MiR-663 inhibits radiation-induced bystander effects by targeting *TGFB1* in a feedback mode

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The mechanisms of radiation-induced bystander effects (RIBE) have been investigated intensively over the past two decades. Although quite a few reports demonstrated that cytokines such as TGF-β1 are induced within the directly irradiated cells and play critical roles in mediating the bystander effects, little is known about the signaling pathways that occur in bystander cells. The crucial question as to why RIBE signals cannot be infinitely transmitted, therefore, remains unclear. In the present study, we showed that miR-663, a radiosensitive microRNA, participates in the regulation of biological effects in both directly irradiated and bystander cells via its targeting of TGF-β1. MiR-663 was downregulated, while *TGFB1* was upregulated in directly irradiated cells. The regulation profile of miR-663 and *TGFB1*, on the other hand, was reversed in bystander cells, in which an elevated miR-663 expression was exhibited and led to downregulation of TGF-β1. Further studies revealed that miR-663 interacts with *TGFB1* directly and that through its binding to the core regulation sequence, miR-663 suppresses the expression of *TGFB1*. Based on the results, we propose that miR-663 inhibits the propagation of RIBE in a feedback mode, in which the induction of TGF-β1 by reduced miR-663 in directly irradiated cells leads to increased level of miR-663 in bystander cells. The upregulation of miR-663 in turn suppresses the expression of TGF-β1 and limits further transmission of the bystander signals.

Introduction

Radiation-induced bystander effects (RIBE), which are biological responses in cells that are not themselves in the path of ionizing radiation but receive signals transmitted from directly hit cells, will amplify or exaggerate the action of low dose radiation and thus can significantly increase radiation risk and tissue damage.[1] In view of RIBE, existing radiotherapy models should be reconsidered and the carcinogenesis risk of low-dose radiation should be re-assessed. Therefore, a better understanding of the mechanisms underlying RIBE is essential.

It has been demonstrated that radiation-induced bystander effects can only be propagated within limited distance. Cells located as far as 1 mm in 3D cultured tissue[2] and 7.5 mm in 2D cultured cells[3] from the directly irradiated cells were shown to display cytotoxic and genotoxic effects that were believed to be the consequences of RIBE. It was proposed that both gap junction intercellular communication (GJIC)[4] and soluble factors such as reactive oxygen species (ROS),[5] nitric oxide (NO),[6] calcium fluxes,[7] and cytokines such as interleukin-8 (IL-8),[8] tumor necrosis factor-α (TNF-α),[9] and transforming growth factor-β1 (TGF-β1)[10-12] generated by directly irradiated cells participate

in the bystander effects. However, most of the studies were focused on signals exiting irradiated cells and likely detected by bystander cells. Questions as to whether bystander cells also send out signals and why RIBE can only be transmitted within a certain distance remain largely unanswered.

In recent years, accumulating evidences have shown that microRNAs (miRNAs) can act as modulators of biological processes including cellular responses to radiation.[13] It was also demonstrated that miRNAs may participate in RIBE. For instance, miR-194 was demonstrated to be upregulated in bystander spleen.[14] Irradiation of rat cranium triggered gender-specific deregulation of the microRNAome in the non-irradiated bystander spleen.[15] Moreover, Kovalchuk et al. showed that the microRNAome profile is altered in bystander 3D human tissue models.[16] Several major bystander endpoints were suggested to be mediated by changed expression of miRNAs. Since miRNAs are secreted to the outside of the cells and circulated in blood and body fluid,[17,18] they may serve as long-distance signal transmitters of RIBE. However, the exact function of miRNAs in RIBE is yet to be determined.

TGF-β1 is a well-established sensor and transducer of radiation stress.[19] Since the observation that TGF-β1 was increased in

a dose-dependent manner in irradiated rat liver,[20] multiple studies have demonstrated the involvement of TGF-β1 in direct radiation responses[21-28] and as an important mediator in RIBE.[10-12] It was suggested that TGF-β1 is induced by radiation at both transcriptional[23] and post-transcriptional levels.[29] However, the role of miRNAs in TGF-β1-mediated responses toward radiation is still largely unknown.

In a previous study, we observed altered miRNA expression profile in human cervical carcinoma cells (HeLa) irradiated with X-rays, suggesting a regulatory role of miRNAs upon radiation treatment.[30] Here, we focused our studies on miR-663 which was previously shown to be downregulated in renal carcinoma tissues exposed to X-ray irradiation[31] and showed that miR-663 is significantly downregulated upon irradiation but increased in bystander cells. Further, the miRNA targets *TGFB1* in a feedback mode.

Results

MiR-663 was downregulated in directly irradiated cells

A previous microRNA chip analysis showed that miR-663 was significantly downregulated in renal carcinoma tissues exposed to X-ray irradiation, regardless of the dosage used.[31] These changes were validated in HeLa cells using qRT-PCR. Consistent with chip results, a significant decrease of miR-663 expression was observed following irradiation of cells with 4 Gy of X-rays. Interestingly, miR-663 level in cells irradiated with 8 Gy X-rays was similar with that of the control, non-irradiated cells (**Fig. 1A**). In addition, alteration of miR-663 expression was time-dependent. Its level was decreased by 40% of the sham control two hours after irradiation, remained relatively unchanged till 24 h post-irradiation and started to increase afterwards. Expression of miR-663 was returned back to control level by 48 h after irradiation (**Fig. 1B**).

To investigate the biological significance of miR-663 downregulation, we measured cell survival and apoptosis of cultured cells transfected with miR-663 inhibitors. As shown in **Figure 1C**, cell survival rate was significantly decreased in miR-663 inhibitor transfected cells. When cells were treated with 4 Gy X-rays, the survival rate was decreased to $5.88 \pm 1.05\%$ ($P < 0.01$) and the level of apoptosis increased from $3.68 \pm 0.83\%$ to $8.66 \pm 1.27\%$ ($P < 0.05$). However, when cells were simultaneously transfected with miR-663, the survival rate was increased (to $11.38 \pm 1.76\%$) while the level of apoptosis reduced (from $8.66 \pm 1.27\%$ to $4.79 \pm 1.03\%$) (**Figs. 1C and D**). To further verify these effects on molecular levels, Bcl-2, Ki67 and caspase-3 were detected. It is shown that miR-663 restored Bcl-2 and Ki67 expression while inhibited
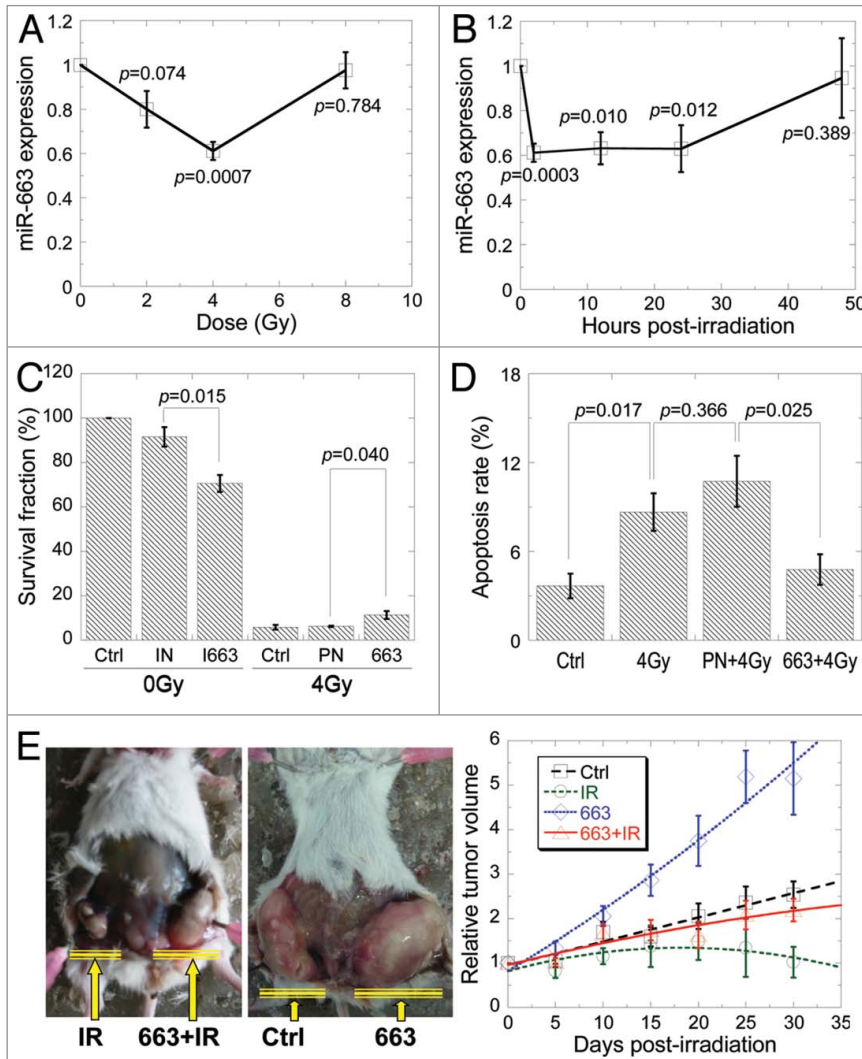

Figure 1. MiR-663 was downregulated in directly irradiated cells. (**A**) qRT-PCR was performed to detect miR-663 expression in HeLa cells 2 h after exposure to different doses of X-rays. (**B**) MiR-663 levels in HeLa cells exposed to 4 Gy X-ray irradiation at different time-points were detected using qRT-PCR. (**C**) Colony forming assay was performed on HeLa cells subjected to different treatments, after which cells were allowed to grow for 13 d. 663, miR-663 mimics; I663, miR-663 inhibitors; PN, negative control for miR-663 mimics; IN, negative control for miR-663 inhibitors. (**D**) Apoptosis rates of HeLa cells subjected to 4 Gy X-ray irradiation plus transfection with either miR-663 mimics or negative control (PN) were assayed with Hoechst33342/PI double staining. (**E**) Tumors generated by a stable HeLa cell line with inducible miR-663 expression in NOD/SCID mice 1 mo after 5 Gy X-ray irradiation and/or the induction of miR-663 expression. Tumor volumes were measured every 5 d and normalized to those obtained immediately before irradiation. The tumor volumes were measured using the formula $ab^2 \times \pi/6$, whereby a represents the length and b is the width. n = 10 flanks. Data were obtained from at least three independent experiments, and presented as means ± SE P values between the indicated samples and sham control (Ctrl) are additionally presented.

caspase-3 activity in directly hit cells (**Figs. S1–3**). Moreover, to confirm these findings *in vivo*, we established an animal model with inducible HeLa-TetR-663 cell lines based on the tetracycline operator system (**Fig. S4**). Our results showed that tumors overexpressing miR-663 grow significantly faster than control tumors (**Fig. 1E**). X-ray irradiation led to significant suppression of tumor growth up to 30 d in our study. However, overexpression of miR-663 abolished the anti-tumor effects of radiation (**Fig. 1E**). These results implied that miR-663 is likely an oncogene and that ionizing radiation may suppress its expression, which suggests a possible involvement of this microRNA in cellular radiosensitivity.

MiR-663 upregulation was observed in bystander cells

Since miR-663 was downregulated in directly irradiated HeLa cells, we next wanted to see if this is also the case in bystander cells. To our surprise, miR-663 level was increased instead in cells treated with the medium harvested from the cultures for directly irradiated cells (conditioned medium, CM) (**Fig. 2A**). 53BP1 foci and MN formation analysis both showed that upon conditioned medium treatment, miR-663 inhibitor transfection led to a greater DNA damage effect than those cells that were transfected with miR-663 mimics (**Figs. 2B and 2C**), indicating a reversed relationship between miR-663 level in bystander cells and the radiation-induced bystander effect. Such a case was also observed in co-culture experiments, in which bystander cells were situated in the same medium together with the directly irradiated cells (**Fig. 2D**). Besides, we also detected the effects of miR-663 on DNA damage in directly hit cells and obtained the similar results (**Fig. S5**).

TGF-β1 is a mediator of radiation-induced bystander effects

As mentioned previously, TGF-β1 acts as a signal molecule in RIBE. Experiments were performed with HeLa cells to confirm the role of TGF-β1 in RIBE. *TGFB1* transcript level was increased more than 2-folds in HeLa cells exposed to 4 Gy X-rays. However, similar as that of miR-663, *TGFB1* level was unchanged upon 8 Gy irradiation (**Fig. 3A**). The upregulation of *TGFB1* was time-dependent, peaked at 2 h and reduced back to sham control level 24 h post-irradiation (**Fig. 3B**). TGF-β1 protein level was also induced evidently after exposure to the indicated X-ray doses (**Fig. 3C**). For cells treated with 4 Gy X-rays, TGF-β1 increased marginally till 12 h after irradiation and declined back to non-irradiated control level by 48 h. TGF-β1 concentration in the CM was increased from 19.4 ±

4.8 pg/mL to 61.2 ± 5.6 pg/mL two hours after 4 Gy irradiation ($P < 0.01$) and remained unchanged till 48 h post-irradiation. It was suppressed by miR-663 expression while increased by miR-663 inhibition (**Fig. 3D**). We further measured CM-induced DNA damage in bystander cells with 53BP1 foci and micronucleus formation assays. The number of 53BP1 foci increased significantly in bystander cells ($P < 0.001$) but was greatly suppressed by TGF-β1 neutralization (**Fig. 3E**). Additionally, MNF increased dramatically in bystander cells after CM transfer ($P < 0.001$). Again, addition of TGF-β1 neutralization antibody could reduce such an increment ($P < 0.01$) (**Fig. 3F**).

MiR-663 directly targets *TGFB1* and suppresses its expression

The findings that miR-663 level was altered both in directly irradiated and bystander cells suggested that this miRNA may play an important role in cellular response to X-ray irradiation. According to online software such as TargetScan, miRanda, *etc.*, *TGFB1* was universally predicted to be a possible target of miR-663. In addition, our results presented above seemed to indicate that expression levels of *TGFB1* and miR-663 were negatively
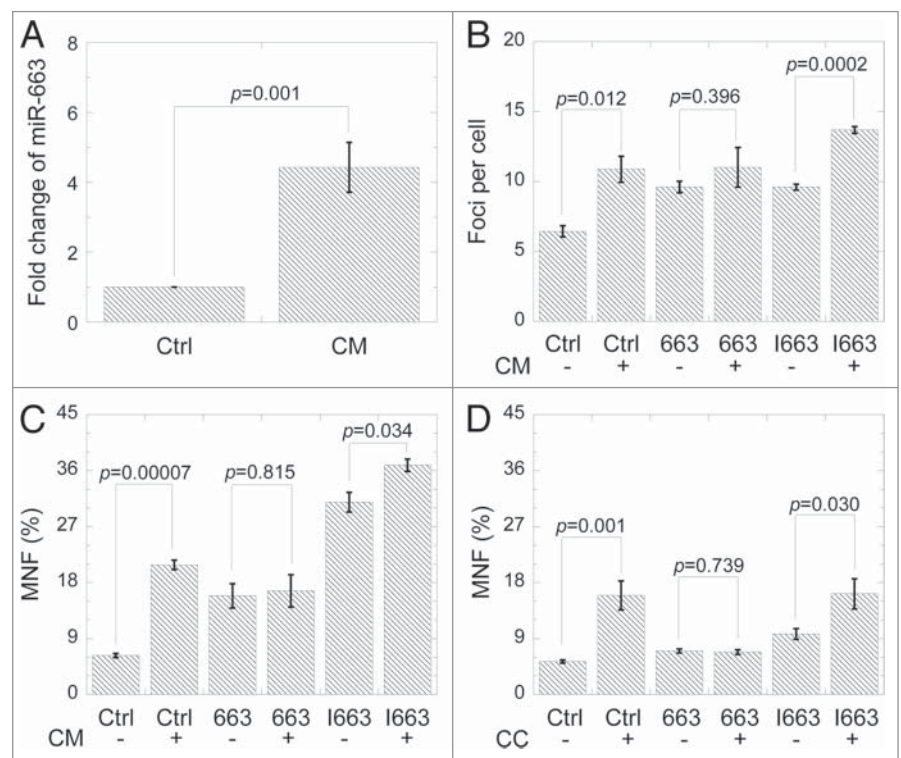

Figure 2. MiR-663 upregulation was observed in bystander cells. (**A**) MiR-663 expression in bystander HeLa cells 12 h after receiving conditioned medium from HeLa cells subjected to 4 Gy X-ray irradiation. (**B**) 24 h after transfection with miR-663 mimics or inhibitors, yields of 53BP1 foci in bystander HeLa cells cultured for 2 h in medium conditioned by HeLa cells for 24 h after 4 Gy X-ray irradiation. (**C**) 24 h after transfection with miR-663 mimics or inhibitors, MNF in bystander HeLa cells cultured in medium conditioned by HeLa cells for 24 h after treatment with 4 Gy X-ray irradiation. (**D**) 24 h after transfection with miR-663 mimics or inhibitors, MNF in bystander HeLa cells co-cultured with HeLa cells exposed to 4 Gy X-rays. Data were obtained from at least three independent experiments, and presented as means ± SE P values between the indicated samples and sham control are also presented.
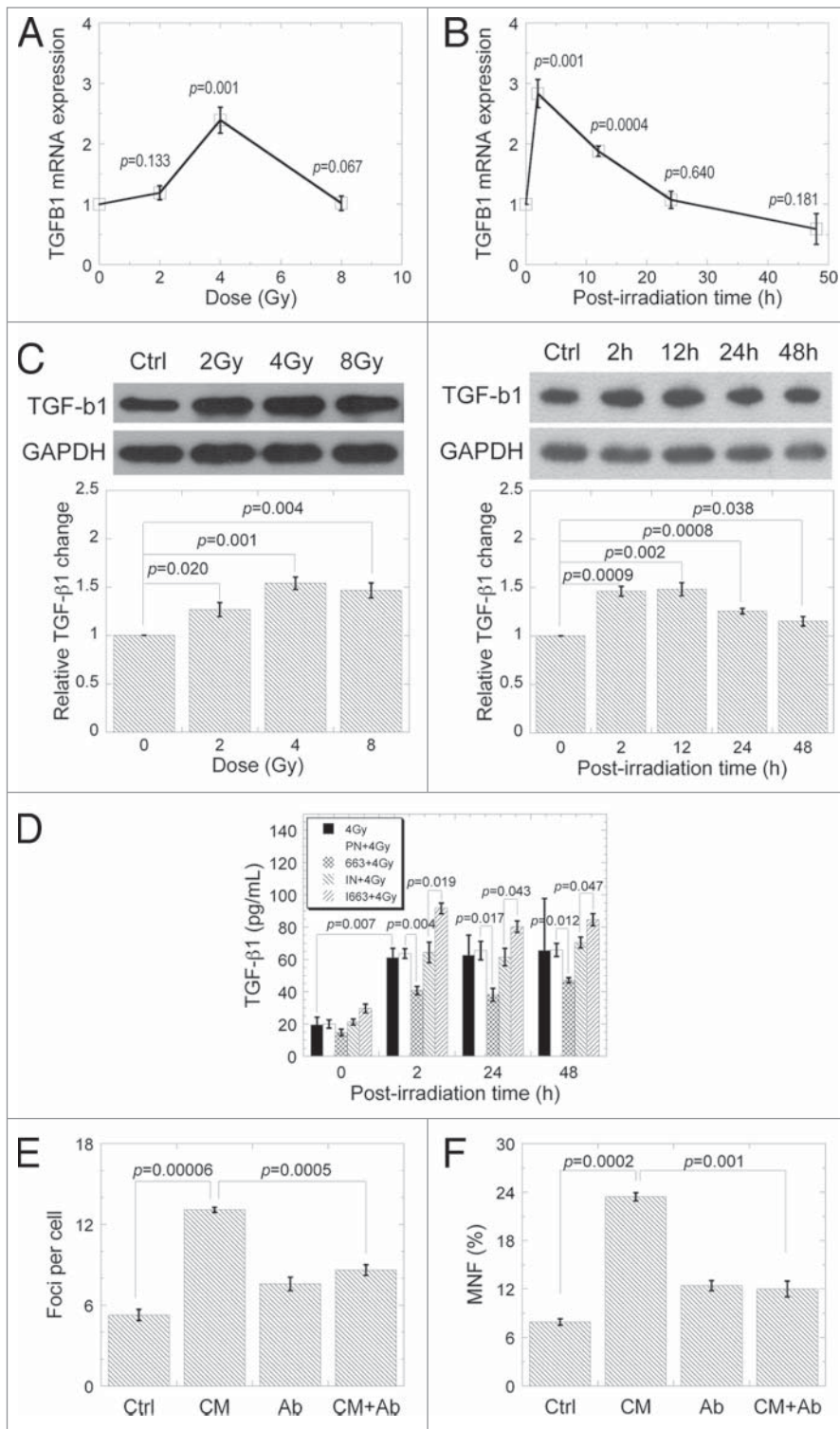
Figure 3. TGF-β1 is a mediator of radiation-induced bystander effects. *TGFB1* mRNA levels were detected using qRT-PCR in HeLa cells 2 h after exposure to different doses of X-ray irradiation. (**B**) *TGFB1* mRNA levels were detected in HeLa cells exposed to 4 Gy X-ray irradiation at different time-points. (**C**) TGF-β1 protein levels were examined by western blot in HeLa cells 2 h after exposure to different doses of X-ray irradiation, or in HeLa cells exposed to 4 Gy X-ray irradiation at different time-points. Western blot results from three independent experiments were quantified using ImageJ and a representative result is shown. (**D**) TGF-β1 protein level in the culture medium. After exposure to 4 Gy X-rays, HeLa cell culture medium was harvested at the indicated time-points for measurement of TGF-β1 protein with an ELISA kit. (**E**) Yields of 53BP1 foci in bystander HeLa cells cultured for 2 h in medium harvested from HeLa cells 24 h after exposuse to 4 Gy X-rays. (**F**) Frequency of micronuclei (MNF) in bystander HeLa cells cultured in medium harvested from HeLa cells 24 h after exposure to 4 Gy X-rays. Data from at least three independent experiments are presented as means ± SE.

correlated. Therefore, we further examined whether the alteration in miR-663 expression indeed had an effect on the expression of *TGFB1*. As shown in **Figures 4A** and B, overexpression of miR-663 led to suppression of *TGFB1* at both mRNA and protein levels. On the other hand, its inhibition resulted in upregulation of both *TGFB1* mRNA and protein. Furthermore, overexpression
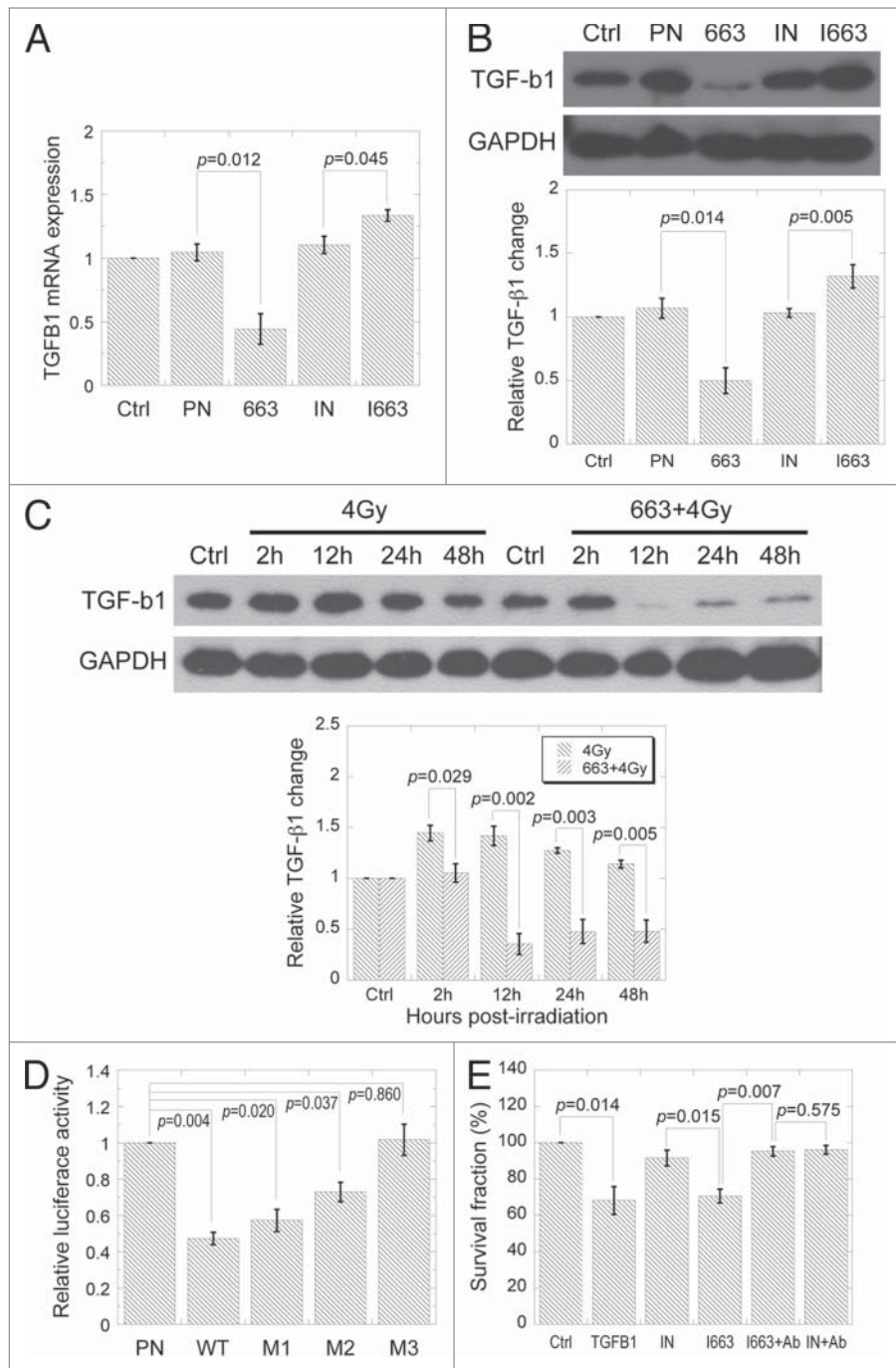
of miR-663 in HeLa cells markedly abolished the induction of TGF-β1 with 4 Gy irradiation (**Fig. 4C**).

Since online tools predicted 5 overlapping miR-663 binding sites distributed within two clusters in the 3′-UTR of *TGFB1* mRNA (**Fig. S6**), we postulated that there may be a direct interaction between miR-663 and *TGFB1*. HeLa cells were co-transfected with miR-663 mimics and pMIR-TGFB1–3′-UTR that contained the 3′-UTR of *TGFB1* transcript downstream of the luciferase gene. As shown in **Figure 4D**, luciferase activity was decreased by more than 50% compared with cells transfected with mimics negative control and pMIR-TGFB1–3′-UTR. The introduction of seed sequence mutations in the second or fourth miR-663 binding sites in *TGFB1* transcript 3′-UTR, of which also resulted in mutations in other binding sites, rescued luciferase activity to various degrees (M1 and M2 in **Figure 4D**). Mutation of both seed sequences totally rescued luciferase activity up to the control level (M3 in **Figure 4D**). Moreover, though addition of TGF-β1 as well as transfection of miR-663 inhibitors reduced cell survival rate, the combination of miR-663 inhibitor transfection and TGF-β1 neutralizing antibody treatment (a double inhibition effect) led to a cell survival fraction that

Figure 4. MiR-663 directly targets *TGFB1* and suppresses its expression. (**A**) *TGFB1* mRNA levels were detected using qRT-PCR in HeLa cells transfected with either miR-663 mimics (663) or inhibitors (I663). PN, negative control for miR-663 mimics; IN, negative control for miR-663 inhibitors. (**B**) TGF-β1 protein levels in HeLa cells transfected by either miR-663 mimics or inhibitors were examined by western blot. Western blot results from three independent experiments were quantified using ImageJ and a representative result is shown. (**C**) TGF-β1 protein levels in HeLa cells exposed to 4 Gy X-ray irradiation only or irradiation plus transfection with either miR-663 mimics or negative control (PN) were examined with western blot. Western blot results from three independent experiments were quantified using ImageJ and a representative result is shown. (**D**) Luciferase reporter assay. HeLa cells were co-transfected with pMIR-Report-Luciferase vectors containing either wild-type (WT) or mutated *TGFB1* 3′-UTR (M1 for mutant-1, M2 for mutant-2, M3 for mutant-3), the renilla luciferase vectors pGL4.74 (as an internal control), and either miR-663 mimics or negative control (PN). Firefly luciferase activity was normalized to that of Renilla. (**E**) Colony forming assay was performed on HeLa cells subjected to different treatments, after which cells were allowed to grow for 13 d. Ab, antibody against TGF-β1. Data from at least three independent experiments are presented as means ± SE.

was comparable to that of the non-treated control (**Fig. 4E**), suggesting an interplay exists between miR-663 and TGF-β1.

MiR-663 regulates RIBE mediated by TGF-β1

Since *TGFB1* is a direct target of miR-663, we aimed to determine whether miR-663 plays a role in TGF-β1-mediated RIBE. The stably inducible cell line HeLa-TetR-663 was used to increase miR-663 expression in directly irradiated cells. As shown in **Figures 5A and B**, upon miR-663 induction by tetracycline treatment in directly irradiated cells from which CM was generated, a significant suppression of 53BP1 foci and MNF was observed in bystander HeLa cells whose miR-663 expression level was not artificially changed, suggesting a role of miR-663 in mediating RIBE.

It has been demonstrated that TGF-β1 regulates expression of several miRNAs.[32-35] Therefore, we want to investigate whether TGF-β1 also exhibits a regulatory effect on miR-663. MiR-663 expression was significantly enhanced 12 h after TGF-β1 administration (**Fig. 5C**), but was reduced back to untreated control level 24 h post-treatment. As shown in **Figure 2A**, conditioned medium significantly induced miR-663 expression in bystander cells. However, when TGF-β1 neutralizing antibody was added into the conditioned medium, miR-663 induction in bystander cells was completely abolished (**Fig. 5D**). These results implicated that TGF-β1 may regulate miR-663 in a feed-back mode. The TGF-β1 signaling strength was also evaluated in both directly hit cells and bystander cells by analysis of Smad2 phosphorylation, the results showed that CM-induced miR-663 upregulation did attenuate the TGF-β1 signaling in bystander cells (**Fig. S7**).

Since miR-663 showed inhibitory effect on TGF-β1 expression and an induction of miR-663 was observed in bystander
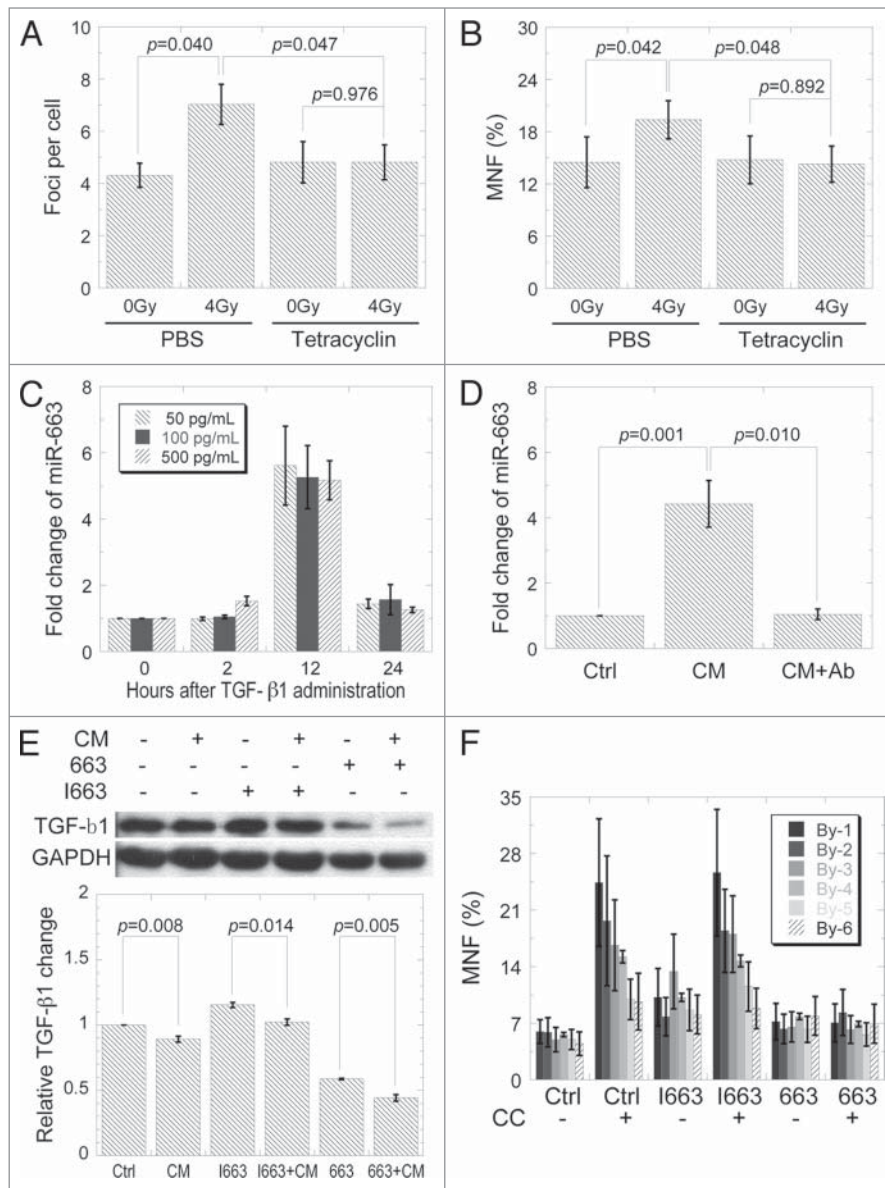
Figure 5. MiR-663 regulates RIBE mediated by TGF-β1. (**A**) Yields of 53BP1 foci in bystander HeLa cells cultured for 2 h in the medium harvested from HeLa cells with inducible miR-663 treated with/without tetracycline for 24 h and post-incubated for 24 h after exposure to 4 Gy X-rays. (**B**) MNF in bystander cells receiving conditioned medium harvested from cells, as indicated in panel A. (**C**) Induction of miR-663 in HeLa cells by TGF-β1. Indicated amounts of recombinant human TGF-β1 were added directly into the culture medium and expression levels of miR-663 measured with qRT-PCR at the indicated time-points. (**D**) MiR-663 expression in bystander HeLa cells 12 h after receiving conditioned medium from HeLa cells subjected to 4 Gy X-ray irradiation with or without TGF-β1 neutralizing antibody. (**E**) 12 h after CM transfer, TGF-β1 protein levels in HeLa cells transfected with either miR-663 mimics or inhibitors were examined with western blot. Western blot results from three independent experiments were quantified using ImageJ and a representative result is shown. (**F**) 24 h after transfection with miR-663 mimics or inhibitors, MNF in bystander HeLa cell populations co-cultured with HeLa cells exposed to 4 Gy X-ray irradiation. Three 12 × 12 mm coverslips were put in a line and divided equally into 6 parts according to different distances from directly irradiated cells, which were named By-1 to By-6. By-1 is the closest (3 mm) while By-6 is the farthest (33 mm). Data were obtained from at least three independent experiments and presented as means ± SE.

cells, we measured TGF-β1 level in bystander cells and found that TGF-β1 level was marginally decreased upon CM treatment. Addition of miR-663 mimics seemed to enhance such an effect while miR-663 inhibitors attenuated the reduction (**Fig. 5E**). Further, we wanted to see if such a reduced level of TGF-β1 was related with the limited transmitted distance of RIBE. As shown in **Figure 5F**, a distance dependency of bystander effects was observed in co-cultured cells. Cells that were transfected with miR-663 inhibitors displayed similar effects as those observed in the un-transfected control; while no such phenomenon was observed in cells that were transfected with miR-663 mimics.

Discussion

In this study, we showed that miR-663 is radiosensitive and significantly downregulated upon irradiation, which is consistent with earlier study with fibroblasts.[36] This implicated that miR-663 may play an important role in cellular response toward radiation. Further studies revealed that miR-663 directly regulates level of *TGFB1* in HeLa cells, an effect that was shown before in human SW480 colon cancer cells.[37] In addition, we demonstrated for the first time that miR-663 functions both in directly irradiated cells and bystander cells through its direct interaction with *TGFB1*.

In the current study, we showed that TGF-β1 is a bona fide target of miR-663. MiR-663 appears to suppress the *TGFB1* transcript by binding to two groups of overlapping miRNA responsive elements (MRE) located in the 3′-UTR of *TGFB1* mRNA. Such an observation is consistent with a previous report related with colon cancer cells.[37] Overexpression of miR-663 was found to attenuate the reduction of cell survival after X-ray treatment, suggesting a protective role of this microRNA toward irradiation. Its suppressive effect on TGF-β1 seems to support such a role because TGF-β1 induces cytostatic and apoptotic responses.[38] These observations concur with the finding that *TGFB1*(+/−), particularly *TGFB1*(−/−) cells, are significantly more radio-resistant than *TGFB1*(+/+) cells, indicating that the

TGF-β1 level may positively correlate with radio sensitivity.[39] Based on our results, we speculated that the overexpression of miR-663 inhibits TGF-β1 production after X-ray irradiation, which in turn results in reduced transportation of TGF-β1 precursor out of the directly irradiated cells and further leads to lower level of existing TGF-β1 precursors in extracellular medium[40] and thus limits the cytostatic effect of radiation. These findings were confirmed in NOD/SCID mice with xenografts derived from HeLa cells. Overexpression of miR-663 in xenografts also significantly suppressed the cytostatic effect of radiation. This may be due to the fact that the ectopic expression of miR-663 rescued radiation-induced miR-663 downregulation and inhibited TGF-β1 expression, and then counteracted its cytostatic effects in xenografts. In addition, we notice that the 3′-UTRs of Smad3 and Smad4 also contain potential miR-663 binding sites, implying that the miRNA simultaneously suppresses more than one component in the TGF-β1 signaling pathway. Taken together, miR-663 is an important modulator of the TGF-β1 signaling pathway.

Besides its involvement in the direct radiation response, TGF-β1 was also identified as a critical signal molecule in radiation-induced bystander effects. Compared with ROS or NOS, TGF-β1 is a relatively stable factor that can diffuse freely in the medium and induce RIBE in non-targeted cells. The downregulation of miR-663 in directly irradiated HeLa cells may contribute to the increased level of TGF-β1 in the culture medium (conditioned medium), of which subsequently participates in the bystander effects. In the present study, it was demonstrated that bystander effects generated by conditioned medium were abrogated by TGF-β1 neutralizing antibody. This result, together with the increased concentration of TGF-β1 detected in conditioned medium, suggested that TGF-β1 plays an important role in the transmission of RIBE. An interesting finding of our current study was that overexpression of miR-663 in directly irradiated cells diminished RIBE, possibly through its inhibitory effect upon TGF-β1 expression. Furthermore, the effects of miR-663 on cell migration, invasion, and EMT, which are all biological effects mediated by TGF-β1 signaling, were also studied in both directly hit cells and bystander cells. It is interesting that both irradiation and CM treatment induced migration, invasion, and EMT in HeLa cells, while overexpression of miR-663 inhibited these effects in both irradiated and bystander cells, and its inhibition aggravated them (**Figs. S8–10**).

In addition to the finding that miR-663 directly inhibits TGF-β1, we found out that TGF-β1 also has a regulatory effect on miR-663 expression. TGF-β1 has been shown to modulate the expression of several miRNAs.[32-35] Our results showed that miR-663 expression was upregulated in HeLa cells by conditioned medium that contained higher level of TGF-β1 released from the directly hit cells and that addition of TGF-β1 neutralizing antibody completely abrogated such an induction. Inhibition of miR-663 in bystander cells strongly aggravates the DNA damage caused by transfer of conditioned medium, indicating that loss of miR-663 enhances RIBE damage caused by TGF-β1 signaling. However, miR-663 overexpression in bystander cells abrogates RIBE damage. Co-culture experiments performed to confirm the results revealed that RIBE damage is dependent on the distance from directly irradiated cells. Since TGF-β1 signals are inhibited by miR-663, bystander cells barely secrete TGF-β1, leading to loss of signals. A distance-dependent concentration gradient is formed by TGF-β1 secreted mainly by directly irradiated cells, which provides an explanation for the distance-dependency of bystander effects.

Based on our results, a model for the interplay between miR-663 and TGF-β1 in directly irradiated and bystander cells was proposed (**Fig. 6**). In irradiated cells, miR-663 downregulation upon irradiation leads to de-repression of *TGFB1* transcript, partially results in increased TGF-β1 level in the conditioned medium. TGF-β1 in culture medium diffuses to bystander cells to trigger intracellular signaling, including induction of ROS and NO production, which cause DNA damage.[10,11] Theoretically, these increased NO and ROS should contribute to either the production or activation of TGF-β1 in the bystander cell population[11,40] and lead to infinite transmission of RIBE. However, the increased level
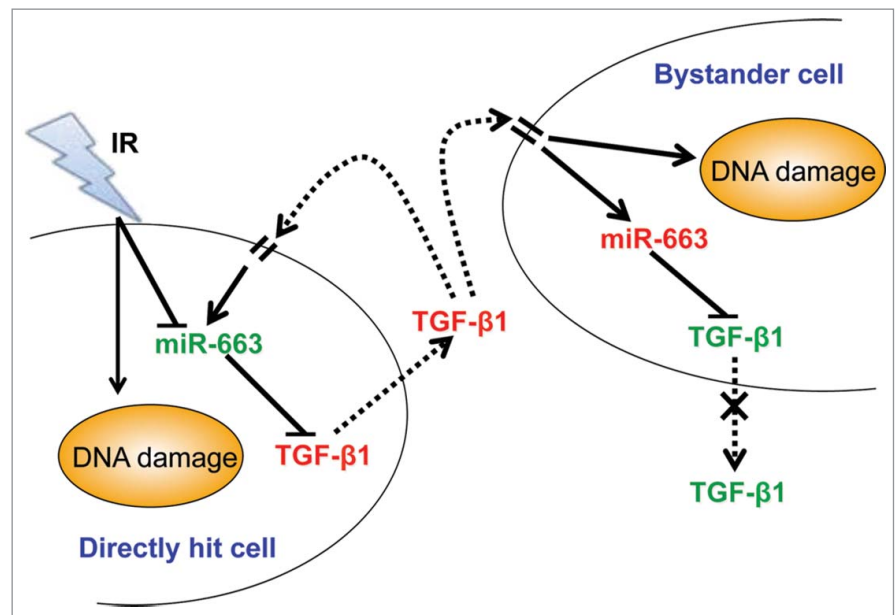

Figure 6. Proposed model for the function of miR-663 in TGF-β1-mediated RIBE. In irradiated cells, miR-663 downregulation leads to derepression of the *TGFB1* transcript, partially resulting in increased TGF-β1 protein levels in the culture medium. TGF-β1 in culture medium diffuses to bystander cells to trigger intracellular signaling, including induction of DNA damage. In bystander cells, miR-663 is upregulated via TGF-β1 signaling, and in turn, inhibits expression of TGF-β1, resulting in lower TGF-β1 production and secretion. Thus, bystander effects are observed in a distance-dependent manner.

of TGF-β1 in conditioned medium upregulates miR-663, which leads to inhibition of *TGFB1* expression in bystander cells. This negative feedback loop adds an important negative regulation element to the TGF-β1 signaling pathway and exerts suppressive effects on transmission of RIBE mediated by TGF-β1, resulting in a distance-dependent effect of bystander effects.

Conclusion

Radiosensitive miR-663 has been identified as an important participant in both direct irradiation effects and RIBE in HeLa cells by targeting *TGFB1*. Secretion of TGF-β1 signals by bystander cells is inhibited via a feedback regulation by overexpressed miR-663, which may explain the limited distance transmission of bystander effects. Although the precise mechanism underlying the modulation of miR-663 by TGF-β1 remains unclear at present, the current findings pave the way for further research on miRNA functions in RIBE. Moreover, this study may provide new insights to the development of radioprotectants or radiosensitizers based on miR-663 in clinical radiotherapy.

Materials and Methods

Cell culture

Human cervical cancer cells (HeLa) were grown in RMPI-1640 culture medium (Gibco) supplemented with 10% fetal bovine serum (Hyclone), 100 units/mL penicillin and 100 mg/mL streptomycin and maintained at 37°C in 5% CO_2 in a humidified incubator (Thermo Scientific).

Irradiation

HeLa cells plated in 35 mm diameter tissue culture dishes (Corning) were irradiated at a dose rate of 1.2 Gy/min using a cabinet X-ray generator (Faxitron) at room temperature. Non-irradiated cells were used as the sham control. Radiation doses were measured with a calibrated semiconductor detector (Diados). The NOD/SCID mice bearing xenografts of HeLa cells were subjected to half-body irradiation at room temperature at a dose rate of 1 Gy/min using the Varian CL2100 medical linear accelerator (Varian) at the Radiotherapy Center of Lanzhou General Hospital.

Quantitative real-time polymerase chain reaction (qRT-PCR)

Cells were harvested with TRIzol reagent (Invitrogen). RNA was isolated following the manufacturer's protocol. Total RNA was reverse-transcribed using the GoScript Reverse Transcription System (Promega) and amplified with qPCR master mix from Promega. Primers for both reverse transcription and PCR were listed in Table S1. The miR-663 PCR was performed with a Chromo4 system (Bio-Rad) under the following conditions: initiation for 2 min at 95°C, followed by 40 thermal cycles each at 95°C for 30 s and at 62°C for 40 s. PCR program for *TGFB1* was set as follows: 2 min at 95°C, followed by 40 thermal cycles

at 95°C for 15 s, 57°C for 20 s, and 72°C for 30 s. All data were analyzed with the C(t) value comparison method.

Clonogenic survival assay

HeLa cells were plated in 12-well plates at a density of 2×10^5 cells per well. Cells were transfected with 30 nM miR-663 mimics or inhibitors (Ambion) using Lipofectamine 2000 (Invitrogen) on the next day. Irradiation with 4 Gy X-rays was performed 6 h after the transfection. Immediately after irradiation, cells were harvested, counted, plated into φ60 mm dishes, and returned to the incubator. Plated cell numbers varied, depending on the different treatments. In terms of TGF-β1 or anti-TGF-β1 treatment, 10 ng/mL recombinant human TGF-β1 (Sigma-Aldrich) or 200 pg/mL anti-TGF-β1 antibody (Merck) was introduced into the culture. Cells were grown for 13 d, fixed with 70% ethanol for 5 min and stained with crystal violet. Colonies containing >50 cells were counted as survivors. At least 3 parallel dishes were scored for each treatment.

Luciferase reporter assay

The 3′-untranslated region (3′-UTR) of the human *TGFB1* transcript was cloned downstream of the luciferase gene between the *Spe*I and *Hin*dIII sites of pMIR-Report-Luciferase vector (Ambion). We additionally generated a construction that contained a mutated seed sequence of miR-663. HeLa cells (2×10^5) on a 12-well plate were co-transfected with 300 ng DNA (pMIR-Reporter-Luciferase-TGFB1–3′-UTR constructs or derived mutants) and 30 nM miR-663 mimics or miR-663 inhibitors and 300 ng renilla luciferase control vector pGL4.74 (Promega) using Lipofectamine 2000. Luciferase activity was measured 48 h later using the Dual Luciferase Reporter Assay System (Promega) with a Tecan Infinite M200 Pro microplate reader (Tecan).

Enzyme-linked immunosorbent assay (ELISA)

Human TGF-β1 ELISA kits were purchased from Boster Corporation (Boster). Cell culture medium was harvested at the indicated time-points for ELISA according to the manufacturer's instruction. Absorbance at 450 nm was measured using a Tecan Infinite M200 Pro microplate reader. According to the ELISA kit manual, all samples were acidulated before ELISA by the addition of 1 N HCl (0.2 volume of sample) to the samples for 10 min and neutralized with 1.2 N NaOH containing 0.5 M HEPES (0.2 volume of sample). This step facilitates TGF-β1 dissociation from the latency-associated peptide (LAP) and activation.

Western blot

Cells were collected and lysed using RIPA buffer. Samples were sonicated (120 W, 3 s followed by 8 s cooling) and centrifuged at 12 000 g for 15 min at 4°C. The total protein concentration was determined by using DC Protein Assay Kit I (Bio-Rad, Richmond). Samples were denatured at 100°C for 5 min, resolved using SDS-PAGE, and transferred to polyvinylidene difluoride (PVDF) membranes (GE Healthcare, Piscataway, NJ, USA). After blockage with PBST (PBS with 0.1% Tween-

20) containing 5% skimmed milk for 1 h at room temperature, the membrane was incubated with primary antibody for 2 h at room temperature, washed three times with PBST for 5 min each, and then incubated with HRP-conjugated secondary antibody for 1 h, washed three times with PBST. Protein bands were visualized using the chemiluminescence system (Millipore) and exposed to X-ray medical films (Kodak/Carestream Health). GAPDH was used as a loading control. Anti-TGF-β1 was purchased from Cell Signaling Technology (Beverly) and anti-GAPDH was from Santa Cruz Biotechnology.

Apoptosis assay

HeLa cells were transfected with 30 nM miR-663 mimics or miRNA precursor negative control 24 h after plated in φ35 mm dishes (4×10^5). Subsequently, cells were subjected to 4 Gy X-ray irradiation. Following a 24 h period, cells were trypsinized and collected via centrifugation at 130 g for 5 min. After washing once with PBS, cells were stained with Hoechst33342 (10 μg/mL) and propidium iodide (PI) (50 μg/mL) at 37°C for 15 min. Cells were then observed under a Leica AF6000 microscope (Leica). At least 500 cells were scored for each sample.

MiR-663 construct and cell line generation

A 300 bp sequence containing miR-663 precursor or negative control sequence was cloned into the pcDNA™6.2-GW/EmGFP-miR (Invitrogen), and the expression cassette transferred into pT-REx-DEST30 (Invitrogen) containing a tetracycline operator through gateway reaction. After verified via sequencing, the construct was transfected into HeLa cells stably expressing the tetracyclin repressor (TetR). The stable cell lines, designated HeLa-TetR-663, and the negative control HeLa-TetR-Neg were established via selection with G418 (Invitrogen) for 14 d. Inducible miR-663 expression was verified using qRT-PCR.

In vivo experiments

HeLa-TetR-663 or HeLa-TetR-Neg cells (1.6×10^6) were injected subcutaneously into the flanks of 5-wk-old NOD/SCID mice (n = 10 flanks). One month later, all mice developed solid tumors. Half of the mice were exposed to 5 Gy X-ray irradiation. Subsequently, miR-663 expression was induced continuously for 7 d by injecting tetracycline (Sigma-Aldrich) intraperitoneally at a daily dose of 4 mg/mouse, and tumor volumes measured every 5 d using a caliper for 1 mo. All tumor volume data were normalized to those obtained just before irradiation. Mice were housed in the Institute of Tumor, Gansu Academy of Medical Sciences, under the approval of the Institutional Review Board or Animal Care and Use Committee.

Conditioned medium (CM) transfer, co-culture, immunocytochemistry, and micronucleus assay

For CM transfer, HeLa cells (8×10^4) were plated in φ35 mm dishes for direct irradiation, while bystander HeLa cells (4×10^4) were plated on 12 × 12 mm coverslips placed in 12-well plates. After irradiation, cell medium was immediately removed and substituted with fresh RPMI-1640 medium. The conditioned medium was harvested 24 h after irradiation, spun at 2500 rpm for 5 min to exclude cells and cellular fragments, and transferred to bystander cells. Two hours after CM transfer, the bystander population on coverslips was fixed for 10 min in 4% paraformaldehyde for immunostaining or incubated with 2.5 μg/mL cytochalasin B (Sigma-Aldrich) for 30 h for the micronucleus (MN) assay. Immunostaining was conducted according to a previous report by our group.[41] Cells were incubated with 53BP1 (Cell Signaling Technology) and subjected to Alexa Fluor® 546 anti-rabbit antibody or Alexa Fluor® 488 anti-mouse antibody (Molecular Probes). 53BP1 foci were counted using an Olympus BX51 fluorescence microscope (Olympus). At least 100 cells were scored for each sample. As for the MN assay, cells were fixed in Carnoy's solution for 15 min. Air-dried cells were stained with acridine orange (30 μg/mL) before observation. At least 500 binucleated cells were counted for each sample. In some experiments, cells were treated with TGF-β1 mouse monoclonal antibody to neutralize the TGF-β1 induced by irradiation. For co-culture, HeLa cells for direct irradiation were plated on 12 × 12 mm coverslips placed in φ35 mm dishes at a concentration of 1×10^5 cells/dish. HeLa cells (3×10^5) were plated in φ60 mm dishes as bystander populations in which six 6 × 12 mm coverslips were pre-located. At 18 h after plating, cells were transfected with either miR-663 mimics (663) or miR-663 inhibitors (I663). Twenty-four hours after transfection, cells in φ35 mm dishes were irradiated and placed into the predetermined sites in the φ60 mm dishes that contained bystander cells. Subsequently, co-culture cells were incubated with 2.5 μg/mL cytochalasin B for 30 h for the MN assay as described above. Bystander cells were divided into six populations, based on distance from directly irradiated cells.

Supplemental Materials

Supplemental materials may be found here: www.landesbioscience.com/journals/rnabiology/article/34345/

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