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Haematologica 2020 [Epub ahead of print]

Citation: Mengjia Hu, Yukai Lu, Hao Zeng, Zihao Zhang, Shilei Chen, Yan Qi, Yang Xu, Fang Chen, Yong Tang, Mo Chen, Changhong Du, Mingqiang Shen, Fengchao Wang, Yongping Su, Song Wang, and Junping Wang. MicroRNA-21 maintains hematopoietic stem cell homeostasis through sustaining the NF-κB signaling pathway in mice. Haematologica. 2020; 105:xxx

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MicroRNA-21 maintains hematopoietic stem cell homeostasis through sustaining the NF-κB signaling pathway in mice


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Running title: MicroRNA-21 maintains HSC homeostasis

Abstract word count: 193

Text word count: 3576

Number of figures: 7

Number of tables: 0

Number of supplemental files: 2
Abstract
Long-term hematopoietic output is dependent on hematopoietic stem cell (HSC) homeostasis which is maintained by a complex molecular network. Among these, microRNAs play crucial roles, while the underlying molecular basis has not been fully elucidated. Here, we show that miR-21 is enriched in murine HSCs, and mice with conditional knockout of miR-21 exhibit an obvious perturbation in normal hematopoiesis. Moreover, significant loss of HSC quiescence and long-term reconstituting ability are observed in the absence of miR-21. Further studies reveal that miR-21 deficiency markedly decreases the NF-κB pathway, accompanied by increased expression of PDCD4, a direct target of miR-21, in HSCs. Interestingly, overexpression of PDCD4 in wild-type HSCs generates similar phenotypes as those of miR-21-deficient HSCs. More importantly, knockdown of PDCD4 can significantly rescue the attenuation of NF-κB activity, thereby improving the defects in miR-21-null HSCs. On the other hand, we find that miR-21 is capable of preventing HSCs from ionizing radiation-induced DNA damage via activation of the NF-κB pathway. Collectively, our data demonstrate that miR-21 is involved in maintaining HSC homeostasis and function, at least in part, by regulating the PDCD4-mediated NF-κB pathway and provide a new insight into the radioprotection of HSCs.

Key Words: hematopoietic stem cells, miR-21, homeostasis, NF-κB, irradiation
**Introduction**

Hematopoiesis is a well-organized developmental process in which hematopoietic stem cells (HSCs) can self-renew and differentiate into all kinds of blood cells\(^\text{1,2}\). Under steady-state conditions, most adult HSCs are retained in a relatively undifferentiated and quiescent state in the bone marrow (BM) microenvironment, which is necessary for sustaining long-term hematopoietic function\(^\text{3,4}\). In contrast, the perturbation of HSC homeostasis may result in hematopoietic failure, immunodeficiencies or hematological malignancies\(^\text{5,6}\). It was known that HSC homeostasis is tightly modulated by a complicated molecular network\(^\text{7}\). In the past decades, many factors, including cell cycle proteins, transcription factors, surface receptors, epigenetic regulatory factors, metabolic regulators, long non-coding RNA and cytokines, have been found to be involved in the control of HSC homeostasis\(^\text{1,3,8,9}\). However, the underlying molecular mechanism is still not completely uncovered.

MicroRNAs (miRNAs) are small non-coding RNAs that participate in a wide range of biological processes by negatively controlling the expression of their target genes through post-transcriptional regulation\(^\text{10,11}\). Previous studies have reported that miRNAs have distinct expression patterns in the hematopoietic system, and specific miRNAs can affect the development of different blood-cell lineages\(^\text{12,13}\). In recent years, several miRNAs, such as miR-22, miR-29a, miR-125a, miR-126, and the miR-132/122 cluster\(^\text{7,14-17}\), have been shown to play an important role in HSC biology.

miR-21, a well-known short RNA, has multiple physiological functions in mammals. It was found that miR-21 is involved in Gfi1-mediated modulation of myelopoiesis and regulates macrophage polarization and anti-inflammatory effect\(^\text{18-20}\). Besides, mice lacking miR-21 display reduced eosinophil levels in the peripheral blood (PB) and impaired eosinophil colony-forming capacity in the BM\(^\text{21}\).
Furthermore, another study revealed that miR-21 mediates hematopoietic suppression in myelodysplastic syndromes by targeting smad7, which is a negative regulator of the TGF-β/smad pathway. In particular, recent studies have defined miR-21 as an oncomiRNA, which is frequently upregulated in many kinds of tumors, including hematological malignancies. Taken together, these findings demonstrate that miR-21 plays significant roles in the hematopoietic system. On the other hand, miR-21 was reported to be implicated in the biology of several types of stem cells, including mesenchymal stem cells, cancer stem cells and embryonic stem cells. However, the exact role of miR-21 in HSC populations is largely unclear.

In the present study, we first found that mouse HSCs express high levels of miR-21 and that targeted deletion of miR-21 leads to abnormal hematopoiesis. Furthermore, the quiescence and long-term reconstituting function of HSCs are significantly impaired with miR-21 deficiency. Then, we demonstrated that miR-21 is involved in the maintenance of HSC homeostasis and function through modulation of the NF-κB pathway by regulating PDCD4. Of note, miR-21 is also able to mitigate radiation-induced DNA damage in HSCs. Collectively, these data indicate a key role of miR-21 in HSC biology and therefore broaden our knowledge of the physiological functions of miR-21.

Methods

Animals. Normal (wild-type) WT C57BL/6J mice were purchased from the Institute of Zoology (Chinese Academy of Sciences, Beijing, China). miR-21^{flx/+} (miR-21^{fl/+}) mice and Mx1-Cre mice were obtained from Shanghai Model Organisms Center (China). miR-21^{fl/fl};Mx1-Cre mice were generated by crossing miR-21^{fl/fl} mice with Mx1-Cre mice. Unless otherwise stated, miR-21 deletion was induced by intraperitoneally injecting 4- to 6-week-old miR-21^{fl/fl};Mx1-Cre+ mice with 250 μg of
polyinosinic:polycytidylic acid (pIpC) (Sigma, St. Louis, MO, USA) every other day for a total of 7 doses. Four weeks after pIpC treatment, these mice were used in subsequent experiments. Identically treated miR-21fl/fl;Mx1-Cre− littermates were served as controls. Congenic C57Bl/6 SJL CD45.1+ mice were kindly provided by Prof. Jinyong Wang (Guangzhou Institutes of Biomedicine and Health, Chinese Academy of Science, Guangzhou, China). All animal experiments were approved by the Animal Care Committee of The Third Military Medical University (Chongqing, China).

**Flow Cytometry.** Single-cell suspensions of BM, spleen and PB were prepared as we described. The detailed information for antibodies used for flow cytometric analysis is provided in Supplementary Table S1. The cell cycle, apoptosis, *in vivo* 5-bromodeoxyuridine (BrdU) incorporation and intracellular protein staining were analyzed as we reported. Samples were detected using a FACSverse or FACSCanto flow cytometer (BD Biosciences, San Jose, CA, USA) and data was analyzed using FlowJo10.0 software (TreeStar, San Carlos, CA, USA). Cell sorting was performed using a FACSAliaII or FACSAliaIII sorter (BD Biosciences).

**Lentiviral transduction.** The recombinant lentivirus carrying PDCD4 gene or specific small hairpin RNA (shRNA) against PDCD4, as well as the corresponding negative controls, were obtained from Hanbio Co.LTD (Shanghai, China). Then, the lentivirus was transducted into HSCs as we reported. Subsequently, transduced LSKs (6×10³) were purified with GFP expression through flow cytometry, and transplanted into 10.0 Gy-irradiated CD45.1+ WT recipients along with 5×10⁵ CD45.1+ competitor BM cells. The sequence of sh-PDCD4 is as follows: 5’-GAGCTTTGTATATGAAGCCATTGTAA-3’.
Microarray analysis. Total RNA was isolated from freshly sorted miR-21^fl/fl or miR-21^∆/∆ Lin^- Sca1^+ c-Kit^+ cells (LSKs). After that, samples were hybridized on the Mouse Clariom D arrays (Affymetrix, Santa Clara, CA, USA) in triplicate following the User Manuals. Raw data were then normalized using the Robust Multi-array Average (RMA). Those (fold change >1.4 and P value <0.05) were defined as differentially expressed genes that have been provided in Supplementary Table S3. Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment were used for microarray data analysis. The data have been deposited in the Gene Expression Omnibus (GEO) database (accession number no. GSE131603).

Statistical analysis. All results were analyzed using the GraphPad Prism 6.0 software (La Jolla, CA, USA). Data between two groups and multiple groups were analyzed by two-tailed Student’s t test and one-way analysis of variance (ANOVA), respectively. The survival rates were compared by log-rank nonparametric test and displayed as Kaplan-Meier survival curves. Unless otherwise stated, data were obtained from at least 3 independent experiments. P value < 0.05 was defined as statistically significant.

Results

miR-21 is enriched in HSCs and its conditional ablation skews hematopoietic differentiation.

Previous miRNA expression profiling studies have shown that miR-21 is highly expressed in mouse BM^{12,27,28}. To evaluate the role of miR-21 in hematopoiesis, we first measured the expression of miR-21 in murine hematopoietic stem and progenitor cells (HSPCs). It was found that miR-21 was relatively enriched in HSCs compared with that of committed hematopoietic progenitors (Figure 1A), which hints that miR-
21 may play a potential role in HSC biology. Then, we generated a conditional knockout mouse model (miR-21\textsuperscript{fl/fl};Mx1-Cre) by crossing miR-21\textsuperscript{fl/fl} mice with Mx1-Cre mice (Figure 1B). The deletion of miR-21 was induced in the hematopoietic compartment by seven injections of pIpC (hereafter, miR-21\textsuperscript{fl/fl};Mx1-Cre\textsuperscript{−} and miR-21\textsuperscript{fl/fl};Mx1-Cre\textsuperscript{+} mice are referred to as miR-21\textsuperscript{fl/fl} and miR-21\textsuperscript{ΔΔ} mice, respectively), which was confirmed by genomic PCR and quantitative RT-PCR (qRT-PCR) analysis (Figure 1C-D; Supplementary Figure S1D). However, we did not find significant differences in total cell number in the BM and spleen, as well as the counts of white blood cells, red blood cells and platelets in the peripheral blood (PB), after miR-21 knockout (Supplementary Figure S1E-F). Interestingly, miR-21\textsuperscript{ΔΔ} mice exhibited a significant increase in the percentage of myeloid cells but a marked decrease in the percentage of B cells in the BM and PB (Figure 1E; Supplementary Figure S1G). Consistently, we found that the percentage of granulocyte monocyte progenitors (GMPs) was obviously enhanced, while the frequencies of common myeloid progenitors (CMPs), megakaryocyte erythroid progenitors (MEPs) and common lymphoid progenitors (CLPs) were reduced in the BM after miR-21 deletion (Figure 1F-G). These findings suggest that miR-21 is crucial for steady-state hematopoiesis.

**Targeted deletion of miR-21 generates an aberrant HSC pool.**

To determine how miR-21 affects hematopoiesis, we next analyzed the phenotypes of HSCs from miR-21\textsuperscript{ΔΔ} mice by flow cytometry. There were evident increases in the percentage and number of LSKs, but not lineage (Lin\textsuperscript{−}) cells and myeloid progenitors (MPs), in the BM from miR-21\textsuperscript{ΔΔ} mice compared with those of miR-21\textsuperscript{fl/fl} mice (Figure 2A). Further analysis revealed that the frequency of long-term HSCs (LT-HSCs) was increased, whereas the proportion of multipotent progenitors (MPPs) was decreased in the LSK compartments in the absence of miR-21 (Figure 2B). Indeed,
the numbers of three LSK subpopulations, especially the LT-HSC, were markedly increased in the BM after miR-21 knockout (Figure 2C). A similar result was obtained by staining LSKs with another set of HSC surface markers, CD150 and CD48 (Figure 2D; Supplementary Figure S2A). We also observed a dramatic increase in the percentage of LSKs in the spleen, but not the PB, when miR-21 was deleted (Figure 2E; Supplementary Figure S2B). These results indicate that miR-21 is responsible for sustaining the normal HSC pool.

**Loss of miR-21 impairs the quiescence and facilitates the proliferation of HSCs.**

We then set out to explore the possible reasons for the accumulation of phenotypic HSCs after miR-21 knockout. Actually, we found a minor but not significantly different decrease in the apoptosis of HSCs upon miR-21 ablation (Supplementary Figure S3A). Importantly, cell cycle and *in vivo* BrdU incorporation analysis revealed that miR-21 deficiency strikingly reduced the quiescence and increased the proliferation of HSCs, but not MPs (Figure 3A-B; Supplementary Figure S3B-C), which is in line with the expression pattern of miR-21. Moreover, qRT-PCR data showed that cyclin-dependent kinase inhibitors (p21 and p27) were downregulated, whereas cell cycle-associated genes (Cyclin E1, Cyclin E2, Cyclin A2 and CDK6) were upregulated in LT-HSCs in the absence of miR-21 (Figure 3C).

Given that proliferating active cells are highly sensitive to genotoxic stress, we therefore administered miR-21^{fl/fl} and miR-21^{ΔΔ} mice with a single dose of 5-fluorouracil (5-FU). As anticipated, miR-21ΔΔ mice displayed more severe myelosuppression compared with than of control mice (Figure 3D-E). Likewise, the survival rate of miR-21^{ΔΔ} mice was significantly reduced after weekly 5-FU injections (Figure 3F). Taken together, these data demonstrate that miR-21 is required to maintain HSCs in a quiescent state.
miR-21 deficiency intrinsically compromises HSC function in long-term reconstitution.

It has been well accepted that the maintenance of HSC quiescence is closely connected with their long-term reconstituting capability. To this end, we first conducted non-competitive bone marrow transplantation (BMT) assays (Figure 4A). As a result, mice transplanted with miR-21∆/∆ BM cells died earlier than those transplanted with miR-21^{fl/fl} BM cells in secondary non-competitive BMT (Figure 4B-C). To further determine the function of miR-21∆/∆ HSCs, we next performed competitive BMT assays (Figure 4D). It was observed that miR-21 deficiency led to a reduced percentage of donor-derived cells in recipients’ PB and LSK compartments after primary and secondary transplantsations, accompanied by myeloid-biased hematopoietic differentiation (Figure 4E-H; Supplementary Figure S4A-B).

To substantiate these findings, we performed another competitive BMT. BM cells from miR-21^{fl/fl};Mx1-Cre- or miR-21^{fl/fl};Mx1-Cre+ mice without pIpC administration were transplanted, and at 4 weeks after transplantation, miR-21 ablation was induced by injecting pIpC into the recipients (Figure 4I). Consistently, the compromised function and biased differentiation were also observed (Figure 4J-K). Further, the reciprocal BMT experiment reflects that BM microenvironmental changes are not enough to mediate the functional defects in miR-21∆/∆ HSCs (Supplementary Figure S4C-E). Collectively, these data confirm that miR-21 intrinsically modulates the function of HSCs.

Specific knockout of miR-21 dramatically decreases the NF-κB pathway in HSCs.

To gain insight into the underlying mechanisms by which miR-21 regulates HSC homeostasis and function, we conducted a microarray analysis of sorted LSKs from miR-21^{fl/fl} and miR-21∆/∆ mice. As shown in Figure 5A, we identified 1050
differentially expressed genes, among which 495 genes were upregulated and 555 genes were downregulated in HSCs when miR-21 was deleted. Then, GO enrichment analysis showed that the upregulated genes were markedly enriched in nucleosome assembly, mitotic nuclear division, cell division, DNA replication-dependent nucleosome assembly, cell cycle and chromosome segregation (Figure 5B), which was in accordance with the findings that miR-21 knockout reduced the quiescence and promoted the proliferation of HSCs. Besides, consistent with the myeloid bias that manifested in miR-21Δ/Δ mice, we noticed a robust increase in the expression of myeloid differentiation genes, such as Mpo, Fcgr3, Csflr, Igsf6, Ly6c1 and Ms4a330,31, in HSCs after miR-21 knockout (Supplementary Figure S5).

Importantly, KEGG pathway analysis revealed that miR-21 ablation dramatically decreased the NF-κB pathway in HSCs (Figure 5C), which has been reported to play a critical role in maintaining hematopoietic homeostasis30,32. In fact, a marked downregulation of NF-κB downstream genes was observed in miR-21Δ/Δ LT-HSCs (Figure 5D), including p21 (Figure 2C). The attenuation of NF-κB activity was further verified by western blotting, immunofluorescence and flow cytometry (Figure 5E-G). Notably, the defects displayed in miR-21Δ/Δ mice, including myeloid bias, accumulation of HSCs in the BM and spleen, and reduced quiescence and impaired reconstituting capacity, resemble such aspects of hematopoietic p65-null mice30. Altogether, these results suggest that miR-21 deficiency evidently inhibits the NF-κB pathway in HSCs, which may contribute to the defects in HSCs.

**The upregulation of PDCD4 is responsible for the defects in miR-21-null HSCs.**

Considering that miRNAs negatively regulate gene expression at the post-transcriptional level, we then deeply analyzed the expression of miR-21 target genes. Of the miR-21 target genes that have a potential role in affecting NF-κB activity25,33-35,
programmed cell death protein 4 (PDCD4) but not PTEN, Spry1 or Spry2 was markedly upregulated in HSCs with miR-21 deficiency (Figure 6A-C; Supplementary Figure S6A). Then, the binding between miR-21 and PDCD4 3’UTR was confirmed by luciferase reporter assay (Supplementary Figure S6B). Intriguingly, we observed that PDCD4 expression was abundant in HSCs in mouse BM (Supplementary Figure S6C), which is consistent with the specific role of miR-21 in HSCs.

We next sought to determine whether the upregulation of PDCD4 in HSCs contributes to the decreased NF-κB activity, and defective phenotype and function observed in miR-21∆/∆ mice. For this purpose, we transduced normal HSCs from WT mice with a lentivirus expressing PDCD4 and found that NF-κB activity was significantly inhibited after overexpression of PDCD4 (Figure 6D). As a consequence, ectopic expression of PDCD4 reduced the quiescence and long-term repopulation potential of HSCs, as well as biased differentiation (Figure 6E and F; Supplementary Figure S6D-E). To further verify this notion, miR-21∆/∆ HSCs were transduced with a lentivirus carrying shRNA against PDCD4 (Supplementary Figure S6F). As expected, knockdown of PDCD4 partly rescued the defects in miR-21-null HSCs (Figure 6H and I; Supplementary Figure S6G-I). These data underscore a critical role of miR-21 in supporting the NF-κB pathway by targeting PDCD4, which is essential for the maintenance of HSC homeostasis.

miR-21 protects HSCs from irradiation-induced damage by activating the NF-κB pathway.

Evidence has indicated a vital role of NF-κB signaling in mitigating irradiation-induced hematopoietic injury, which promoted us to speculate that miR-21 may affect irradiation-induced biological processes in HSCs. To confirm this, miR-21fl/fl and miR-21∆/∆ mice were simultaneously subjected to ionizing radiation. We found
that the loss of miR-21 led to distinctly declined DNA damage repair, accompanied by increased apoptosis, in HSCs undergoing irradiation exposure (Figure 7A-B; Supplementary Figure S7A). Moreover, several NF-κB target genes (Ier3, Xrcc5 and Gadd45b) involved in DNA damage repair were significantly decreased in HSCs with miR-21 deficiency (Figure 7C). Additionally, a more serious decrease in the number of HSCs and white blood cells and an increased death rate were observed in miR-21^−/^− mice after irradiation (Figure 7D-E; Supplementary Figure S7B). Based on the finding that NF-κB signaling is implicated in thrombopoietin (TPO)-promoted DNA damage repair in HSCs, we administered mice with TPO and observed that it did not work in miR-21^−/^− HSCs (Supplementary Figure S7F-G).

Finally, we determined whether the addition of exogenous miR-21 can protect HSCs from irradiation by activating the NF-κB pathway. As a result, treatment of mice with a miR-21 agomir, which is an engineered miRNA mimic, increased NF-κB activity in HSCs by suppressing PDCD4 (Figure 7H), thereby significantly alleviating irradiation-induced damage (Figure 7J; Supplementary Figure S7C). Specifically, these effects were abrogated by a specific NF-κB inhibitor, QNZ^38 (Figure 7J; Supplementary Figure S7C). In conclusion, our findings demonstrate that miR-21 can also act as a radioprotector for HSCs.

**Discussion**

The maintenance of HSC homeostasis contributes to its function in the continuous supplementation of blood cells throughout the lifetime^1,6. In the past few decades, studies aimed at researching hematopoiesis have revealed many factors capable of controlling the homeostasis of HSCs. Recently, growing evidence has suggested that miRNAs play prominent roles in hematopoietic cells, including HSCs^17,27. In this
work, we showed for the first time that miR-21 is required to maintain HSC homeostasis and function by sustaining the NF-κB pathway.

As a multi-faceted non-coding RNA, miR-21 is present in multiple tissues and implicated in various physiological and pathological processes\textsuperscript{39}. Here, we observed that miR-21 is relatively enriched in HSCs in adult mouse BM, implying that miR-21 may be involved in HSC biology. As a consequence, we found that specific knockout of miR-21 in the hematopoietic system causes an abnormal expansion of HSC pool in the BM and spleen. Besides, mice with conditional deletion of miR-21 display a distinctly myeloid-skewed differentiation, along with the decrease of B cells. The reason might be that miR-21 deficiency affects the differentiation of HSPCs, which is consistent with a previous study\textsuperscript{18}. In addition, a previous study reported that miR-21 can inhibit the apoptosis of diffuse large B-cell lymphoma cells through upregulating BCL-2\textsuperscript{40}. Thus, miR-21 may also play a direct role in regulating B cell survival. Taken together, our results reveal that miR-21 is essential to maintain normal hematopoiesis in mice.

Recently, miR-21 has been increasingly regarded as an oncogene due to its function in promoting tumor cell proliferation and inhibiting apoptosis\textsuperscript{10}. In the present study, we found that miR-21 deficiency leads to a marked impairment in HSC quiescence, accompanied by increased proliferation, which is very different from the functions of miR-21 in other types of cells. Additionally, deletion of miR-21 in HSCs had no effect on cell apoptosis at a steady state. These results suggest that miR-21 regulates the cell cycle and apoptosis, most likely depending on the cellular contexts. It was well recognized that the loss of HSC quiescence can bring about a transient augmentation of phenotypic HSCs but eventually compromises their function\textsuperscript{6}. These results could explain our observation that miR-21\textsuperscript{∆A} HSCs have a diminished long-
term reconstituting capacity. Notably, the reciprocal transplantation assay validated that the defects manifested in miR-21-deficient HSCs are cell-intrinsic. In addition, we observed a myeloid bias in recipients transplanted with miR-21Δ/Δ BM cells, which is consistent with the changes in non-transplanted miR-21Δ/Δ mice.

In an effort to characterize the molecular mechanisms by which miR-21 regulates HSC homeostasis, we performed a microarray analysis. Notably, we observed a marked downregulation of the NF-κB pathway when miR-21 was deleted. It was well established that the NF-κB transcription factor family plays a key role in various physiological processes, including cell proliferation, apoptosis, inflammation and immune responses41. Current studies using mouse genetic models have indicated that, although aberrant activation of NF-κB is not beneficial for hematopoiesis, the basal NF-κB signaling is indispensable for HSC homeostasis42. Interestingly, miR-21Δ/Δ HSCs showed similar phenotypes to those of p65-null HSCs30. However, it is unknown whether miR-21 regulates HSC homeostasis and function via the NF-κB pathway. Further investigations revealed that a previously recognized target of miR-21, the tumor suppressor PDCD433,43, was obviously upregulated in HSCs with miR-21 deficiency. However, there is a controversy about the function of PDCD4 in regulating NF-κB activity. Most studies have shown that PDCD4 is involved in promoting NF-κB activation44,45. However, in our study, we found that overexpression of PDCD4 in WT HSCs by lentiviral transfection inhibited NF-κB activity, while knockdown of PDCD4 expression significantly improved the defects in miR-21-null HSCs. These results elucidate that miR-21 sustains the NF-κB pathway, at least partly, by directly targeting PDCD4, which is consistent with a previous study33. However, whether miR-21 can regulate other target genes still needs further researches. In addition, there is an opposing viewpoint that miR-21 inhibits NF-κB
activity\textsuperscript{45}, which may involve miR-21 regulation of physiological functions in a cellular context-dependent manner.

As we know, ionizing radiation-caused hematopoietic cell death primarily attributes to the generation of double strand breaks (DSBs) that are the most serious form of DNA damage\textsuperscript{2}. At present, the acute myelosuppression induced by irradiation can be temporarily treated using various hematopoietic growth factors that are able to accelerate the recovery of hematopoietic function by stimulating HSPC proliferation and differentiation\textsuperscript{46}. However, the effective strategy to protect DNA from irradiation-induced damage in HSCs is still lacking. Previous studies have revealed that miR-21 mediates radioresistance in many types of tumor cells, which is a great challenge for cancer radiotherapy\textsuperscript{47}. Further, it has been shown that miR-21 can be stimulated by irradiation via the EGFR/STAT3 or ATM pathways\textsuperscript{48,49}, which is line with our data (Supplementary Figure S7D). However, the role of miR-21 in HSCs in the context of irradiation remains obscure. In the present research, we discovered that miR-21 can prevent HSCs from irradiation-induced DNA damage through activating the NF-\kappa B pathway, which is consistent with a recent finding that miR-21 promotes hematopoietic cell survival after irradiation exposure\textsuperscript{50}. Moreover, our study revealed that miR-21 may also be involved in TPO-mediated NHEJ repair in HSCs. Nevertheless, we do not deny that there may be other NF-\kappa B-independent mechanisms that mediate the radioprotective effects of miR-21 in HSCs\textsuperscript{47}.

On the other hand, some studies have reported that miR-21 is also a downstream target gene of NF-\kappa B pathway\textsuperscript{44}. Indeed, overexpression of PDCD4 or treatment with a NF-\kappa B inhibitor (QNZ) significantly inhibited the expression of miR-21 in HSCs from normal WT mice (data not shown). These findings lead us to hypothesize that there may exist a positive regulatory feedback loop that regulates HSC function.
In summary, our findings reveal a novel function of miR-21 as an important regulator of HSC homeostasis via modulation of the NF-κB pathway by targeting PDCD4, thereby extending our understanding of the biological function of miR-21. At the same time, we demonstrate that targeting miR-21 may be a promising avenue for safeguarding HSCs against irradiation damage.

**Acknowledgements**

We thank Prof. Jinyong Wang for gifting CD45.1 mice, Yang Liu for technical support in flow cytometry, Liting Wang for technical assistance in immunofluorescence microscopy. This work was supported by grants from the National Natural Science Fund of China (No. 81725019, 81930090, 81573084, 81500087) and the Scientific Research Project of PLA (AWS16J014).

**Author contributions**

M.H. designed the study, performed experiments, analyzed data and wrote the paper. Y.L., Z.Z. and H.Z. performed experiments and analyzed data. S.C., Y.Q., Y.X., F.C. and Y.T. participated in the animal experiments and data analysis. M.C., C.D. and M.S. contributed to the *in vitro* experiments. F.W. and Y.S. participated in the initial experimental design and discussed the manuscript. J.W. and S.W. conceived and supervised the study, and revised the manuscript.

**Conflict-of-interest disclosure**

The authors declare no conflict of interest.

**References:**

Figure Legends

Figure 1. miR-21 is enriched in HSCs and its conditional ablation skews hematopoietic differentiation. (A) QRT-PCR analysis of miR-21 expression in long-term HSCs (LT-HSCs), short-term HSCs (ST-HSCs), multipotent progenitors (MPPs), common myeloid progenitors (CMPs), common lymphoid progenitors (CLPs), granulocyte monocyte progenitors (GMPs) and megakaryocyte erythroid progenitors (MEPs) isolated from normal 8-week-old WT mice (n = 4 mice). The relative miR-21 expression was compared with that in LT-HSCs. Gating strategies are provided in Supplementary Figure S1A-B. (B) The strategy for the generation of the conditional miR-21 knockout mouse model. The detailed genotyping see Supplementary Figure S1C. (C) Schematic for plpC-inducible deletion of miR-21 in the hematopoietic system. (D) PCR-based analysis of genomic DNA from the bone marrow (BM) and spleen (Sp) of miR-21^{fl/fl} and miR-21^{Δ/Δ} mice. (E) Flow cytometric analysis of the percentages of T cells (CD3ε+), B cells (B220+) and myeloid cells (Gr-1+ and Mac-1+) in the BM of miR-21^{fl/fl} and miR-21^{Δ/Δ} mice (n = 6 mice per group). (F, G) Flow cytometric analysis of the percentages of (F) CMPs, MEPs, GMPs and (G) CLPs in the BM of miR-21^{fl/fl} and miR-21^{Δ/Δ} mice (n = 6 mice per group). All data are shown as means ± SD. *P < 0.05, **P < 0.01.

Figure 2. Targeted deletion of miR-21 generates an aberrant HSC pool. (A) Flow cytometric analysis of the percentages and number of Lin− cells, MPs and LSKs in miR-21^{fl/fl} and miR-21^{Δ/Δ} BM (n = 6 mice per group). (B) Flow cytometric analysis of the proportions of LT-HSCs, ST-HSCs and MPPs in LSKs from miR-21^{fl/fl} and miR-21^{Δ/Δ} mice (n = 6 mice per group). (C) The number of LT-HSCs, ST-HSCs and MPPs in miR-21^{fl/fl} and miR-21^{Δ/Δ} BM (n = 6 mice per group). (D) Flow cytometric analysis
of the number of CD150⁺ CD48⁻ LSKs in miR-21^{fl/fl} and miR-21^{Δ/Δ} BM (n = 6 mice per group). (E) Flow cytometric analysis of the percentage of LSKs in the Spleen (Sp) miR-21^{fl/fl} and miR-21^{Δ/Δ} mice (n = 6 mice per group). All data are shown as means ± SD. *P < 0.05, **P < 0.01.

**Figure 3. Loss of miR-21 impairs the quiescence and facilitates the proliferation of HSCs.** (A) Cell cycle analysis of LSK and LT-HSC in the BM of miR-21^{fl/fl} and miR-21^{Δ/Δ} mice (n = 6 mice per group). (B) miR-21^{fl/fl} and miR-21^{Δ/Δ} mice were intraperitoneally injected with BrdU (1 mg/6 g mouse weight). Twelve hours later, the BrdU incorporation in LSK and LT-HSC from these mice were analyzed by flow cytometry (n = 6 mice per group). (C) QRT-PCR analysis of the relative expression of cyclin-dependent kinase inhibitors and cell cycle-associated genes in sorted miR-21^{fl/fl} and miR-21^{Δ/Δ} LT-HSCs (n = 3 mice per group). (D, E) The number of (D) BM cells and (E) LSKs from miR-21^{fl/fl} and miR-21^{Δ/Δ} mice after a single dose of 5-FU injection (n = 6 mice per group). (F) The survival rates of miR-21^{fl/fl} and miR-21^{Δ/Δ} mice after sequential 5-FU injections (n = 10 mice per group). All data are shown as means ± SD. *P < 0.05, **P < 0.01.

**Figure 4. miR-21 deficiency intrinsically compromises HSC function in long-term reconstitution.** (A-C) CD45.2⁺ miR-21^{fl/fl} or miR-21^{Δ/Δ} BM cells were transplanted into 10.0 Gy-irradiated CD45.1⁺ recipients. Sixteen weeks later, BM cells harvested from primary recipients were transplanted into 10.0 Gy-irradiated secondary CD45.1⁺ recipients. The survival rates of these recipient mice after (B) primary and (C) secondary non-competitive bone marrow transplantation (BMT) were monitored (n = 10 mice per group). (D-H) CD45.2⁺ miR-21^{fl/fl} or miR-21^{Δ/Δ} BM cells, mixed (1:1) with CD45.1⁺ competitor BM cells, were transplanted into 10.0 Gy-
irradiated CD45.1+ recipients. Sixteen weeks later, BM cells harvested from primary recipients were transplanted into 10.0 Gy-irradiated secondary CD45.1+ recipients. At indicated time, CD45.2+ donor chimerism and lineage distribution in recipients’ PB after (E, F) primary and (G, H) secondary competitive BMT were analyzed by flow cytometry (n = 8 mice per group). The data of lineage distribution shown were obtained from at 16 weeks after primary and secondary competitive BMT. (I-K) CD45.2+ BM cells from miR-21fl/fl;Mx1-Cre− or miR-21fl/fl;Mx1-Cre+ mice without pIpC treatment, together (1:1) with CD45.1+ competitor BM cells, were transplanted into 10 Gy-irradiated CD45.1+ WT recipients. Eight weeks after transplantation, miR-21 deletion was induced by injecting the recipients with pIpC. At indicated time, the (J) donor chimerism and (K) lineage distribution in recipients’ PB after transplantation were analyzed by flow cytometry. The data of lineage distribution shown were obtained from at 24 weeks after transplantation. The schematic diagram is shown in the left panel. All data are shown as means ± SD. *P < 0.05, **P < 0.01.

Figure 5. Specific knockout of miR-21 dramatically decreases the NF-κB pathway in HSCs. (A-C) LSKs sorted from miR-21Δ/Δ and miR-21fl/fl mice were used for microarray analysis. (A) Heatmap of differentially expressed genes between miR-21fl/fl and miR-21Δ/Δ LSKs. (B) Gene Ontology (GO) analysis of upregulated genes in LSKs after deletion of miR-21. The data shown are the top 15 enriched GO terms. (C) KEGG pathway analysis of downregulated genes in miR-21Δ/Δ LSKs relative to miR-21fl/fl LSKs. The top 5 enriched pathways are shown. The microarray data were gathered from one experiment with 3 biological replicates (A-C). (D) QRT-PCR analysis of the relative expression of NF-κB target genes (including Egr1, Tnf, Birc3, Nr4a2, Tnfaip3 and Nfkbia) in miR-21fl/fl and miR-21Δ/Δ LT-HSCs (n = 3 mice per group). (E, F) The expression of p-p65 in LSKs from miR-21fl/fl and miR-21Δ/Δ BM (n
= 5 mice per group), determined by (E) western blotting and (F) immunofluorescence, respectively. DAPI indicates the nucleus of cells. Scale bar represents 5 μm. (G) Flow cytometric analysis of the expression of p-p65 in LSKs and LT-HSCs from miR-21^fl/fl and miR-21^Δ/Δ BM (n = 5 mice per group). MFI, mean fluorescence intensity. All data are shown as means ± SD. **P < 0.01.

**Figure 6. The upregulation of PDCD4 is responsible for the defects in miR-21-null HSCs.** (A, B) The expression of PDCD4 in LSKs from miR-21^fl/fl and miR-21^Δ/Δ BM (n = 5 mice per group), determined by (A) western blotting and (B) immunofluorescence, respectively. DAPI indicates the nucleus of cells. Scale bar represents 5 μm. (C) Flow cytometric analysis of the expression of PDCD4 in LSKs and LT-HSCs from miR-21^fl/fl and miR-21^Δ/Δ BM (n = 5 mice per group). MFI, mean fluorescence intensity. (D) Western blotting analysis of the expression of PDCD4, p-p65 and p65 in LSKs after transduced with control or PDCD4 (n = 5 mice per group). (E, F) Normal LSKs from CD45.2^+ WT mice were transduced with the lentivirus carrying control or PDCD4, then transduced cells (6×10^3), mixed with CD45.1^+ competitor BM cells (5×10^5), were transplanted into 10.0 Gy-irradiated CD45.1^+ recipients. At 12 weeks after transplantation, (E) the cell cycle of CD45.2^+ donor-derived LT-HSCs in recipients’ BM, and (F) the CD45.2^+ donor chimerism in recipients’ PB were analyzed by flow cytometry (n = 6 mice per group). (G-I) CD45.2^+ miR-21^fl/fl or miR-21^Δ/Δ LSKs (6×10^3) transduced with the lentivirus carrying control or shRNA against PDCD4 (sh-PDCD4), mixed with CD45.1^+ competitor BM cells (5×10^5), were transplanted into 10.0 Gy-irradiated CD45.1^+ recipients. At 12 weeks after transplantation, (H) the cell cycle of CD45.2^+ donor-derived LT-HSCs in recipients’ BM, and (I) the CD45.2^+ donor chimerism in
recipients’ PB were analyzed by flow cytometry (n = 6 mice per group). All data are shown as means ± SD. **P < 0.01.

Figure 7. miR-21 protects HSCs from irradiation-induced damage by activating the NF-κB pathway. (A) Flow cytometric analysis of the percentage of γ-H2AX+ cells in at 0 (No irradiation) or 2, 6, 12, 24 hours after 4.0 Gy total body irradiation (TBI) (n = 6 mice per group). (B) Representative immunofluorescence plots showing the expression of γ-H2AX in LSKs from miR-21^{fl/fl} and miR-21^{Δ/Δ} mice 24 hours after 4.0 Gy TBI. DAPI indicates the nucleus of cells. Scale bar represents 5 μm. (C) QRT-PCR analysis of the relative expression of NF-κB target genes (Ier3, Xrcc5 and Gadd45b) in LSKs from miR-21^{fl/fl} and miR-21^{Δ/Δ} mice 5 hours after 4.0 Gy TBI (n = 3 mice per group). (D) The number of LSKs in the BM of miR-21^{fl/fl} and miR-21^{Δ/Δ} mice after 4.5 Gy TBI (n = 6 mice per group). (E) The survival rates of miR-21^{fl/fl} and miR-21^{Δ/Δ} mice after 6.5 Gy TBI (n = 10 mice per group). (F, G) miR-21^{fl/fl} and miR-21^{Δ/Δ} mice were treated with saline or TPO (8 μg/kg) 30 minutes before 4.0 Gy TBI. Then, (F) the expression of p-p65 in LSKs were measured 40 minutes after irradiation (n = 6 mice per group), and (G) the percentage of γ-H2AX+ LSKs in these mice were measured by flow cytometry 24 hours after irradiation (n = 6 mice per group). (H) Normal WT mice were treated with 3 doses of miR-21 agomir or negative control (NC) on consecutive days by tail intravenous injection. Twenty-four hours after last injection, the relative miR-21 expression in LSKs from these mice was detected by qRT-PCR (n = 6 mice per group), and the expression of PDCD4 and p-p65 in LSKs from these mice was analyzed by flow cytometry (n = 6 mice per group). (J) Mice were treated with miR-21 agomir or NC as described before. Twenty-one hours after last injection, mice were intraperitoneally injected with one dose of QNZ or control. Three hours later, mice were subjected to 4.0 Gy TBI. The percentage of γ-H2AX+
cells in LSKs from these mice was detected 24 hours after irradiation by flow cytometry (n = 6 mice per group). All data are shown as means ± SD. *P < 0.05, **P < 0.01.
Figure 1
Figure 2
Figure 4
Figure 5
Figure 6
Figure 7
Supplementary Methods

**Hematological analysis and irradiation.** Hematological analysis and irradiation were conducted as we described\(^1\).

**5-fluorouracil (5-FU) treatment.** Mice were given a single dose of 5-FU (150 mg/kg; Sigma) by intraperitoneal injection. At each indicated time after injection, mice were sacrificed and their BM cells and LSKs were measured. In another experiment, mice were weekly injected with 5-FU (150 mg/kg) for 3 weeks, and then the survival rates were monitored.

**miR-21 agomir and NF-κB inhibitor treatment.** Mice were given with 3 doses of miR-21-5p agomir (30mg/kg for each dose; GenePharma, Shanghai, China) on consecutive days by tail intravenous injection. Mismatched oligos (GenePharma Company) was served as negative control (NC) and used at similar doses. For NF-κB inhibition, mice were administrated with a specific NF-κB inhibitor, QNZ (1 mg/kg; MedChem Express, NJ, USA), by intraperitoneal injection.

**Transplantation assays.** For non-competitive bone marrow transplantation (BMT), \(1 \times 10^6\) CD45.2\(^+\) BM cells from miR-21\(^{fl/fl}\) or miR-21\(^{Δ/Δ}\) mice were transplanted into 10.0 Gy-irradiated CD45.1\(^+\) recipients. Sixteen weeks later, \(1 \times 10^6\) BM cells harvested from primary recipients were transplanted into 10.0 Gy-irradiated secondary CD45.1\(^+\) recipients. The survival rates were monitored after transplantations.

For competitive BMT, \(5 \times 10^5\) CD45.2\(^+\) BM cells from miR-21\(^{fl/fl}\) or miR-21\(^{Δ/Δ}\) mice, mixed with \(5 \times 10^5\) competitor BM cells from CD45.1\(^+\) WT mice, were transplanted into 10.0 Gy-irradiated CD45.1\(^+\) recipients. Sixteen weeks later, \(1 \times 10^6\) BM cells harvested from primary recipients were transplanted into 10.0 Gy-irradiated
secondary CD45.1+ recipients. In another competitive BMT, 5×10^5 CD45.2+ BM cells from miR-21^{fl/fl};Mx1-Cre− or miR-21^{fl/fl};Mx1-Cre+ mice without plpC treatment, mixed with 5×10^5 competitor BM cells from CD45.1+ WT mice, were transplanted into 10.0 Gy-irradiated CD45.1+ recipients. Eight weeks later, all recipients were injected with plpC. For reciprocal BMT, 1×10^6 CD45.1+ BM cells were transplanted into 10.0 Gy-irradiated CD45.2+ miR-21^{fl/fl} or miR-21^{Δ/Δ} recipients. At each indicated time after transplantation, PB or BM samples obtained from recipients were analyzed by flow cytometry. The transplantations after lentiviral transduction were performed as described below.

**Luciferase reporter assay.** The fragment of wild type (WT)-PDCD4 containing the predicted miR-21 binding site, as well as the corresponding mutant (MUT)-PDCD4, were amplified and cloned into the pmirGLO vector (Promega Corporation, Madison, WI, USA). After that, miR-21 mimic or miRNA-negative control (miR-NC), together with WT-PDCD4 or MUT-PDCD4, were co-transfected into 293T cells using lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA). Forty-eight hours later, the relative luciferase activity was measured.

**Quantitative RT-PCR (qRT-PCR).** RNA was extracted from freshly sorted cells using the RNAqueous kit (Ambion, Darmstadt, Germany). The expression of mRNAs or miR-21-5p was detected as we described^2,3. The primers sequences are provided in Supplementary Table 2.

**Western blotting.** Western blotting was performed as we reported^4. The following antibodies were used: anti-PDCD4 (Abcam, Cambridge, UK), anti-Spry1 (Novus, Littleton, CO, USA), anti-Spry2 (Abcam), anti-PTEN (Abcam), anti-p-p65 (Cell Signaling Technology, Danvers, MA, USA), anti-p65 (Cell Signaling Technology)
and anti-β-actin (Cell Signaling Technology). The detailed information for antibodies used for western blotting analysis is provided in Supplementary Table S1.

**Immunofluorescence microscopy.** Freshly sorted LSKs were placed on Poly-L-lysine coating slides. After fixation, permeabilization and blocking, samples were stained with anti-PDCD4 (Abcam), anti-p-p65 (Cell Signaling Technology) and γ-H2AX<sup>S139</sup> (Abcam) antibodies. Then, some samples were incubated with secondary antibodies (Invitrogen) and 4',6-diamidino-2-phenylindole (DAPI, Sigma), and imaged using a Zeiss LSM800 confocal microscope (Carl Zeiss, Jena, Germany). The detailed information for antibodies used for western blotting analysis is provided in Supplementary Table S1.

**Supplementary References**

Supplementary Figure S1. The conditional deletion of miR-21 disturbs normal hematopoiesis in mice. (A) Flow cytometric gating strategies for identifying Lin− cells, MP (Lin− Sca1− c-Kit+), LSK (Lin− Sca1+ c-Kit+), LT-HSC (Lin− Sca1+ c-Kit+ CD34− Flk2−), ST-HSC (Lin− Sca1+ c-Kit+ CD34+ Flk2+) and MPP (Lin− Sca1+ c-Kit+ CD34− Flk2−) in the BM. (B) Flow cytometric gating strategies for identifying CMP (Lin− CD127− Sca1− c-Kit+ CD16/32− CD34+), GMP (Lin− CD127− Sca1− c-Kit+ CD16/32− CD34+), MEP (Lin− CD127− Sca1− c-Kit+ CD16/32− CD34+) and CLP (Lin−
CD127+ Sca1+ c-Kit+ in the BM. (C) Genotyping for the conditional deletion of miR-21. Primers P1 and P2 were used to amplify wild-type (WT) band (758 bp), floxed band (982 bp) and deleted band (480 bp). (D) QRT-PCR analysis of miR-21 expression in the BM and spleen (Sp) from miR-21fl/fl and miR-21Δ/Δ mice (n = 3 mice per group). (E) The counts of white blood cell (WBC), red blood cell (RBC) and platelet (PLT) in the peripheral blood (PB) of miR-21fl/fl and miR-21Δ/Δ mice (n = 8 mice per group). (F) BM and spleen (Sp) cell numbers in miR-21fl/fl and miR-21Δ/Δ mice (n = 6 mice per group). (G) Flow cytometric analysis of the percentages of T cells (CD3e+), B cells (B220+) and myeloid cells (Gr-1+ and Mac-1+) in the PB of miR-21fl/fl and miR-21Δ/Δ mice (n = 6 mice per group). All data are shown as means ± SD. **P < 0.01.
Supplementary Figure S2

**Supplementary Figure S2.** Loss of miR-21 leads to an obvious increase in HSC percentage in the BM but not the peripheral blood of mice. (A) Flow cytometric analysis of the percentage of CD150⁺ CD48⁻ LSKs in miR-21⁺⁻ and miR-21Δ/Δ BM (n = 6 mice per group). (B) Flow cytometric analysis of the percentage of LSKs in the peripheral blood (PB) of miR-21⁺⁻ and miR-21Δ/Δ mice (n = 6 mice per group). All data are shown as means ± SD. **P < 0.01.
Supplementary Figure S3. The effects of miR-21 deficiency on HSPC apoptosis, quiescence and proliferation. (A) Flow cytometric analysis of the percentage of apoptotic cells (Annexin V+ and 7-AAD−) in LT-HSC, ST-HSC and MPP from the BM of miR-21fl/fl and miR-21Δ/Δ mice (n = 6 mice per group). (B) Cell cycle analysis of ST-HSC, MPP and MP in the BM of miR-21fl/fl and miR-21Δ/Δ mice (n = 6 mice per group). (C) Flow cytometric analysis of the BrdU incorporation in ST-HSC, MPP and MP from miR-21fl/fl and miR-21Δ/Δ BM (n = 6 mice per group). All data are shown as means ± SD. **P < 0.01.
Supplementary Figure S4. miR-21 deficiency intrinsically compromises HSC function in long-term reconstitution. (A, B) The chimerism of LSKs in CD45.1+ recipients’ BM at 16 weeks after (A) primary and (B) secondary competitive BMT (n = 8 mice per group). (C-E) CD45.1+ BM cells were transplanted into 10.0 Gy-irradiated CD45.2+ miR-21^{fl/fl} or miR-21^{Δ/Δ} recipients. At 16 weeks after transplantation, the (D) percentage and (E) lineage distribution of CD45.1+ donor-derived cells in the PB of miR-21^{fl/fl} and miR-21^{Δ/Δ} recipients were analyzed by flow cytometry (n = 8 mice per group). The schematic diagram of reciprocal BMT assay is shown in the left panel. All data are shown as means ± SD. **P < 0.01.
Supplementary Figure S5

Supplementary Figure S5. Loss of miR-21 results in increased expression of myeloid differentiation-associated genes in HSCs. QRT-PCR analysis of the relative expression of myeloid differentiation-associated genes (including Mpo, Fcgr3, Csf1r, Igsf6, Ly6c1 and Ms4a3) in LT-HSCs from miR-21^{f/f} and miR-21^{Δ/Δ} BM (n = 3 mice per group). Data are showed as mean ± SD. All data are shown as means ± SD. **P < 0.01.
Supplementary Figure S6. The upregulation of PDCD4 is responsible for the defects in miR-21-null HSCs. (A) Western blot analysis of the expression of PTEN, Spry1 and Spry2 in miR-21^{fl/fl} and miR-21^{Δ/Δ} LSKs (n = 5 mice per group). (B) 293T cells were co-transfected with WT-PDCD4 (3’UTR) or MUT-PDCD4 (3’UTR) and miR-21 or miR-NC, and then the relative luciferase activity was detected. (C) QRT-PCR analysis of the relative PDCD4 expression in LSKs and BM cells from normal 8-week-old WT mice (n = 4 mice per group). (D) Schematic diagram of lentiviral transduction of PDCD4 and competitive transplantation. (E) The lineage distribution of CD45.2^{+} donor-derived cells in recipients’ PB 12 weeks after PDCD4 overexpression and competitive transplantation (n = 6 mice per group). (F) QRT-PCR
analysis of the relative PDCD4 expression in miR-21^{Δ/Δ} LSKs after transduced with control or sh-PDCD4 (n = 4 mice per group). (G-I) CD45.2^{+} miR-21^{0/0} or miR-21^{Δ/Δ} LSKs transduced with control or sh-PDCD4, mixed with CD45.1^{+} competitor BM cells, were transplanted into 10.0 Gy-irradiated CD45.1^{+} recipients. At 12 weeks after transplantation, the expression of (G) PDCD4 and (H) p-p65 in CD45.2^{+} donor-derived LT-HSCs from recipients’ BM, and (I) the lineage distribution of CD45.2^{+} donor-derived cells in recipients’ PB were detected by flow cytometry (n = 6 mice per group). All data are shown as means ± SD. *P < 0.05, **P < 0.01.
Supplementary Figure S7. miR-21 is involved in protecting HSCs from irradiation-induced damage. (A) Flow cytometric analysis of the percentage of apoptotic cells (Annexin V+ and 7-AAD-) in LSKs from the BM of miR-21^fl/fl and miR-21^Δ/Δ mice 24 hours after 6.0 Gy TBI (n = 6 mice per group). (B) WBC count in the PB of miR-21^fl/fl and miR-21^Δ/Δ mice after 4.5 Gy TBI (n = 6 mice per group). (C) Normal WT mice were treated with 3 doses of miR-21 agomir or negative control (NC) on consecutive days by tail intravenous injection. Twenty-one hours after last injection, mice were intraperitoneally injected with one dose of QNZ or control. Three hours later, mice were subjected to 6.0 Gy TBI. The percentage of apoptotic cells in LSKs from these mice was detected 24 hours after irradiation by flow cytometry (n = 6 mice per group). (D) The relative expression of miR-21 in LSKs from normal WT mice at 0, 6 and 24 hours after 4.5 Gy TBI (n = 4 mice per group). The relative miR-21 expression was compared with that in 0 hour group. All data are shown as means ± SD. *P < 0.05, **P < 0.01.
**Supplementary Table S1**

 Antibodies used in flow cytometry, immunofluorescence and western blotting

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FC, flow cytometry; WB, western blot; IF, immunofluorescence.
### Supplementary Table S2

**Primers for mRNA expression analysis**

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Supplementary Table S3

The differentially expressed genes between miR-21^{fl/fl} and miR-21^{Δ/Δ} LSKs (uploaded as separate excel file).