hRAD9 functions as a tumor suppressor by inducing p21-dependent senescence and suppressing epithelial–mesenchymal transition through inhibition of Slug transcription

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Senescence and epithelial–mesenchymal transition (EMT) are opposing roles in tumor progression, in that, one is a barrier against tumorigenesis, whereas the other is required for invasive malignancies. Here, we report that the DNA damage response (DDR) protein hRAD9 contributes to induction of senescence and inhibition of EMT. Our data show that hRAD9 is frequently downregulated in breast and lung cancers. Loss of hRAD9 expression is associated with tumor stage in breast and lung cancers, as well as with acquisition of an invasive phenotype. Ectopic hRAD9 expression in high invasive cancer cell lines, H1299 and MDA-MB 231, with low endogenous hRAD9 induced senescence by upregulation of nuclear p21, independent of the p53 status. Ectopic expression of hRAD9 also significantly attenuated cellular migration and invasion in vitro and tumor growth in a xenograft mouse model in vivo. In contrast, silencing hRAD9 in lower invasive cancer cell lines, A549 and MCF7, with high endogenous hRAD9 dramatically increased their migration and invasion abilities, and simultaneously activated EMT. Knockdown of hRAD9 increased, whereas ectopic expression of hRAD9 decreased, the expression of Slug. Moreover, hRAD9 directly bound to the promoter region of Slug gene and repressed its transcriptional activity. Taken together, these results suggest that hRAD9 is a potential tumor suppressor in breast and lung cancers and that it is likely to function by upregulating p21 and inhibiting Slug to regulate tumorigenesis.

Introduction

Senescence is an irreversible form of cell cycle arrest that can be triggered by various forms of stress, including DNA damage, which induces the DNA damage response (DDR) pathway, and oncogene activation. Persistent DDR signaling is essential for both the initiation and maintenance of senescence, whereas oncogene-induced senescence relies on the activation of tumor suppressors that mediate cell cycle arrest (1). Thus, senescence is a crucial tumor-suppressive mechanism and a natural barrier to malignant progression. On the other hand, epithelial–mesenchymal transition (EMT) is known to facilitate invasion and metastatic dissemination of tumors and, therefore, is a major mechanism of tumor progression. Recently, growing evidence suggests that EMT and senescence are crossed during tumor progression (2). For example, a number of key senescence-associated factors, such as p53, p21 and RB, have been found to affect EMT (3–5). Several distinct transcription factors, which contribute to activating EMT such as Zeb1, Twist and Snail, can concomitantly suppress senescence (6–9). Human RAD9 (hRAD9) is a structural homolog of Schizosaccharomyces pombe Rad9 and belongs to the DDR protein family. hRAD9 can modulate checkpoints to protect cells from incomplete replication or DNA damage, and it also regulates various biological processes, including DNA repair, cell cycle regulation, apoptosis, inhibition of androgen receptor, transactivation of p21 and genomic integrity (10–17). In addition, deletion of mouse RAD9 results in embryonic lethality in mice, indicating that mammalian RAD9 is essential for embryogenesis and development (18). Because hRAD9 plays a critical role in DNA checkpoint/repair, growth control and genomic stability, it seems clear that hRAD9 is important for preventing tumorigenesis. However, investigations of hRAD9 expressions in various cancers and of the role of hRAD9 in tumor progression have yielded paradoxical results (12,19–24). Upregulation of hRAD9 mRNA and protein level was reported in breast (19), prostate (21) and thyroid cancers (22), and accumulation of hRAD9 in nucleus of tumors was also reported in breast (25) and lung cancers (20). In addition, silencing hRAD9 in prostate cancer cell lines reduced their tumorigenicity in vivo (21), and a cancer-promoting function for hRAD9 was also demonstrated in prostate cancer cell lines (26). In contrast to the aforementioned studies indicating a function for tumor promotion, downregulation of hRAD9 mRNA was also reported in prostate (12) and testicular (23) cancers although the limited sample sets were presented in these studies. The tumor-suppressive role of hRAD9 was further supported by the observation that mice lacking RAD9 in keratinocytes are prone to the development of genotoxin-induced skin tumors (27). Clearly, the mechanism by which the multifunctional hRAD9 protein acts as an oncogene and a tumor suppressor, respectively, are unclear.

The aim of this study was to evaluate the role of hRAD9 in tumor progression. In this report, we showed that hRAD9 expression was downregulated in breast and lung cancers. The results of functional studies using both gain-of-function and loss-of-function experimental models identified hRAD9 as a novel putative tumor suppressor for breast and lung cancers.

Materials and methods

Patients and tissue samples
This study was approved by the Institutional Review Boards of National Cheng Kung University Hospital, Tainan, Taiwan. A total of 55 paired tissue samples (tumor and adjacent normal tissue) were collected from 30 patients with breast cancer diagnosed and 25 patients with lung cancer diagnosed. All included patients were diagnosed and/or treated at the National Cheng Kung University Hospital between 2008 and 2013. All studies involving human subjects was conducted in strict compliance with and approved by the Institutional Review Board of National Cheng Kung University Hospital. All patients signed informed consent for tissue collection and analysis. Freshly removed tumor and adjacent non-tumor tissue samples were immediately frozen in liquid nitrogen and stored at liquid nitrogen freezer until further analysis. The tumor stage was defined according to the American Joint Committee classification.

Cell culture
Human embryonic kidney (HEK293T), lung cancer (A549 and H1299) and breast cancer (MCF7 and MDA-MB 231) cell lines were maintained in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 10% fetal bovine serum and grown in an atmosphere of 5% CO2 at 37°C.

siRNA knockdown studies
siRNA transfection was performed using Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer’s instructions. The siRNA sequences
were listed below: scrambled, 5′-CUCUAUCAUGAAGAGUU-3′; siRad9, 5′-GCUUUCCUGUCCUGUUC-3′; siSlug, 5′-GCAUUGCA GACAGGGCA-3′ and sip21, 5′-GCCUUAUGUCUAGUUUGUGU-3′.

Proliferation, colony formation, apoptosis and senescence assays

Proliferation was measured by trypan blue staining. Cells were plated at a concentration of 1 x 10^5 cells in 10 cm dishes. Every 24 h, cells were trypsinized and counted. To analyze colony formation ability, 1 x 10^3 cells per well were cultured in six-well dishes for 2 weeks, the colonies were stained with 0.5% crystal violet and were counted. The percentages of apoptotic cells were determined using fluorescein isothiocyanate-annexin V apoptosis detection kit (BD Biosciences) according to the manufacturer’s instructions. Senescent cells were examined by senescence-associated β-galactosidase (SA-β-Gal) staining. Briefly, cells were fixed in 0.2% (wt/vol) glutaraldehyde and then stained with the staining solution [5 mM K,Fe(CN)6, 5 mM K,Fe(CN)6, 30 mM sodium phosphate buffer, 150 mM NaCl, 2 mM MgCl2, 1 mM/ml X-Gal at pH 6.0] at 37°C for 16–24 h. The percentages of senescent cells were calculated in three independent representative fields.

Mouse xenograft

The 6- to 8-week-old NOD/SCID mice were purchased from National Cheng Kung University Laboratory Animal Center and maintained under pathogen-free conditions. The 1 x 10^9 empty vector- or hRAD9-expressing H1299 or MDA-MB 231 cells were suspended in phosphate-buffered saline and subcutaneous injected into the right flank of NOD/SCID mice (n = 5). The injected NOD/SCID mice were killed 45 or 60 days after inoculation of H1299 or MDA-MB 231 cells, and tumors were isolated and fixed in 10% natural-buffered formalin (Merck) for further immunohistochemical staining. The tumor volume was calculated as L x W x H x 0.52, where L is the length, W is the width and H is the height.

Luciferase assay

The luciferase reporter assays were conducted as described (28) (see also Supplementary Materials, available at Carcinogenesis Online).

Chromatin immunoprecipitation assay

Chromatin immunoprecipitation (ChIP) was performed using a magna ChIP assay kit (Millipore), according to the manufacturer’s directions. The protein-chromatin complexes were immunoprecipitated with 3 μg anti-Myc antibody (Millipore) or anti-rabbit isotype-matched immunoglobulin (IgG; Abcam, ChIP grade). The binding region of the Slug promoter was amplified by PCR with the following primers: ChIP-slug-Pro-F: 5′-TCTCTTGCGCGACACTGTGT-3′, ChIP-slug-Pro-R: 5′-GAGGTAAACTCCTCGGTAGAG-3′.

Data analysis

For quantification of western blots of each specimen, hRAD9 protein levels were normalized by β-actin using NIH ImageJ software. The relative protein expression was calculated by comparing the normalized protein level in each tumor tissue to its corresponding normal tissue from the same patient. Results were presented as mean ± SD. The one- or two-tailed Student’s t-test was performed to compare differences between two groups. Differences between groups were analyzed by one-way analysis of variance. Fisher exact test was used to assess the relationship between hRAD9 expression and clinicopathological features. Statistical significance was set at P ≤ 0.05.

Results

hRAD9 expression is significantly downregulated in breast and lung cancer tissues and cell lines

To understand the role of hRAD9 in tumor progression, we first examined hRAD9 protein levels in breast and lung cancer specimens (Figure 1A). Our data showed that levels of hRAD9 protein were reduced (decreased to <50%, compared with adjacent normal tissues) in 24/30 (80%) of breast cancer and 19/25 (76%) of lung cancer (Figure 1B). Notably, hRAD9 expression was significantly downregulated in breast and lung cancers (Figure 1C; P < 0.0001 and P = 0.0007, respectively). In addition, a negative correlation was observed between hRAD9 expression and tumor stage in breast and lung cancers (Figure 1D and Supplementary Table 1, available at Carcinogenesis Online). The clinical significance of hRAD9 expression is also seen in survival analysis using the publicly available Kaplan–Meier plotter database (29), revealing that high hRAD9 expression was associated with longer relapse-free survival (hazard ratio = 0.55, 95% confidence interval = 0.49–0.63; P < 0.001) in 3364 breast cancer patients and longer overall survival (hazard ratio = 0.46, 95% confidence interval = 0.23–0.90; P = 0.0198) in 226 of lung cancer patients (Supplementary Table 2, available at Carcinogenesis Online).

We next examined hRAD9 protein levels in breast and lung cancer cell lines. In concordance with the clinical findings, relatively high levels of hRAD9 expression were detected in lower invasive breast and lung cancer cell lines, MCF7 and A549, whereas low levels of hRAD9 expression were detected in highly invasive breast and lung cancer cell lines, MDA-MB 231 and H1299 (Figure 1E). These data indicate that downregulation of hRAD9 occurs in most breast and lung cancer specimens, especially in patients with invasive breast and lung cancers and in the highly invasive cancer cell lines, suggesting that hRAD9 may play a negative role in tumor progression.

Ectopic hRAD9 expression induces senescence in highly invasive cancer cells

To evaluate the functional role of hRAD9 in tumor progression, we ectopically expressed hRAD9 in MDA-MB 231 and H1299 cells (Figure 2A). In agreement with previous reports (20,25), immunofluorescent staining indicated that exogenous hRAD9 was mainly localized in the nucleus in hRAD9-expressing cells (Supplementary Figure 1A, available at Carcinogenesis Online). Because hRAD9-expressing cells exhibited a decrease in proliferation (Figure 2B), these cells were evaluated by annexin V/propidium iodide and Ki67 to gain further insight into the effect of hRAD9 on proliferation. Surprisingly, staining for apoptosis revealed no significant difference between hRAD9-expressing cells and controls (Figure 2C); however, the number of Ki67-positive cells significantly decreased in hRAD9-expressing cells compared with controls (Figure 2D). In addition, most hRAD9-expressing cells had a senescent morphology, including the enlarged and flattened shape (Figure 2E), and were stained positive for SA-β-Gal (Figure 2F). Moreover, disoriented accumulation of actin filaments was also observed in hRAD9-expressing cells (Supplementary Figure 1B, available at Carcinogenesis Online). These results indicate that restoring hRAD9 expression is sufficient to induce senescence in highly invasive cancer cells.

Ectopic hRAD9 expression induces senescence by nuclear upregulation of p21, independent of the p53 status

Because increased expression of cyclin-dependent kinase (Cdk) inhibitor p21 can induce senescence in certain cells, and previous studies indicate that hRAD9 can activate p21 transcription (13,30,31), we next investigated whether hRAD9-induced senescence was mediated by enhanced p21 expression. We found that p21 expression was upregulated in hRAD9-expressing H1299 and MDA-MB 231 cells, predominantly in the nucleus (Figure 3A and B). In addition, we also examined the expression of two other Cdk inhibitors, p16 and p27 in hRAD9-expressing H1299 and MDA-MB 231 cells. The results showed no significant difference in the levels of p16 and p27 protein expression between hRAD9-expressing cells and the respective control cells (Supplementary Figure 2, available at Carcinogenesis Online). Furthermore, silencing hRAD9 expression profoundly decreased most of the hRAD9-induced p21 expression (Figure 3C), and silencing hRAD9 or p21 expression decreased senescence (Figure 3D) in hRAD9-expressing cells. Collectively, these results suggest that hRAD9-induced senescence is mediated, at least in part, by p21 upregulation in the nucleus.

Although p21 is largely responsible for p53-dependent G1 arrest in response to genotoxic stress, it should be noted that the H1299 and MBA-MB 231 cells used in the above ectopic hRAD9 expression experiments were p53-null or p53-defective. We therefore investigated whether hRAD9 could also induce senescence in cancer cells with wild-type p53. We found that expression of hRAD9 in A549 cells induced morphological characteristics of senescence, increased SA-β-Gal activity and enhanced p21 expression (Figure 3E). Silencing hRAD9 or p21 in A549 hRAD9-expressing cells also decreased the SA-β-Gal activity (Figure 3F). Taken together, these results indicate that hRAD9 could promote senescence by upregulating p21 in a p53-independent manner.
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Because hRAD9 restoration significantly diminished colony formation, migration and invasion in H1299 and MDA-MB 231 cells (Figure 4A–C), the effect of hRAD9 expression on tumorigenesis was examined in vivo. The NOD/SCID mice were subcutaneously transplanted with empty vector- or hRAD9-expressing H1299 or MDA-MB 231 cells, and tumorigenesis was monitored. The average tumor volume in mice bearing hRAD9-expressing H1299 or MDA-MB 231 cells was decreased to 15 and 47%, respectively, compared with controls (Figure 4D). Notably, immunohistochemical staining revealed that hRAD9-expressing tumors had significantly increased nuclear p21 staining and decreased Ki67 staining compared with empty vector-expressing tumors (Figure 4E), strongly indicating the in vivo tumor suppression driven by hRAD9. Collectively, these results indicate that restoring hRAD9 expression suppresses tumorigenicity of highly invasive cancer cells in vitro and in vivo.

Silencing hRAD9 in cancer cells with lower invasive capacity activates EMT and increases migratory and invasive potential

To evaluate whether downregulation of hRAD9 could promote tumor progression in lower invasive cancer cells, hRAD9 expression was

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**Fig. 1.** hRAD9 expression is significantly downregulated in breast and lung cancer tissues and cell lines. (A) Representative western blots showing hRAD9 protein levels in paired normal (N) and tumor (T) specimens of breast and lung cancers. β-Actin was used as a loading control. (B) Accumulated percentage of low or high relative hRAD9 protein levels (tumor/normal) in paired normal and tumor specimens. (C) Box and whisker plot showing the normalized hRAD9 protein levels in normal or tumor tissues of breast and lung cancers. The horizontal line represents the median, the box is the interquartile range (25–75%) and the whiskers extend to 1.5 times the interquartile range. (D) Relative hRAD9 protein levels of breast and lung cancer patients grouped by pathological stages. The horizontal lines represent the median values. (E) Endogenous hRAD9 protein levels in MCF7, MDA-MB 231, A549 and H1299 cells were measured by western blot analysis (left). hRAD9 protein levels were quantified using ImageJ software and normalized to β-actin levels (right). β-Actin was used as a loading control. *P < 0.05, **P < 0.001.
silenced in MCF7 and A549 cells, and their cellular behaviors were examined. Knockdown of hRAD9 expression in breast, lung and prostate cancer cells inhibited cell proliferation (19,26,32). Consistent with these previous studies, hRAD9-knockdown A549 and MCF7 cells displayed decreased cell proliferation (Figure 5A). In addition, the decreased cell proliferation was also found in both the hRAD9-knockdown H1299 and MDA-MB 231 cells (Supplementary Figure 3, available at Carcinogenesis Online). Furthermore, the hRAD9-knockdown cells also exhibited decreased anchorage-dependent colony formation ability (Figure 5B). However, the hRAD9-knockdown cells exhibited dramatic changes in their morphologies resembling those that occur during the EMT (Figure 5C). A corresponding decrease in epithelial markers, E-cadherin and plakoglobin, and an increase in mesenchymal markers, N-cadherin and vimentin, in these hRAD9-knockdown cells were observed (Figure 5D). Immunofluorescent staining also indicated that E-cadherin was profoundly decreased in both the A549- and MCF7-hRAD9-knockdown cells, whereas vimentin was significantly increased in A549 hRAD9-knockdown cells and was slightly
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increased in MCF7 hRAD9-knockdown cells (Figure 5E). Moreover, hRAD9-knockdown cells became less adherent than controls (Figure 5F) and had dramatically increased migration (Figure 5G) and invasion (Figure 5H) abilities. These data indicate that hRAD9 depletion in lower invasive cancer cells inhibits cell growth but activates EMT and dramatically increases their migration and invasion abilities.

**hRAD9 inhibits EMT through suppression of Slug**

The EMT competence of hRAD9-knockdown cells prompted us to examine the expression of EMT inducers, and among these, only Slug was significantly upregulated (Figure 6A). To confirm that Slug is the major factor contributing to EMT induction in hRAD9-knockdown cells, siRNA-mediated knockdown of Slug was performed. The results indicated that A549 and MCF7 hRAD9-knockdown cells with silenced Slug did not show significant changes in EMT markers, suggesting that Slug is the key regulator of EMT induction by hRAD9 depletion.

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Fig. 3. Ectopic hRAD9 expression induces senescence by nuclear upregulation of p21, independent of the p53 status. (A) Western blot analysis of hRAD9 and p21 in empty vector- or hRAD9-expressing H1299 and MDA-MB 231 cells. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a loading control. (B) Empty vector- or hRAD9-expressing H1299 and MDA-MB 231 cells were doubly immunostained for hRAD9 and p21 (×40 magnification). hRAD9 was shown in green, and p21 was shown in red. Nuclei were counterstained with 4′,6-diamidino-2-phenylindole (blue). Arrowhead indicates the same cell in each image. (C and D) Western blot analysis (C) and SA-β-Gal assay (D) were performed in hRAD9-expressing H1299 and MDA-MB 231 cells transfected with scrambled, siRad9 or sip21 siRNA. β-Actin was used as a loading control of western blots. SA-β-Gal staining (×40 magnification) was shown in bright-field images (left). Scale bars = 200 µm. The percentage of SA-β-Gal-positive cells was presented in the right histogram. (E) Stable A549 cells generated by lentivirus infection that overexpressed green fluorescent protein (GFP) or hRAD9 were used for western blot analysis of hRAD9, GFP and p21 (right). A549 cells that stably overexpressed GFP were used as control cells. β-Actin was used as a loading control. Cell morphology and SA-β-Gal activity were analyzed by phase-contrast (×10 magnification) and bright-field microscopy (×40 magnification) (left). Scale bars = 200 µm. (F) hRAD9-expressing A549 cells transfected with scrambled, siRad9 and sip21 siRNA were subjected to SA-β-Gal assay to determine the percentage of the senescent population. ***P < 0.001.
exhibited a compact appearance and had restored E-cadherin expression (Supplementary Figure 4, available at Carcinogenesis Online).

Because silencing hRAD9 in lower invasive cancer cells enhanced Slug expression, we investigated whether ectopic hRAD9 expression could reduce Slug expression. Indeed, Slug expression was obviously downregulated in hRAD9-expressing H1299 and MDA-MB 231 cells (Figure 6B). Similarly, a marked downregulation of Slug with a concomitant upregulation of E-cadherin was detected in both the A549 and MCF7 cells with transient expression of hRAD9 (Figure 6C), further suggesting that hRAD9 inhibits EMT through suppression of Slug in breast and lung cancer cell lines.

hRAD9 directly binds the Slug promoter and represses its transcriptional activity

Based on above data, we speculated that hRAD9 might be involved in the transcriptional regulation of Slug. To investigate this, the reporter constructs, Slug Pro-D1 (from −400 to +175) and Slug Pro-D2 (from −200 to +175) containing different lengths of the 5′-flanking sequence of slug were generated from wild-type Slug promoter construct (from −807 to +175) (33) and then, promoter assay was performed by cotransfection of the reporter construct with the hRAD9 expression plasmid or the empty vector control. As shown in Figure 6D, reporter activity was significantly decreased in HEK293T cells containing Slug Pro-D2 (reporter construct) in the presence of hRAD9 expression, compared with that of cells cotransfected with the vector control, suggesting that hRAD9 is able to repress Slug transcription via the −400/−200 sequence of Slug promoter. The direct binding of hRAD9 to the −345/−199 fragment of Slug promoter was further demonstrated by ChIP assays (Figure 6E). Furthermore, ChIP validated that the interaction of hRAD9 with −345/−199 Slug promoter fragment could be enhanced when the anti-Myc antibody was added. Although the target sequence of hRAD9 has not been well defined, previous study
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indicated that hRAD9 could recognize and regulate p21 expression via binding to the p53 consensus sequence (GGGCATG) in the promoter region of p21 (13). In the sequence of −345/−199 Slug promoter, we identified the sequence, GGGCAGG (from −286 to −280), which is similar to the aforementioned hRAD9-binding sequence. To verify this putative sequence is the target of repression by hRAD9, the point mutants (5'GAAAAGG3', instead of GGGCAGG) were generated and used for luciferase analysis. The results of promoter assay revealed that in contrast to the promoter activity that was profoundly decreased in cells transfected with wild-type Slug promoter, the promoter activity was unaffected in cells transfected with Slug promoter mutant (Figure 6F). These results indicate that the −400/−200 fragment covering the putative hRAD9-binding site is involved in the downregulation of slug transcription by hRAD9. Taken together, the above results show that hRAD9 directly binds its target site in slug promoter region and downregulates the expression of Slug, further suggesting that hRAD9-mediated downregulation of Slug could be one of the mechanisms by which hRAD9 functions as a tumor suppressor.

Fig. 5. Silencing hRAD9 in cancer cells with lower invasive capacity activates EMT and increases migratory and invasive potential. (A and B) Stable knockdown of hRAD9 in A549 and MCF7 cells was achieved by lentiviral-mediated delivery of the shRNA for hRAD9. Cells infected with luciferase shRNA lentivirus (shLuc) were used as control cells. shLuc- and shRad9-expressing A549 and MCF7 cells were counted after a 72 h incubation (A) and were used for anchorage-dependent colony formation assay (B). (C) shRad9-expressing A549 and MCF7 cells exhibited fibroblast-like and scattered morphology. Phase-contrast images indicating the morphological changes of A549 and MCF7 cells (×10 magnification). (D) Western blot analysis of epithelial (E-cadherin and Plakoglobin) and mesenchymal (N-cadherin and vimentin) markers in shLuc- or shRad9-expressing A549 and MCF7 cells. β-Actin was used as a loading control. (E) shLuc- or shRad9-expressing cells were immunostained for E-cadherin and vimentin (×20 magnification). E-cadherin and vimentin were shown in green. Nuclei were counterstained with 4′,6-diamidino-2-phenylindole (blue). (F) shLuc- or shRad9-expressing A549 and MCF7 cells were subjected to cell adhesion assay. Results were presented as a percentage of adhesion relative to control cells. (G and H) Transwell migration (G) and invasion (H) assays of shLuc- or shRad9-expressing A549 and MCF7 cells were performed. Graph represented the mean number of migrated or invaded cells obtained for each cells. *P < 0.05, **P < 0.01, ***P < 0.001.
Discussion

In this report, we present data showing that hRAD9 expression is frequently downregulated in breast and lung cancers. Loss of hRAD9 expression was associated with the tumor stages, as well as with acquisition of an invasive phenotype in breast and lung cancer cells. The results of functional studies suggest that hRAD9 is a potential tumor suppressor in breast and lung cancers and that it is likely to function by potently upregulating p21 and inhibiting Slug to regulate tumorigenesis. Furthermore, survival analysis using Kaplan–Meier plotter database revealed that high hRAD9 expression was associated with significantly decreased risk for relapse-free survival of breast cancer patients and for overall survival of lung cancer patients, further supporting the role of hRAD9 in tumor suppression.

Accumulating evidence suggests that senescence-inducing signals are established and maintained by either the p53 and/or p16/pRb pathways. The p53-mediated senescence is mainly caused by induction of its target gene p21, and the main role of p21 is to inhibit the Cdkks and thereby, dephosphorylation of pRb, allowing growth arrest and senescence (34). Another key senescence mediator, p16, can dephosphorylate pRb independently of p53 by inhibiting Cdk4 and Cdk6. Thus, p21 and p16 are likely to cooperate to keep pRb in a hypo-phosphorylated
form during senescence. Intriguingly, senescence induction by hRAD9 restoration in the cells that we examined here was largely dependent on p21, but independent of p53, p16 and p27. Indeed, among the tested cells, H1299 (p53-null and methylated p16), MBA-MB 231 (mutant p53 and deleted p16) and A549 (wild-type p53 and deleted p16) cells retain the capacity to undergo senescence via the hRAD9/ p21 signaling pathway. In addition, we also found that the expressions of p16 and p27 were unaffected in hRAD9-expressing cells when compared with the control cells (Supplementary Figure 2, available at Carcinogenesis Online).

The present study shows for the first time that ectopic hRAD9 expression in highly invasive cancer cells did not increase apoptosis but led to senescence, and this effect was mediated by p21 upregulation. Importantly, we found that p21 is predominately located in the nucleus of hRAD9-expressing cells, which is consistent with the hypothesis that the presence of p21 in nucleus is necessary for cell cycle arrest and senescence (35). Because previous investigations have shown that overexpression of hRAD9 resulted in induced apoptosis in various cell lines (11,16), we initially predicted that hRAD9 overexpression would cause the apoptosis in H1299 and MBA-MB-231 cells. But unexpectedly, our results showed that overexpression of hRAD9 did not enhance the apoptosis in H1299 and MBA-MB-231 cells. As we presented in this work, staining for apoptosis revealed no significant difference between hRAD9-expressing cells and control (Figure 2C). To further confirm these observations, we fixed hRAD9-expressing cells (and controls) in 3.7% formaldehyde, stained with 4′,6-diamidino-2-phenylindole and counted the cells with apoptotic nuclear morphology and those that had normal nuclear morphology by the method of Komatsu et al. (11). The results revealed that a small proportion of hRAD9-expressing cells (3.85% of hRAD9-expressing H1299 cells (1.46% of mock cells) and 2.74% of hRAD9-expressing MBA-MB-231 cells (2.46% of mock cells)) showed apoptotic nuclear morphology (data not shown). In addition, the western blots revealed that the cleavage form of PARP and active from of caspase-3 were not detected in hRAD9-expressing H1299 and MBA-MB-231 cells (Supplementary Figure 5A, available at Carcinogenesis Online). In contrast to previous studies showing that overexpression of hRAD9 in 293, MCF7, U937, MBA-MB 468 and HeLa cells induced apoptosis via an association with antiapoptotic Bcl-2 or Bcl-XL in cytosol (11,16), we found that hRAD9 was mainly located in the nucleus of hRAD9-expressing cells, which is associated with p21 upregulation in nucleus, resulting in induction of senescence. Whether different subcellular localizations of hRAD9 and/or p21 and/or different signaling contexts of cell types relate to these different effects mediated by upregulated hRAD9 remain to be elucidated.

The EMT process is a major mechanism of metastasis because it not only induces increased cell motility and invasiveness after EMT but also enables cancer cells to avoid apoptosis and senescence (2,36). Slug overexpression has been described in many types of cancers and is important for tumor progression toward invasion and metastasis through repression of E-cadherin (37–39). On the other hand, the senescence-driving protein p21 acts unquestionably as an EMT inhibitor, and the absence of p21 enables the proliferation of DNA damaged cells and promotes tumor progression (2). In this study, we found that ectopic hRAD9 expression in highly invasive cancer cells, which were p53-null or -deficient, led to upregulation of p21, with concomitant downregulation of Slug, and thereby, suppressed the tumorigenicity in vitro and in vivo. These results suggest that the hRAD9 downregulation might simultaneously favor malignant conversion through the upregulation of Slug and escape from senescence due to low p21 levels in p53-null or -deficient cells. Inversely, our data also show that silencing of hRAD9 in poorly invasive cancer cells results in activation of EMT via upregulation of Slug, and migration and invasion abilities were stimulated concomitantly, despite these hRAD9-knockdown cells exhibited decreased cell proliferation and anchorage-dependent colony formation ability. Although hRAD9 is a direct activator of p21, we did not observe significant alterations of p21 expression in hRAD9-knockdown cells (data not shown), probably due to the maintenance of basal p21 levels by other transcription factors such as wild-type p53 (in MCF7 and A549). Although the mechanism for the hRAD9 silencing-mediated reduction of cell proliferation remains to be elucidated, it seems clear that reduction of cell growth and anchorage-dependent colony formation ability would be advantageous for tumor dissemination and invasion. Thus, our data demonstrate that hRAD9 could repress Slug expression by directly binding to the putative target site of the Slug promoter region and the results that silencing hRAD9 led to a reduced expression of E-cadherin and plakoglobin, and a concomitant increase of N-cadherin and vimentin, indicating that the hRAD9 inhibits EMT through suppression of Slug may be one of key underlying mechanisms behind its antitumor functions. In addition, considering that hRAD9 is a DDR protein that can modulate G1 and G2 checkpoints to protect cells from incomplete replication or DNA damages, a decrease in DNA repair and an accumulation of unrepaired DNA damage, promoted by silencing of hRAD9, subsequently leading to an invasive and aggressive cancer phenotype cannot be excluded.

Previous studies have reported the hRAD9 overexpression in a variety of tumor specimens (19–21), leading some to propose that hRAD9 may function as an oncogenic protein in these tumors. Immunohistochemical analysis of 48 non-small-cell lung carcinoma revealed that 33% of specimens displayed elevated levels of hRAD9 expression and accumulation of the protein in the nucleus (20). However, in a subsequent study by quantitative reverse transcription–PCR method, the same author (Maniya et al.) showed no statistical difference in the level of hRAD9 mRNA between the tumor and normal tissues in 30 specimens (40). In one study of breast cancer, although Cheng et al. (19) found that hRAD9 mRNA level is elevated in 52.1% (25/48) of breast cancer tissue, hRAD9 protein expression was not evaluated in this study. However, in a subsequent study by immunohistochemical analysis of 37 breast cancer tissues, the same group reported that hRAD9 protein was detected in similar amounts in the cytoplasm of both normal and cancer tissues, whereas an increased nuclear accumulation of hRAD9 in cancer tissues was observed. One possible explanation for the discrepant protein expressions of hRAD9 between previous studies and our present study may due to the specificity of hRAD9 antibody. In fact, we found that the hRAD9 antibody used in the present study [also used in the previous studies of prostate cancer (21,26)] is more effective than those used in the aforementioned breast and lung cancer studies (Supplementary Figure 5B, available at Carcinogenesis Online). Importantly, although functional assays of previous studies showed that knockdown of hRAD9 expression in MCF7 and A549 cells resulted in reduced cell proliferation (19,32), which is consistent with results presented here, experimental data to support hRAD9 as an oncogenic protein—that is, overexpression of hRAD9 in breast or lung cancer cell lines could promote the tumor progression—have not been provided. Nevertheless, a recent study demonstrated that hRAD9 knockdown led to decreased cell proliferation, increased apoptosis, impaired in vitro migration and invasion of prostate cancer DU145 and PC3 cells. Moreover, restoration of mRAD9 (the mouse homolog) in hRAD9-knockdown DU145 cells restored their cell motility, invasion, anchorage-independent cell growth and anoikis resistance (26). These results indicated that hRAD9 seems to act as a promoter of tumor progression in prostate cancer, contradictory to the results of our study in breast and lung cancers. At present, it is difficult to reconcile these discordant conclusions in different cancers. Whether hRAD9 functions differently depending on the cell and tissue types warrants further studies.

In summary, our results indicate that hRAD9 is an essential tumor suppressor in breast and lung cancers. hRAD9 not only represses tumor growth by activation of p21 expression but also inhibits EMT by Slug repression. During tumor progression, hRAD9 downregulation might repress tumor growth, nevertheless, upregulate Slug expression to activate EMT and promote cell migration and invasiveness in breast and lung cancer cell lines. A model for this proposed pathway is outlined (Supplementary Figure 6, available at Carcinogenesis Online). Senescence is a barrier against tumor progression in vivo, and the EMT inducer Slug is overexpressed in various cancers and is important for tumor progression toward invasion and metastasis. In
addition, senescence induction by hRAD9 is p53 and p16 independent, and most human cancers have defective p53 and frequent loss of the INK4a/p16 locus. Our data imply the strategies such as the identification of compounds that robust induction of hRAD9 may be an effective therapeutic approach in selected tumors.

Supplementary material

Supplementary Tables 1 and 2 and Figures 1–6 can be found at http://carcin.oxfordjournals.org/

Funding

National Science Council, Taiwan (NSC99-2314-B006-021-MY3).

Acknowledgements

The authors thank Dr Chao-Chung Chen, Cheng-Kai Wang, Min-Jai Huang and Shu-Yi Wu from National Cheng Kung University for technical assistance and the National RNAi Core Facility located at the Institute of Molecular Biology/Genomic Research Center, Academia Sinica for the help of lentivirus production. We are grateful to Sheng-Hsiang Lin and Shang-Chi Lee for providing the statistical consulting services from the Biostatistics Consulting Center, National Cheng Kung University Hospital.

Conflict of Interest Statement: None declared.

References


Received October 7, 2013; revised December 23, 2013; accepted January 4, 2014