 (Santa Cruz), the F10 mAb showed significantly better results. Several groups used ab5363 in Western blot analysis [2, 27, 39]. In this study, using ab5363 we only detected the signals of over-expressed hRad1 protein, but did not detect the endogenous Rad1 protein (Figure 4a and Figure 4b). We could not even detect the over-expressed hRad1 protein using D6 (Figure 4a). This situation occurs often using commercial anti-Rad1 antibodies (our unpublished data). Furthermore, these two mAbs are not usable in immunoprecipitation and immunofluorescence, while the F10 mAb can be used in ELISA, Western blot analysis, immunoprecipitation and immunofluorescence assays (Figure 2, Figure 3, Figure 4 and Figure 6).

The 9-1-1 complex is proposed to play an important role in the maintenance of genomic integrity and this concept was mainly derived from the studies using anti-Rad9 antibodies [5−18]. The knowledge on the response of Rad1 to genotoxins is very limited. Rad1 mediated the resistance to HU in MES cells and schizosaccharomyces pombe cells[10, 29, 40−42]. Thus, Rad1 may participate in the response of HU treatment at molecular level. In this study, we observed significantly increased Rad1 protein expression in wild type MES cells at 12 h after HU treatment using F10 mAb, but did not change in *Rad9*$^{-/-}$ MES cells under the same conditions (Figure 5). Therefore, Rad9 may regulate the response of Rad1 to HU treatment and the mechanism deserves further investigation. Freire *et al* reported that no marked variation in hRad1 protein expression was observed at 1, 3 and 7 h after exposing U2OS cells to UV (50 J/m^2) or ionizing radiation (10 Gy)[12]. Therefore, whether Rad1 response to genotoxic stresses including HU depends on Rad9 needs further studies.

Using immunofluorescence with F10 mAb, we found that endogenous mRad1 was distributed mainly in the cytoplasm of MES cells (Figure 6a). Hirai *et al*. reported that endogenous hRad1 was localized mainly in the nucleus of MDA-MB-468 cells and hRad9 was required for the nuclear localization of the hRad1 protein [43]. Freire *et al*. reported that the endogenous hRad1 was located mainly in the nucleus of HeLa cells and a small portion of hRad1 was cytoplasmic. Therefore, the distribution of endogenous Rad1 protein was controversial. In this study, we used *Rad1*$^{-/-}$ MES cells as the negative control and confirmed that the signals detected using immunofluorescence assay carried out with F10 mAb were indeed from the Rad1 protein (Figure 6a). We noticed that the other two groups used self-made anti-Rad1 polyclonal antibodies [12, 27, 43] and they did not rigorously confirm the immunofluorescence signals as we did in this study. Of note, the cell models we used were different from theirs. Whether the differences in cell models contributed to the different results is inconclusive at the present and more studies will be needed to resolve or reconcile these differences.

It has been reported that genotoxin treatment increased the chromatin binding of Rad9 [44] and exposure of human skin fibroblast cells to IR or HU triggers translocation of hHus1 from the cytosol to the nucleus [22]. Rad9, Rad1 and Hus1 can form a heterotrimer, the 9-1-1complex [3, 4, 45−47]. The 9-1-1 complex may be actually formed in cells challenged by genotoxic stresses based on studies from several laboratories [26−27, 48]. However, the data from these research groups are not exclusive. There is a generally accepted conception that Rad9 is localized in the nucleus and in response to genotoxins Rad1 and Hus1 move to the nucleus and form the 9-1-1 complex with Rad9 which functions in cell cycle checkpoint control, DNA repair and other activities required for genome stability or apoptosis [49−51]. However, in this study, we did not observe the translocation of mRad1 from the cytoplasm to the nucleus after HU treatment(Figure 6b). Our data demonstrated that in response to HU treatment, the majority of Rad1 protein was still located in the cytoplasm and not migrated into the nucleus, thus Rad1 was not likely to join Rad9 and Hus1 to form of 9-1-1 complex in the nucleus. The exact molecular roles of individual molecules of Rad9, Rad1 and/or Hus1 proteins in cells may be more complicated than the model of 9-1-1 complex. F10 mAb will be a powerful tool to reveal the functions of these proteins and the 9-1-1 complex.

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高质量抗体揭示 Rad1 蛋白在细胞中新的潜在活动机理 *

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摘要　一组在进化上(从酵母到人)保守的基因 *Rad9*、*Rad1* 和 *Hus1* 在细胞周期监控点调控和 DNA 损伤修复中发挥重要作用．这三个蛋白可以形成环形异源三聚体，即 9-1-1 蛋白复合体．9-1-1 复合体被认为是 Rad9、Rad1 和 Hus1 行使功能的主要形式．到目前为止，没有一个好的抗 Rad1 的抗体，严重阻碍了对 Rad1 和 9-1-1 复合体的研究．在本研究中，我们成功地制备了一株小鼠抗 Rad1 蛋白的单克隆抗体．这个抗体能够有效地检测小鼠和人的内源 Rad1 蛋白，可以用于酶联免疫吸附、蛋白质免疫印迹、免疫共沉淀和免疫荧光等实验．利用该抗体，我们发现在 DNA 损伤剂羟基脲(HU)的诱导下，小鼠 Rad1 蛋白在 $Rad9^{+/+}$ 小鼠胚胎干细胞中表达明显增加，而在 $Rad9^{-/-}$ 的小鼠胚胎干细胞中没有观察到该现象，这表明 Rad9 对 Rad1 的蛋白表达有调控作用．此外，内源的 Rad1 蛋白主要分布在细胞质中，在 HU 处理后并没有迁移进入细胞核的现象，这与先前广泛被人们所接受的在 DNA 损伤压力下 Rad1 和 Hus1 能够迁移进入细胞核并与 Rad9 形成 9-1-1 蛋白复合体的说法相矛盾．综合看来，Rad1 和 9-1-1 蛋白复合体的分子作用机制比预期的要复杂，我们成功制备的 Rad1 单克隆抗体将成为研究 Rad1 以及 9-1-1 蛋白复合体的强有力的工具．

关键词　Rad9-Hus1-Rad1 (9-1-1) 蛋白复合体，Rad1，Rad1 单克隆抗体，羟基脲 (HU)
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