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MMP9 inhibition increases erythropoiesis in RPS14-deficient del(5q) MDS models through suppression of TGF-β pathways

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Key Points

- MMP9 inhibition improves erythropoiesis in RPS14-deficient del(5q) MDS models.
- Increased MMP9 expression in RPS14-deficient cells activates TGF-β signaling, resulting in defective erythroid developments.

The del(5q) myelodysplastic syndrome (MDS) is a distinct subtype of MDS, associated with deletion of the ribosomal protein S14 (*RPS14*) gene that results in macrocytic anemia. This study sought to identify novel targets for the treatment of patients with del(5q) MDS by performing an in vivo drug screen using an rps14-deficient zebrafish model. From this, we identified the secreted gelatinase matrix metalloproteinase 9 (MMP9). MMP9 inhibitors significantly improved the erythroid defect in rps14-deficient zebrafish. Similarly, treatment with MMP9 inhibitors increased the number of colony forming unit-erythroid colonies and the $CD71^+$ erythroid population from RPS14 knockdown human $BMCD34^+$ cells. Importantly, we found that MMP9 expression is upregulated in RPS14-deficient cells by monocyte chemoattractant protein 1. Double knockdown of MMP9 and RPS14 increased the $CD71^+$ population compared with RPS14 single knockdown, suggesting that increased expression of MMP9 contributes to the erythroid defect observed in RPS14-deficient cells. In addition, transforming growth factor β (TGF-β) signaling is activated in RPS14 knockdown cells, and treatment with SB431542, a TGF-β inhibitor, improved the defective erythroid development of RPS14-deficient models. We found that recombinant MMP9 treatment decreases the $CD71^+$ population through increased SMAD2/3 phosphorylation, suggesting that MMP9 directly activates TGF-β signaling in RPS14-deficient cells. Finally, we confirmed that MMP9 inhibitors reduce SMAD2/3 phosphorylation in RPS14-deficient cells to rescue the erythroid defect. In summary, these study results support a novel role for MMP9 in the pathogenesis of del(5q) MDS and the potential for the clinical use of MMP9 inhibitors in the treatment of patients with del(5q) MDS.

Introduction

The del(5q) myelodysplastic syndrome (MDS) is a distinct subtype of MDS, associated with the deletion of chromosome 5q, and leading to macrocytic anemia, normal or high platelet count with hypolobulated micromegakaryocytes, and risk of progression to acute myelogenous leukemia (AML).[1-4] A commonly deleted region of 5q has been narrowed to a 1.5-Mb locus on chromosome 5 and contains ~40 protein-coding genes, including ribosomal protein S14 (*RPS14*). Small hairpin RNAs (shRNAs) targeting *RPS14* cause a severe erythroid defect, whereas overexpression of RPS14 in del(5q) MDS patient cells rescues erythropoiesis.[5,6] Thus, although haploinsufficiency of genes in the commonly deleted region has been related to the hematological phenotype,[7-10] the deletion of *RPS14* is a key determinant of severe macrocytic anemia in del(5q) MDS.[5]

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For original data, please contact kmsakamo@stanford.edu.

The full-text version of this article contains a data supplement.

Matrix metalloproteinases (MMPs) are a group of extracellular matrix proteolytic enzymes that have been implicated in various pathological conditions, including cancer, tumor invasion, and metastasis.[11,12] MMPs can promote the epithelial–mesenchymal transition by proteolytic activation of latent transforming growth factor β (TGF-β).[13-15] The secreted gelatinase MMP9 was the first implicated in hematopoietic stem cell mobilization through cleavage of hematopoietic stem cell–maintaining factors such as cytokines responsible for stem cell retention.[16-18] However, the literature is inconsistent regarding the role of MMP9 in hematopoietic stem cells.[16,19-21]

Lenalidomide is approved by the US Food and Drug Administration and the first targeted therapy for the treatment of lower risk, transfusion-dependent del(5q) MDS. Lenalidomide promotes p53 degradation by stabilizing the mouse double minute 2 protein.[22-24] p53-dependent apoptosis of del(5q) erythroid progenitors is a key factor in the molecular pathogenesis of del(5q) MDS.[25-29] However, although p53 degradation by lenalidomide treatment is a highly effective therapy to restore effective erythropoiesis in patients with del(5q) MDS, ~50% of patients acquire resistance to lenalidomide within 2 to 3 years.[22] In addition, some patients with p53 mutations do not achieve a complete cytogenetic response to lenalidomide.[30-32]

Given the limitations of lenalidomide treatment, we sought to identify novel targets and possible therapeutic approaches for the treatment of patients with del(5q) MDS by performing an in vivo drug screen using a zebrafish model of MDS. We generated a stable genetic mutation of zebrafish rps14 using CRISPR-Cas9 gene targeting. This zebrafish model mirrors the anemic phenotype observed in patients with del(5q) MDS. The current study reports that MMP9 inhibitors improve the erythroid defect observed in RPS14-deficient del(5q) human hematopoietic stem/progenitor cells and in zebrafish. We found that MMP9 is upregulated in RPS14 knockdown cells and that increased expression of MMP9 activates TGF-β signaling, resulting in defective erythroid development. Our results also show that MMP9 inhibitors reduce the activated TGF-β signaling, resulting in enhanced erythropoiesis. Taken together, these results suggest a novel molecular pathway in the pathogenesis of del(5q) MDS and the potential use of MMP9 inhibitors to treat patients with del(5q) MDS.

Methods

Zebrafish studies

All zebrafish experiments were approved by the Institutional Animal Care and Use Committee at the University of California Los Angeles. The generation and characterization of rps14-deficient zebrafish were previously described.[29] The rps14-deficient zebrafish exhibited an anemic phenotype during development, enabling in vivo assay of small molecules that can improve the phenotype. To identify compounds that alleviate the anemia in this model, we collected embryos from mating heterozygous adults of rps14$^{+/-}$ and placed ~16 embryos in each well of 48-well plates at the tail bud stage (supplemental Figure 1A). Individual wells contained 10 μM of starting compounds from the LOPAC 1280 (MilliporeSigma) or our in-house compound libraries of known bioactive drugs. The LOPAC library contains 1280 bioactive small molecules of pharmacologically active compounds, and our in-house libraries collected commercially available compounds that have defined activities in pathways such as wnt, shh (sonic hedgehog), fgf (fibroblast growth factor), tgf (transforming growth factor), mmp (matrix metalloproteinases), and others. At 2 days postfertilization, the embryos were stained with *o*-dianisidine to assess hemoglobin signal.[29] Using L-leucine and dexamethasone as positive controls, new positive compounds were defined as those that exhibited notably improved hemoglobin levels in morphologically recognizable mutant embryos, in duplicate, followed by genotype confirmation (supplemental Figure 1C). Without drug treatment, approximately one-fourth of 16 embryos would exhibit visible defective erythropoiesis. When a positive hit was identified during the screen, the number of embryos with defective erythropoiesis was clearly less than one-fourth. Primary hits were further validated with commercial compounds, and MMP9 inhibitor I (MMP9-I, 444278) and MMP9 inhibitor II (MMP9-II, 444293) were purchased from MilliporeSigma and dissolved in dimethyl sulfoxide (DMSO). SB431542 (S1067) was purchased from Selleck Chemicals and dissolved in DMSO. Zebrafish embryos were treated with different concentrations of MMP9 inhibitors and SB431542, and dose titrations were determined that ranged from 2 to 20 μM for MMP9 inhibitors and SB431542.

To determine expression of *mmp9* by quantitative reverse transcription polymerase chain reaction (RT-qPCR), adult heterozygous rps14$^{+/-}$ zebrafish were mated to generate embryos. Homozygous mutant embryos were identified as those showing smaller eyes, slight necrosis in the brain and cardiac edema, and were separated from rps14$^{+/+}$ and rps14$^{+/-}$ siblings at 48 hours postfertilization. The accuracy of mutant embryos was also confirmed by using genotyping. For each group, total RNA was prepared at 48 hours postfertilization, and RT-qPCR analysis was performed on Stratagene Mx3005p (SYBR Green, Agilent Technologies) by using FastStart Universal SYBR Green (MilliporeSigma, as instructed). Specific primers used to detect zebrafish *mmp9* by using *β-actin* are presented in supplemental Table 5.

Cell culture

Primary human CD34^{+} hematopoietic stem/progenitor cells were purified from bone marrow aspirates purchased from Allcells and Stemcell Technologies. CD34^{+} cells were purified by using MACS cell separation (Miltenyi Biotec) and cryopreserved. Upon thawing, cells were cultured in x-Vivo 15 medium (Lonza) containing 10% fetal bovine serum, 1× penicillin-streptomycin-glutamine (Thermo Fisher Scientific), FLT-3 (Miltenyi Biotec), thrombopoietin (Miltenyi Biotec), interleukin-3 (Miltenyi Biotec), interleukin-6 (Miltenyi Biotec), granulocyte-macrophage colony-stimulating factor (Miltenyi Biotec), stem cell factor (Miltenyi Biotec), and erythropoietin according to a liquid culture system. Bone marrow from patients with del(5q) MDS was collected through voluntary patient participation at Stanford University in compliance with the institutional review board regulations. Informed consent was obtained from all human subjects in accordance with the Declaration of Helsinki and the Data Protection Directive. HEK 293 cell lines were cultured in Iscove modified Dulbecco medium (Corning) containing 10% fetal bovine serum and 1× penicillin-streptomycin-glutamine.

Colony assays

Hematopoietic cells sorted per GFP+ or mCherry+ were seeded in methylcellulose medium containing only erythropoietin (H4330, Stemcell Technologies), in triplicate, with a density of 1500 to 4000 cells per plate. Colony forming unit-erythroid (CFU-E) colonies were counted 1 week later by an investigator blinded to the conditions.

Fluorescence-activated cell sorter analysis

For cell surface flow cytometry, cells were washed with phosphate-buffered saline and then incubated with indicated antibodies for 20 minutes on ice. After washing, cells were analyzed by using fluorescence-activated cell sorting (FACS).

For intracellular flow cytometry, cells were incubated with GolgiStop/GolgiPlug (BD Biosciences). After 12 hours, cells were fixed in 3.2% paraformaldehyde for 15 minutes on ice and permeabilized with permeabilization solution (BD Biosciences) for 20 minutes on ice. Cells were then incubated with the indicated antibodies. After washing, cells were analyzed by using FACS. Data were collected on a DxP10 (Cytek Development) flow cytometer and analyzed by using FlowJo Software (version 10). All antibodies are listed in the supplemental Methods.

Enzyme-linked immunosorbent assay

Enzyme-linked immunosorbent assay (ELISA) kits for MMP9 (RAB0372) and TFG-β (RAB0460) were purchased from MilliporeSigma. Cell culture media were diluted by 1 to 750 for MMP9 or 1 to 4 for TGF-β. The ELISA kit for monocyte chemoattractant protein 1 (MCP1; KHC1011) was purchased from Invitrogen. Cell culture media were diluted by 1 to 4 for MCP1. MMP9, TGF-β, and MCP1 levels were measured according to the manufacturer's instruction.

Drug treatment

MMP9-I (MilliporeSigma, 444278) and MMP9-II (MilliporeSigma, 444293) were dissolved in DMSO. Cells were treated according to indicated concentrations of each inhibitor for 6 days. SB431542 (S1067) was dissolved in DMSO. Cells were treated by either 0.5 or 2 μM for 4 days. Recombinant MMP9 (911-MP) was purchased from R&D Systems and dissolved in assay buffer (50 nM Tris, 10 mM $CaCl_2$, 150 mM NaCl, 0.05% Brij-35, pH 7.5). Before treatment, recombinant MMP9 (rMMP9) was activated by adding *p*-aminophenylmercuric acetate to a final concentration of 1 mM and incubating for 24 hours at 37°C. rMMP9 activity was confirmed by performing gelatin zymography (supplemental Figure 2). Cells were treated according to the indicated concentrations of activated rMMP9 for 2 or 4 days. Recombinant MCP1 (rMCP1, 279-MC) was purchased from R&D Systems and dissolved in phosphate-buffered saline containing 0.1% bovine serum albumin. Cells were treated according to the indicated concentrations of rMCP1 for 4 days.

Statistical analysis

Data are presented as mean ± standard deviation. *P* values for statistical significance were obtained by using an unpaired Student *t* test or analysis of variance test (Tukey's multiple comparison). $P < .05$ was considered significant. The data are representative of at least 2 independent experiments.

Results

MMP9 inhibitors increase the erythroid development in RPS14-deficient models

To identify novel targets for the treatment of patients with del(5q) MDS, we performed an in vivo drug screening assay using an rps14-deficient zebrafish model. We previously generated a stable genetic mutation in the zebrafish *rps14* using CRISPR-Cas9 gene targeting and showed that this zebrafish model mirrors the anemic phenotype seen in the del(5q) MDS.[29] Approximately 500 compounds were tested, and among the positive hits, we found that inhibitors for MMP9, MMP13, and compound SB431542 (a TGF-β inhibitor) significantly rescued the erythroid defect observed in rps14-deficient zebrafish (Figure 1A; supplemental Figure 1B). The finding suggested that abnormal activation of MMP and TGF-β pathways are causing the erythroid deficiency in our model. To confirm a role for these pathways in human cells, these inhibitors were tested in an in vitro human del(5q) MDS model (Figure 1B-C; supplemental Figures 4-7). Because expression of the *MMP13* gene was not detectable in our human bone marrow cells, we focused on analysis of the MMP9 and TGF-β inhibitors.

We first optimized the concentration of each MMP9 inhibitor for treatment of human bone marrow $CD34^+$ ($BMCD34^+$) cells. We determined that 5 nM/1 μM for MMP9-I and 10 nM/10 μM for MMP9-II are sufficient to observe an effect (supplemental Figure 3). We next examined the effects of the MMP9 inhibitors in an in vitro human del(5q) MDS model. RPS14 knockdown $BMCD34^+$ cells were established by transducing lentivirus expressing RPS14-GFP shRNAs, achieving ~50% knockdown efficiency. Haploinsufficient expression of *RPS14* was confirmed by using RT-qPCR and western blot analysis (supplemental Figure 4). One day after RPS14 knockdown, we added the MMP9 inhibitors; after 6 days, erythroid development was evaluated by performing colony assay and FACS. Treatment with the MMP9 inhibitors at both low and high concentrations was found to significantly increase the number of erythroid colonies in RPS14 knockdown cells compared with DMSO control cells (Figure 1B). Similarly, FACS showed increased $CD71^+$ or $CD235a^+$ erythroid populations in RPS14 knockdown cells after treatment with the MMP9 inhibitors but no change in the $CD11b^+$ myeloid population ($P < .001$; n = 4) (Figure 1C; supplemental Figures 5-7). These data suggest that MMP9 inhibitors have significant effects on erythroid development in RPS14-deficient cells.

MMP9 is upregulated in RPS14-deficient cells, resulting in a decreased erythroid population

To understand the mechanism by which the MMP9 inhibitors affect erythroid development, MMP9 expression was examined in both the human and zebrafish RPS14-deficient models, as well as in $CD34^+$ cells from low-risk del(5q) MDS patients. After transduction of human $BMCD34^+$ cells with lentivirus expressing RPS14 shRNAs, GFP+ cells were sorted and MMP9 levels were analyzed according to RT-qPCR and western blot analysis. MMP9 expression levels were significantly increased in RPS14 knockdown cells compared with control cells ($P < .001$; n = 4) (Figure 2A-B). Similarly, mmp9 mRNA was increased ~twofold in rps14-deficient zebrafish (Figure 2C). In addition, we observed a 350- to 700-fold increase in *MMP9* expression in bone marrow $CD34^+$ cells from low-risk del(5q) MDS patients who had not received lenalidomide compared with healthy control subjects ($P < .0003$, n = 3 or 7) (Figure 2D).

Activated MMP9 protein is secreted and plays a role in the degradation of the extracellular matrix.[11,12] To determine whether the increased MMP9 protein in RPS14-deficient cells is functionally active, RPS14 knockdown cells were cultured for an additional 2 days after sorting cells for GFP+. We collected the culture media to examine the secreted MMP9 level according to ELISA. Similar to the increased intracellular MMP9 observed according to western blot analysis, we also found that secreted extracellular MMP9 was

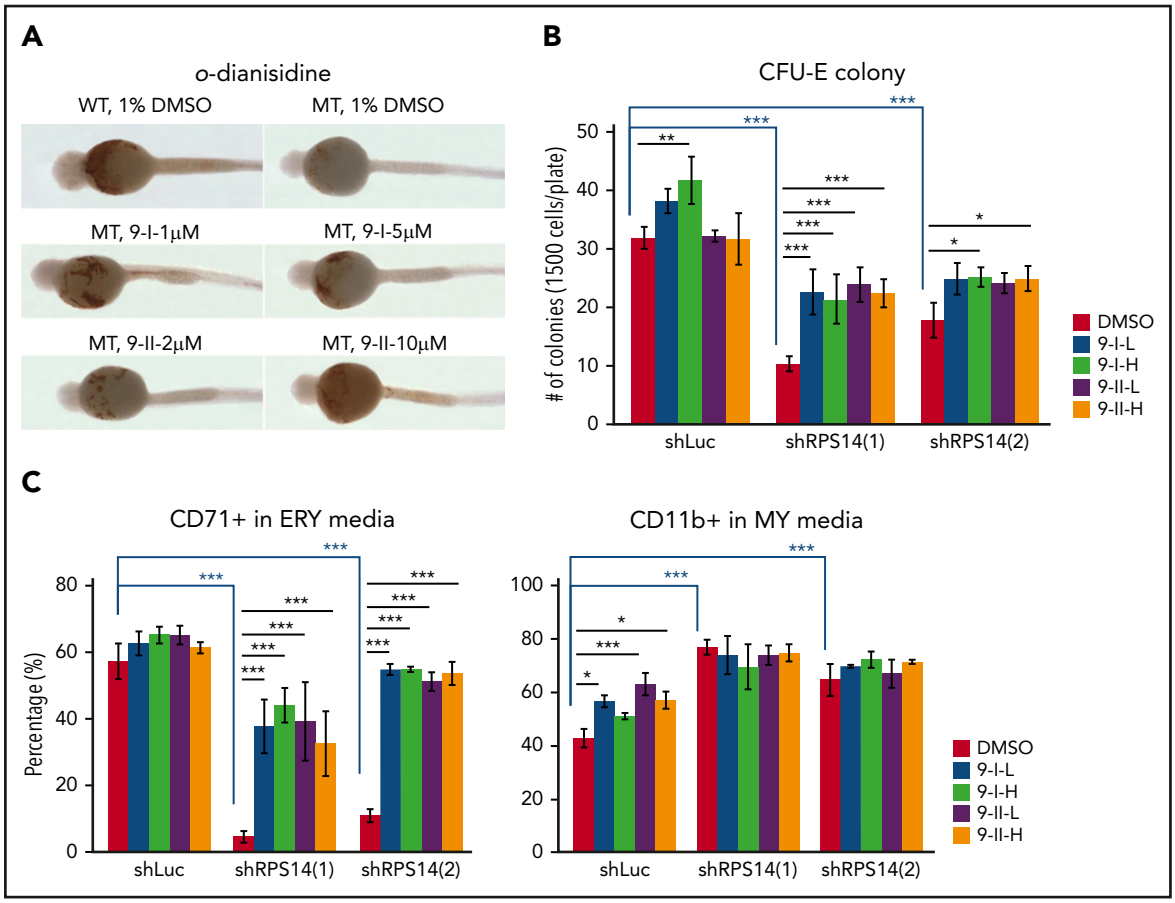
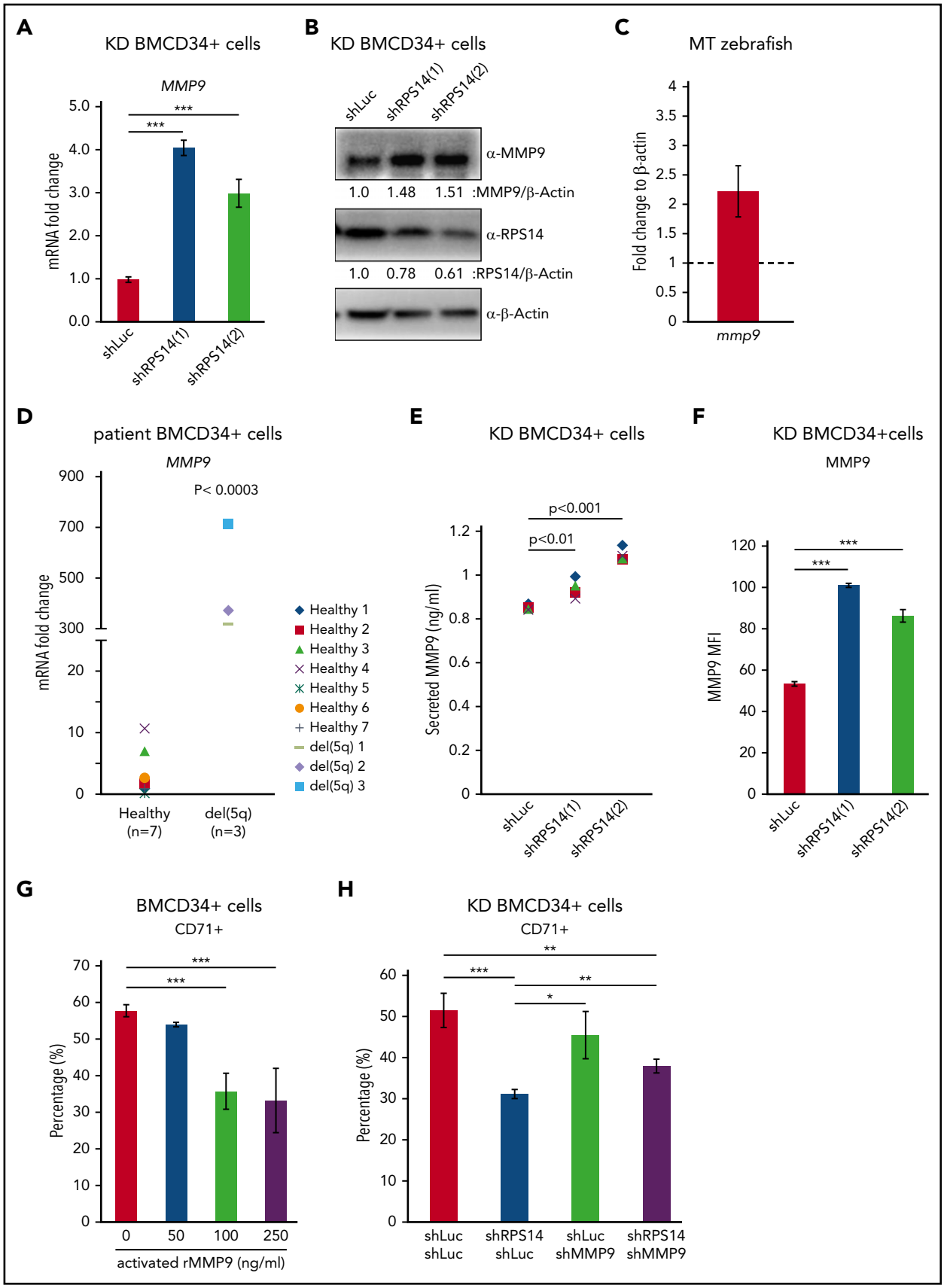


Figure 1. MMP9 inhibitors increase the erythroid development in RPS14-deficient models. (A) Wild-type (WT) embryos stained with *o*-dianisidine showed strong brown signal on the yolk sac, indicating normal hemoglobin levels. rps14$^{-/-}$ mutant embryos (MT) stained with *o*-dianisidine showed weak brown signal on the yolk sac, indicating reduced hemoglobin levels. MT treated with different concentrations of mmp9 inhibitors showed improved staining signal of hemoglobin compared with DMSO-treated rps14$^{-/-}$ MT. All embryos are ventral views. (B-C) Human BMCD34^{+} cells were transduced with lentivirus carrying shRNAs against RPS14 or Luc control. After 1 day of transduction, cells were treated by MMP9 inhibitors. 9-I-L, MMP9-I 5 nM; 9-I-H, MMP9-I 1 μM; 9-II-L, MMP9-II 10 nM; 9-II-H, MMP9-II 10 μM. (B) Cells were sorted for GFP+ at 4 days after treatment. A total of 1500 GFP+ cells were plated in methylcellulose media specific for CFU-E colonies and cultured for 1 week. Colonies were counted by an investigator blinded to the conditions. (C) Cells were analyzed for the CD71 and the CD11b expression by using flow cytometry at 6 days after treatment with MMP9 inhibitors. Data are representative of 3 independent transduction experiments. *$P < .05$, **$P < .01$, ***$P < .001$. ERY media, erythroid media; MY media, myeloid media.

increased in RPS14 knockdown cells (Figure 2E). Furthermore, we observed the total cellular MMP9 level by treating cells with protein transport inhibitors, which block MMP9 secretion. Consistent with our previous assays, a nearly twofold increase was observed in MMP9 protein level in RPS14-deficient cells compared with control cells according to FACS ($P < .001$; n = 4) (Figure 2F).

To determine the specificity of MMP9 inhibitors in effecting erythroid development in RPS14 knockdown cells, RPS19 or RPL11 knockdown cells were tested.[33,34] We observed no increase in the CD71^{+} erythroid population in either group of knockdown cells after treatment of MMP9 inhibitors (supplemental Figure 8). Similarly, MMP9 expression was not significantly increased in RPS19 or RPL11 knockdown cells (supplemental Figure 9). These results suggest that the effect of MMP9 inhibitors is specific to RPS14-deficient del(5q) MDS cells.

To determine the potential role of MMP9 during erythroid development, we examined the MMP9 effect by treating hematopoietic progenitor cells with recombinant MMP9 protein (rMMP9). Human BMCD34^{+} cells were treated by using activated rMMP9 for 2 days, and then erythropoiesis was examined according to FACS. Our results showed that rMMP9 treatment decreased the CD71^{+} or CD235a^{+} erythroid populations by 40% compared with the non-treatment control ($P < .001$; n = 4) (Figure 2G; supplemental Figures 10 and 11). To determine whether increased MMP9 expression is required for the reduced erythroid development in RPS14 knockdown cells, a double knockdown experiment using shRNAs targeting MMP9 and RPS14 was performed. We first transduced a lentivirus expressing MMP9-mCherry shRNA into BMCD34^{+} cells, and confirmed 80% downregulation of MMP9 mRNA by using RT-qPCR (supplemental Figure 12). We then cotransduced lentivirus expressing MMP9 and RPS14 shRNAs and examined erythropoiesis by using FACS. Our results showed that knockdown of both MMP9 and RPS14 partially rescued the decreased CD71^{+} or CD235a^{+} populations in RPS14 knockdown cells ($P < .01$; n = 4) (Figure 2H; supplemental Figures 13 and 14). In contrast, MMP9 knockdown alone did not affect erythroid development.


A
KD BMCD34+ cells
MMP9
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5.0
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shLuc
shRPS14(1)
shRPS14(2)
B
KD BMCD34+ cells
shLuc
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α-MMP9
1.0 1.48 1.51 :MMP9/β-Actin
α-RPS14
1.0 0.78 0.61 :RPS14/β-Actin
α-β-Actin
C
MT zebrafish
Fold change to β-actin
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3.5
3
2.5
2
1.5
1
0.5
0
mmp9
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MMP9
P< 0.0003
mRNA fold change
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700
500
300
20
10
0
Healthy
(n=7)
del(5q)
(n=3)
Healthy 1
Healthy 2
Healthy 3
Healthy 4
Healthy 5
Healthy 6
Healthy 7
del(5q) 1
del(5q) 2
del(5q) 3
E
KD BMCD34+ cells
p<0.001
p<0.01
Secreted MMP9 (ng/ml)
1.2
1
0.8
0.6
0.4
0.2
0
shLuc
shRPS14(1)
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120
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shLuc
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BMCD34+ cells
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Percentage (%)
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Figure 2.

Taken together, our results suggest that the increased expression of MMP9 in RPS14-deficient cells contributes to the erythroid defect observed in these cells.

MCP1 regulates MMP9 expression in RPS14-deficient cells

Previous research in human aortic smooth muscle cells showed that MCP1 stimulates MMP9 expression.[35] Thus, we investigated a role for MCP1 in regulating MMP9 expression in RPS14-deficient BMCD34$^+$ cells. Because inflammatory signaling is dysregulated in del(5q) MDS, we first analyzed MCP1 expression in RPS14 knockdown cells. After 5 days of transduction with lentivirus expressing RPS14 shRNAs, we sorted cells for GFP+ and analyzed MCP1 mRNA expression by using RT-qPCR. MCP1 mRNA expression was significantly increased in RPS14 knockdown cells compared with control BMCD34$^+$ cells not expressing shRPS14 ($P < .05$; n = 4) (Figure 3A). In addition, increased MCP1 protein was observed in RPS14 knockdown cells by using flow cytometry ($P < .05$; n = 4) and ELISA ($P < .04$; n = 3) (Figure 3B-C). As was the case for MMP9 expression, the elevated MCP1 expression was only apparent in RPS14 knockdown cells, not RPS19 or RPL11 knockdown cells (supplemental Figure 15).

We tested if MCP1 regulates MMP9 expression by treating normal BMCD34$^+$ cells with recombinant MCP1 (rMCP1) for 4 days and then measured MMP9 levels by using RT-qPCR and flow cytometry. The data showed that both RNA and protein levels of MMP9 are dose dependently increased with rMCP1 treatment ($P < .01$; n = 4) (Figure 3D-E). We next examined whether MCP1 regulates MMP9 expression in RPS14-deficient cells. A double knockdown experiment was performed with MCP1 and RPS14 shRNAs. After transfecting the lentiviral vector of MCP1-mCherry shRNA to HEK 293 cells, downregulation of *MCP1* expression by 80% was confirmed by using RT-qPCR (supplemental Figure 16). We then cotransduced lentivirus expressing MCP1 and RPS14 shRNAs and examined MMP9 expression by using flow cytometry. Our results showed that double knockdown of both MCP1 and RPS14 expression significantly reduced MMP9 expression in RPS14 knockdown cells, especially in the CD71$^+$ erythroid population ($P < .05$; n = 3) (Figure 3F).

Taken together, our results suggest that MCP1 is required for the increased MMP9 expression in RPS14-deficient cells.

MMP9 function in RPS14-deficient cells is independent of the p53 pathway

It has been well established that haploinsufficiency of some ribosomal proteins leads to p53 activation and subsequent erythroid defects during hematopoiesis.[27,36,37] We have also observed that our RPS14-deficient del(5q) MDS cells exhibit the increased p53-dependent apoptosis and erythroid defects. This scenario suggests that p53 activation may also be involved in MMP9 function in our model cells. To test this theory, we first examined MMP9 expression in cells in which p53 was knocked down by shRNA. A lentivirus expressing p53-mCherry shRNA to BMCD34$^+$ cells decreased p53 mRNA expression by 90% (supplemental Figure 17A).[34,38] We then cotransduced lentivirus expressing p53 and RPS14 shRNAs and examined MMP9 level by using flow cytometry. The increased expression of MMP9 in RPS14 knockdown cells was unaffected by double knockdown of both p53 and RPS14 (supplemental Figure 17B). These data suggest that p53 activation does not affect MMP9 expression in RPS14-deficient cells.

We next tested if MMP9 inhibitors suppress apoptosis in RPS14-deficient cells. We treated RPS14 knockdown cells with MMP9 inhibitors for 6 days and then analyzed apoptosis by staining cells with Annexin V. We observed the increased Annexin V+ cells in RPS14 knockdown cells compared with control cells. However, treatment with MMP9 inhibitors did not affect the percentage of Annexin V+ cells, suggesting MMP9 inhibitors do not affect apoptotic pathways in RPS14 knockdown cells (supplemental Figure 18).

Taken together, these data suggest that MMP9 function in RPS14-deficient cells is p53 independent.

Inhibition of TGF-β signaling increases erythroid development in RPS14-deficient models

TGF-β is an important physiological regulator of cell proliferation and differentiation. Recent reports suggest that inhibition of TGF-β promotes hematopoiesis in samples from patients with MDS.[39-43] In addition, it is known that MMP9 activates latent TGF-β through proteolytic cleavage, resulting in signaling activation.[14,15] These findings suggest that increased MMP9 in RPS14 knockdown cells may activate TGF-β signaling, resulting in defective erythroid development. To test this hypothesis, we first examined whether TGF-β signaling is activated in our RPS14-deficient models. Human BMCD34$^+$ cells were transduced with lentivirus expressing RPS14 shRNA. RPS14 knockdown cells were cultured for an additional 2 days after sorting cells for GFP+, and the culture media were collected to detect secreted TGF-β levels by using ELISA. Interestingly, significantly increased TGF-β levels were found in RPS14 knockdown cells compared with control cells ($P < .001$; n = 3 or 4) (Figure 4A). To verify that increased TGF-β activates downstream signaling, SMAD2/3 phosphorylation (pSMAD2/3) was examined. Total SMAD2/3 protein level was not changed, but a significant increase in pSMAD2/3 was observed, indicating that TGF-β signaling is activated in RPS14 knockdown cells (Figure 4B).

Figure 2. MMP9 is upregulated in RPS14-deficient cells resulting in decreased erythroid populations. Human BMCD34$^+$ cells were transduced with lentivirus carrying shRNAs against RPS14 or Luc control. (A) After 5 days of transduction, cells were sorted for GFP+. RNA was collected and analyzed by using RT-qPCR. (B) Protein was collected from sorted cells and analyzed by using western blot analysis. β-Actin was used as a loading control. (C) RNA was collected from rps14-deficient and wild-type zebrafish embryos and analyzed by using RT-qPCR. (D) RNA was isolated from bone marrow CD34$^+$ cells from patients with del(5q) MDS or healthy control subjects and analyzed by using RT-qPCR. (E) GFP+-sorted cells were cultured for an additional 2 days, and culture media were collected for an MMP9 ELISA. (F) After 7 days of transduction, cells were treated by using GolgiPlug and GolgiStop for 12 hours. Cells were stained with MMP9 antibody and analyzed according to flow cytometry. (G) Human BMCD34$^+$ cells were cultured in liquid culture media. After 5 days of culture, cells were treated by rMMP9 for 2 days and analyzed for the CD71 expression by using flow cytometry. (H) Human BMCD34$^+$ cells were transduced with lentivirus carrying shRNAs against RPS14 and MMP9. After 7 days of transduction, cells were analyzed for the CD71 expression by using flow cytometry. Data are representative of 3 independent transduction experiments. *$P < .05$, **$P < .01$, ***$P < .001$.

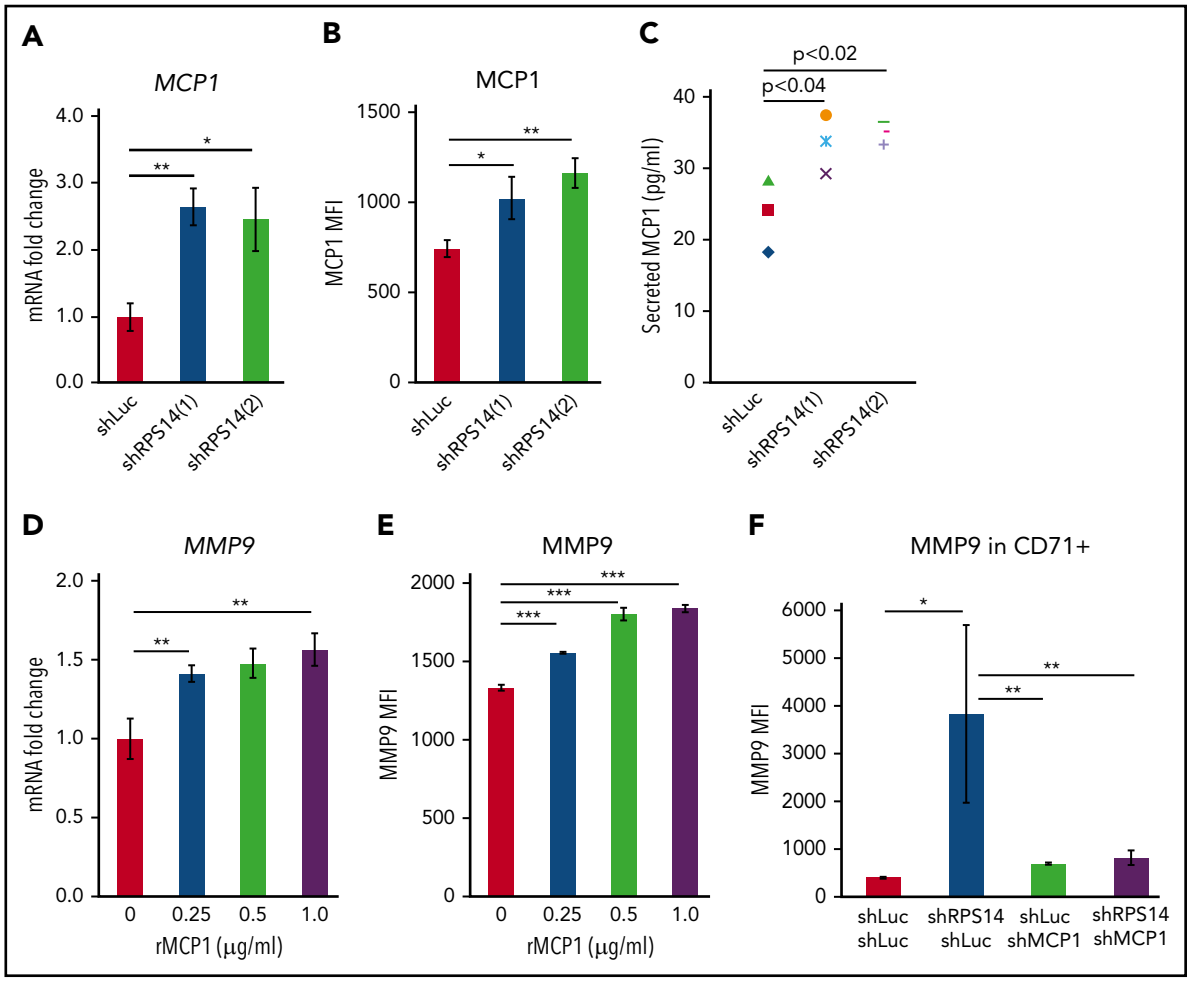

Figure 3. MCP1 regulates MMP9 expression in RPS14-deficient cells. Human BMCD34$^+$ cells were transduced with lentivirus carrying shRNAs against RPS14 or Luc control. (A) After 5 days of transduction, cells were sorted for GFP+. RNA was collected and analyzed by using RT-qPCR. (B) After 5 days of transduction, cells were stained with MCP1 antibody and analyzed by using flow cytometry. (C) Cells sorted per GFP+ were cultured for an additional 2 days, and culture media were collected for an MCP1 ELISA. (D) Human BMCD34$^+$ cells were cultured in liquid culture media. After 1 day of culture, cells were treated by rMCP1 for 4 days. RNA was collected and analyzed by using RT-qPCR. (E) Human BMCD34$^+$ cells were cultured in liquid culture media. After 1 day of culture, cells were treated by rMCP1 for 4 days. Cells were stained with MMP9 antibody and analyzed by using flow cytometry. (F) Human BMCD34$^+$ cells were transduced with lentivirus carrying shRNAs against RPS14 and MCP1. After 7 days of transduction, cells were stained with MMP9 antibody and analyzed by using flow cytometry. Data are representative of 2 independent transduction experiments. *$P < .05$, **$P < .01$, ***$P < .001$.

We next examined whether inhibition of TGF-β signaling rescues the erythroid defect in RPS14-deficient models. RPS14 knockdown cells were treated with a known TGF-β–signaling inhibitor, SB431542, for 4 days, and erythroid development was examined according to FACS and colony assay. These data showed that 2 μM of SB431542 treatment significantly increased the CD71$^+$ population in RPS14 knockdown cells and partially rescued the numbers of CFU-E colonies ($P < .05$; n = 4) (Figure 4C-D; supplemental Figure 19), a result that reflected what we observed with the treatment of MMP9 inhibitors (Figure 1B-C). Strikingly, in our initial screen, we identified that SB431542 treatment rescued the erythroid defect in rps14-deficient zebrafish compared with DMSO control (Figure 4E). To study the effects of TGF-β inhibition on erythroid development, we established a knockdown system for TGF-β type I receptor (ALK5) by transducing lentivirus expressing ALK5-mCherry shRNA. After confirming the downregulated expression of *ALK5* by 80% (supplemental Figure 20), we cotransduced lentiviruses expressing ALK5 and RPS14 shRNAs and examined erythropoiesis according to FACS. Consistent with treatment with SB431542, we observed that double knockdown of ALK5 and RPS14 partially rescued the decreased CD71$^+$ population in RPS14 knockdown cells (Figure 4F; supplemental Figure 21). Taken together, these data indicate that activation of TGF-β signaling induces the erythroid defect observed in RPS14-deficient cells.

MMP9 inhibitors attenuate activated TGF-β signaling in human RPS14-deficient cells

To determine whether increased MMP9 directly activates TGF-β signaling to inhibit erythroid development in RPS14-deficient cells, we examined TGF-β signaling by addition of rMMP9 protein to human BMCD34$^+$ cells. Two days after treatment, the level of pSMAD2/3

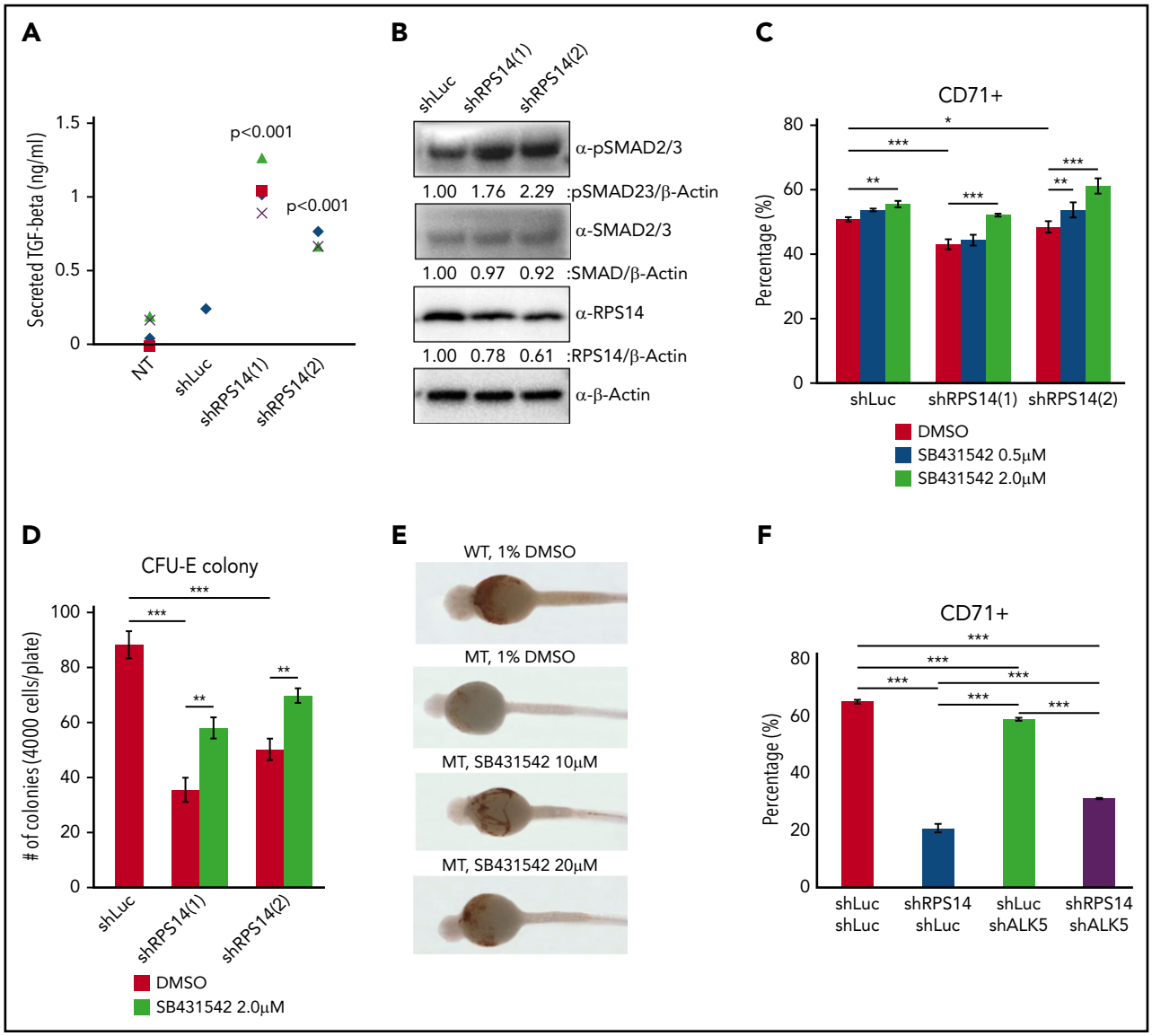

Figure 4. Inhibition of TGF-β signaling increases erythroid development in RPS14-deficient models. Human BMCD34^{+} cells were transduced with lentivirus carrying shRNAs against RPS14 or Luc control. (A) After 5 days of transduction, cells were sorted for GFP+ and were cultured for an additional 2 days. Culture media were collected for a TGF-β ELISA. (B) After 5 days of transduction, cells were sorted for GFP+. Protein was collected and analyzed by using western blot analysis. β-Actin was used as a loading control. (C) After 3 days of transduction, cells were treated by SB431542 at the indicated concentration for 4 days and analyzed for the CD71 expression by using flow cytometry. (D) After 1 day of transduction, cells were treated by 2 μM of SB431542 for 4 days and sorted for GFP+. A total of 4000 cells of GFP+ cells were plated in methylcellulose media specific for CFU-E colonies and cultured for 1 week. Colonies were counted by an investigator blinded to the conditions. (E) Wild-type (WT) embryos stained with *o*-dianisidine showed strong brown signal on the yolk sac, indicating normal hemoglobin levels. rps14$^{-/-}$ mutant embryos (MT) stained with *o*-dianisidine showed weak brown signal on the yolk sac, indicating reduced hemoglobin levels. MT treated with 2 concentrations of SB431542 showed improved staining signal of hemoglobin compared with DMSO-treated rps14$^{-/-}$ MT. All embryos are ventral views. (F) Human BMCD34^{+} cells were transduced with lentivirus carrying shRNAs against RPS14 and ALK5. After 7 days of transduction, cells were analyzed for the CD71 expression by using flow cytometry. Data are representative of 3 independent transduction experiments. *$P < .05$, **$P < .01$, ***$P < .001$.

was examined by using a western blot assay. As observed in RPS14-deficient cells, pSMAD2/3 levels increased with rMMP9 treatment, although the overall total SMAD2/3 protein level was unchanged (Figure 5A). Furthermore, we found that the decreased CD71^{+} or CD235a^{+} populations observed upon addition of rMMP9 was significantly rescued by SB431542 treatment ($P < .001$; n = 4) (Figure 5B; supplemental Figures 22 and 23). Concurrently, the increased pSMAD2/3 level in rMMP9-treated cells was reduced by SB431542 treatment (supplemental Figure 24).

In addition, we examined whether MMP9 inhibitors suppress the activation of TGF-β signaling to rescue the erythroid defect observed in RPS14-deficient cells. We treated RPS14 knockdown cells with MMP9 inhibitors for 2 days, and then examined the pSMAD2/3 level by using FACS. We found that reduced pSMAD2/3 levels were

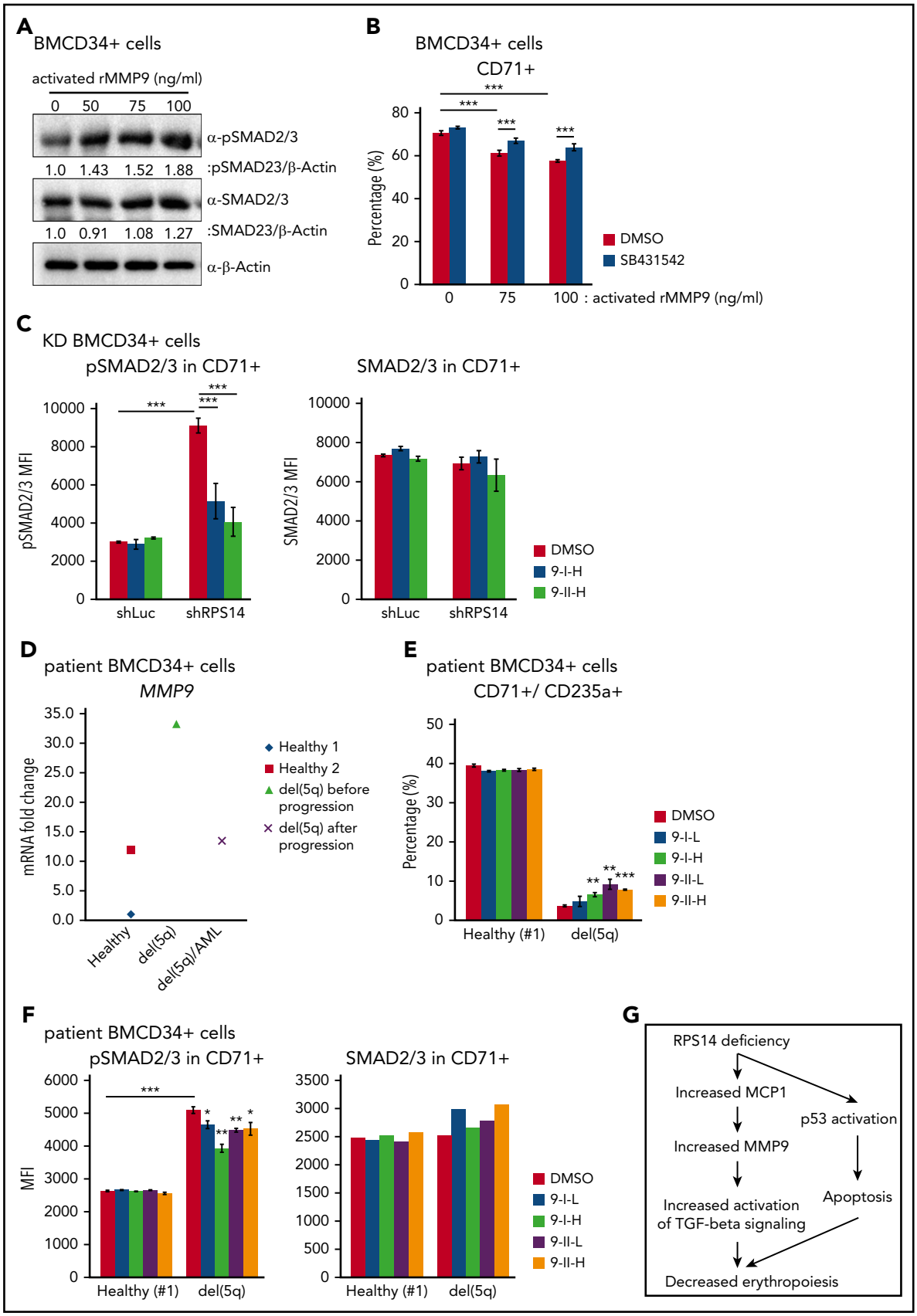
A
BMCD34+ cells
activated rMMP9 (ng/ml)
0 50 75 100
α-pSMAD2/3
1.0 1.43 1.52 1.88
:pSMAD23/β-Actin
α-SMAD2/3
1.0 0.91 1.08 1.27
:SMAD23/β-Actin
α-β-Actin
B
BMCD34+ cells
CD71+
Percentage (%)
DMSO
SB431542
0 75 100 : activated rMMP9 (ng/ml)
C
KD BMCD34+ cells
pSMAD2/3 in CD71+
SMAD2/3 in CD71+
pSMAD2/3 MFI
SMAD2/3 MFI
shLuc shRPS14
DMSO
9-I-H
9-II-H
D
patient BMCD34+ cells
MMP9
mRNA fold change
Healthy 1
Healthy 2
del(5q) before progression
del(5q) after progression
Healthy del(5q) del(5q)/AML
E
patient BMCD34+ cells
CD71+/ CD235a+
Percentage (%)
DMSO
9-I-L
9-I-H
9-II-L
9-II-H
Healthy (#1) del(5q)
F
patient BMCD34+ cells
pSMAD2/3 in CD71+
SMAD2/3 in CD71+
MFI
Healthy (#1) del(5q)
G
RPS14 deficiency
Increased MCP1
p53 activation
Increased MMP9
Apoptosis
Increased activation of TGF-beta signaling
Decreased erythropoiesis

Figure 5.

observed with treatment of MMP9 inhibitors in RPS14 knockdown cells ($P < .001$; n = 4), confirming that MMP9 inhibitors function to inhibit TGF-β activation (Figure 5C).

Finally, we tested that MMP9 inhibitors rescue the erythroid defect in primary BMCD34$^+$ cells from a patient with del(5q) MDS who had multiple cytogenetic abnormalities. We first examined MMP9 expression in BMCD34$^+$ cells from this patient before and after progression to AML by using RT-qPCR. MMP9 expression was increased threefold during the MDS stage compared with that of healthy control subjects (Figure 5D). Interestingly, MMP9 levels decreased after progression to AML, indicating that only del(5q) MDS cells overexpressed MMP9. Next, we treated BMCD34$^+$ cells from this patient from before the AML progression with MMP9 inhibitors and observed erythroid development according to FACS. MMP9 inhibitors partially rescued the CD71$^+$/CD235a$^+$ erythroid population in MDS stage BMCD34$^+$ cells of this patient (Figure 5E) with suppression of pSMAD2/3 (Figure 5F), showing that MMP9 inhibitors attenuate TGF-β activation in a patient with del(5q) MDS.

Thus, these data show that RPS14 deficiency induces MMP9 expression to activate TGF-β signaling, thereby contributing to defective erythropoiesis (Figure 5G).

Discussion

The current study identified that MMP9 inhibitors partially rescue the erythroid defect in RPS14-deficient del(5q) MDS models. MMP9 inhibitors significantly increased erythroid development of RPS14-deficient BMCD34$^+$ cells compared with control cells.

We showed that zebrafish genetic mutant of rps14 can be used as an effective in vivo model of del(5q) MDS anemia to screen for compounds that increase erythroid cell development, offering a novel platform to screen drugs and identify candidate compounds that can be validated in human systems.

It is intriguing that the effects of MMP9 in RPS14-deficient cells are restricted to erythroid progenitors. Preliminary examination of MMP9 expression in various cell populations derived from human normal BMCD34$^+$ cells indicates that MMP9 expression is lower in the CD71$^+$ erythroid population than in other cell populations, including CD11b$^+$ myelocytes, CD11c$^+$ dendritic cells, CD14$^+$ macrophages/monocytes, CD66b$^+$ granulocytes, and CD41a$^+$ platelets. Indeed, CD11b$^+$ cells have fourfold higher expression of MMP9 expressed in CD71$^+$ cells. Combined with the lineage development defects in RPS14-deficient cells being restricted to erythroid cells in which MMP9 is upregulated, we propose that these nonerythroid cell lineages maintain a low basal MMP9 expression through an intrinsic sensitivity to MMP9-mediated TGF-β activation. This theory is supported by our data that MMP9 inhibitors and rMMP9 treatment do not affect nonerythroid lineages.

The function of MMP9 in erythropoiesis or MDS has not been previously characterized. We report that MMP9 is ordinarily a negative regulator of erythroid development. In RPS14-deficient cells, we showed that increased MMP9 expression and activation result in defective erythropoiesis, but it is unclear if basal MMP9 contributes to normal erythroid differentiation. Given that we observed no effect on erythropoiesis of normal BMCD34$^+$ cells when MMP9 was downregulated, we propose that MMP9 expression is not required for normal hematopoiesis. However, expression levels above the threshold of activation are antagonistic to erythropoiesis as rMMP9 treatment of normal BMCD34$^+$ cells and increased expression in human and zebrafish models of del(5q) MDS significantly reduced erythroid development. These observations are consistent with MMP9 regulation in other systems in which MMP9 expression is tightly regulated in normal physiological conditions.

Previous publications have shown that inflammatory signaling is dysregulated in del(5q) MDS.[23,44,45] One mechanism for lenalidomide action is believed to be immune modulation. In this study, we observed that MCP1, a small cytokine that belongs to the CC chemokine family, is upregulated in RPS14-deficient cells. More importantly, we found that MCP1 has a critical role in regulating MMP9 expression. These observations suggest the possibility that treatment of lenalidomide in RPS14-deficient cells corrects the increased MMP9 level through modulation of MCP1 expression. However, our preliminary data show that MMP9 expression was not changed by lenalidomide treatment in RPS14 knockdown cells (supplemental Figure 25). Similarly, MMP9 function in inhibiting erythroid development was shown to be independent of the apoptosis pathway in which lenalidomide exerts selective cytotoxicity on del(5q) clones through its inhibitory effect on mouse double minute 2 protein. We therefore suggest that MMP9 inhibitors could be an alternative to lenalidomide for patients with del(5q) MDS.

Defective ribosome biogenesis due to deletions or mutations of ribosomal proteins has been implicated in the pathophysiology of the anemia that is characteristic of both del(5q) MDS and Diamond-Blackfan anemia. Although deficiencies of various ribosomal proteins are observed in patients with Diamond-Blackfan anemia, RPS14 deficiency is observed only in patients with del(5q) MDS. Our data showed that the effect of MMP9 inhibition on erythropoiesis

Figure 5. MMP9 inhibitors attenuate activated TGF-β signaling in human RPS14-deficient cells and in bone marrow cells from a patient with del(5q) MDS. (A) Human BMCD34$^+$ cells were cultured in liquid culture media. After 5 days of culture, cells were treated by rMMP9 for 2 days, and protein was collected for western blot analysis. (B) Human BMCD34$^+$ cells were cultured in liquid culture media. After 3 days of culture, cells were treated by rMMP9 for 2 days followed by SB431542 treatment for an additional 2 days. At 7 days after culture, cells were analyzed for the CD71 expression by using flow cytometry. (C) Human BMCD34$^+$ cells were transduced with lentivirus carrying shRNAs against RPS14. After 5 days of transduction, cells were treated by MMP9 inhibitors for 2 days and were analyzed for pSMAD2/3 or SMAD2/3 expression by using flow cytometry. (D) RNA was isolated from bone marrow CD34$^+$ cells from a patient with del(5q) MDS or healthy control subjects and analyzed by using RT-qPCR. (E) BMCD34$^+$ cells from a patient with del(5q) MDS or a healthy control subject were cultured in liquid culture media. After 1 day of culture, cells were treated by MMP9 inhibitors for 4 days. Cells were cultured for an additional 4 days without MMP9 inhibitors and then analyzed for CD71 and CD235a expressions by using flow cytometry. (F) BMCD34$^+$ cells from a patient with del(5q) MDS or a healthy control subject were cultured in liquid culture media. After 1 day of culture, cells were treated by MMP9 inhibitors for 4 days and then analyzed for pSMAD2/3 or SMAD2/3 expression by using flow cytometry. (G) Model of the defective erythroid development in RPS14 deficiency through increased MMP9 expression. Data are representative of 3 independent transduction experiments (A-C) or 1 independent experiment (D-F). *$P < .05$, **$P < .01$, ***$P < .001$.

is specific to RPS14-deficient cells, and not RPS19- or RPL11-deficient cells. The data suggest that RPS14 protein specifically regulates MCP1/MMP9 expressions. Several ribosomal proteins have been reported to have extra-ribosomal functions in various cellular processes.[46] Ribosomal protein S3 is identified as an essential subunit of NF-κB complexes selectively to regulate the specific subset of NF-κB target genes.[47] It may be possible that RPS14 plays an extra-ribosomal role to regulate MCP1/MMP9 expressions during erythropoiesis of human bone marrow progenitor cells.

Previous studies indicate that TGF-β signaling is myelosuppressive, suppressing erythroid and myeloid cell developments.[39-43] In addition, increased levels of TGF-β superfamily inhibitors of erythropoiesis (predominantly growth and differentiation factor-11) occur within MDS erythroid cells. Luspatercept (ACE-536) is a novel fusion protein that acts as a ligand trap by binding and blocking signaling from the inhibitory factors SMAD2/3, thus increasing erythropoiesis.[35] Encouraging phase 2 data with ACE-536, treating lower risk MDS patients who are nonresponsive to erythroid-stimulating agents, and predominantly presenting with ring sideroblasts have demonstrated a ~60% erythroid response rate compared with ~25% for those lacking this feature.[48] Our results support a role for TGF-β as a negative regulator of erythroid development. Furthermore, our data indicate that MMP9 is an upstream regulator of TGF-β signaling in erythroid development. MMP9 serves as a critical mediator between increased extracellular TGF-β levels and activation of intracellular TGF-β signaling pathways in erythroid progenitors. Interestingly, it has been reported that MMP9 and TGF-β positively regulate one another during tumor malignancy.[13-15] Because we observed a similar regulatory loop between MMP9 and TGF-β in RPS14 deficiency, we suspect that this mechanism might contribute to rapid disease progression in patients with del(5q) MDS. It would be valuable to correlate MMP9 expression in patient samples representing different stages of del(5q) MDS.

Previous reports also suggest that several molecular pathways are necessary in the development of del(5q) MDS, including p53 activation due to aberrant ribosome biogenesis, loss of the miRNA genes miR-145 and miR-146a, and *CSNK1A1* gene deficiency.[49-51] Because MMP9 inhibitors partially rescued the erythroid defect in RPS14-deficient cells, we propose that MMP9 upregulation contributes in concert with other mechanisms to the molecular pathogenesis leading to macrocytic anemia in patients with del(5q) MDS.

In conclusion, our study shows a novel function of MMP9 in the pathogenesis of RPS14-deficient del(5q) MDS in both zebrafish and human models. Upregulated MMP9 facilitates TGF-β signaling to inhibit erythroid development. In contrast, MMP9 inhibitors partially rescued the erythroid population by decreasing TGF-β activation in one del(5q) patient's $BMCD34^+$ cells as well as RPS14-deficient del(5q) models (Figures 1 and 5). This suggests that MMP9 inhibitors may serve as therapeutic agents for patients with del(5q) MDS, especially patients who have developed resistance to lenalidomide.

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Authorship

Contribution: M.Y., S.K., M.C.W., H.-D.C., and K.J.S. performed the experiments with human hematopoietic cells; H.H. and C.C. performed the experiments with zebrafish; research was designed by M.Y., S.L, and K.M.S.; and the manuscript was written by M.Y., P.L.G, B.G., A.N., S.L., and K.M.S.

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