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Original Article

Iron deficiency accelerates intervertebral disc degeneration through affecting the stability of DNA polymerase epsilon complex

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Abstract: Iron serves as an important cofactor of iron-containing proteins that play critical roles in the maintenance of DNA stability and cell cycle progression. The disturbed iron homeostasis results in the pathogenesis of many diseases such as cancer and anemia. In this study, we found a clear correlation between iron deficiency and intervertebral disc degeneration (IDD). Through microarray experiments, we found that a large number of genes were differentially expressed in tissues with different degrees of degeneration. Among them, an iron-containing gene, *PolE*, the catalytic subunit of DNA polymerase epsilon (Polε), and the other two Polε subunits, including *PolE2* and *PolE3,* were markedly downregulated, while some proteins involved in apoptosis such as *Caspase-3* and *-8* were significantly upregulated. By supplementation with an iron chelator deferoxamine (DFO) or knocking down either iron divalentmetal transporter 1 (DMT1) or transferrin receptor 1 (TfR1) in the nucleus pulposus (NP) cells, we found that the protein levels of PolE complex members were dramatically reduced, whereas the intrinsic apoptotic pathway was activated. Interestingly, overexpression of *PolE* in NP cells knocked down with either DMT1 or TfR could not reverse the stability of PolE complex and apoptosis status. In summary, our study suggests that iron deficiency is an important factor in the aggravation of IDD. Proper iron supplementation may be an effective strategy to alleviate the symptoms of patients with IDD.

Keywords: IDD, iron deficiency, PolE, apoptosis, DMT1, TfR1

Introduction

Iron acts as an essential nutrient for mammalian cells because it is required for the functional maintenance of iron-containing proteins, which participate in a variety of biological processes, including catalyzing the formation of hydroxyl radicals, regulating cell proliferation, DNA replication and cell cycle progression [1, 2]. In mammalian cells, iron mainly exists in two states, ferrous iron (Fe^{2+}) and ferric iron (Fe^{3+}), which enables it to participate in the Fenton reaction through gaining and losing electrons [3]. To sustain the physiologic iron requirement, human iron homeostasis is regulated at two different levels: systemic and cellular levels [4-6]. The systemic homeostasis is controlled by several processes: (1) duodenal enterocytes that localize in the small intestine absorb dietary iron through divalentmetal transporter 1 (DMT1) [7, 8]; (2) macrophages in the liver, spleen, and bone marrow recycle iron from senescent or damaged red blood cells [2]; (3) iron exporter ferroportin (Fpn) releases the stored iron from hepatocytes [9]; and (4) hepcidin controls iron trafficking and efflux by binding to Fpn, causing its internalization and degradation [10]. Cellular iron is mainly taken up by DMT1, transferrin receptor 1 (TfR1), and TfR2, followed by iron transport among different cellular compartments [11]. The newly acquired iron is temporarily stored in the cytosolic "labile iron pool" (LIP) and is only exported when needed [11]. The excess cellular iron is either stored in ferritin or exported by Fpn [11]. In addition, iron homeostasis in mammalian cells is also post-transcriptionally regulated by iron regulatory protein 1 (IRP1) and IRP2 [11]. The human body

and cells need iron homeostasis to maintain iron-containing protein functions, signal transduction and the microenvironment. Iron overload or deficiency can cause a number of diseases such as cancer and anemia [2].

Intervertebral discs (IVDs) are located between adjacent vertebrae in the vertebral column and consist of three parts, including nucleus pulposus (NP), annulus fibrosus (AF) and cartilage endplates [12]. The outer endplates mainly function to absorb small molecules and nutrients required for NP and AF cells, and AF is required for the prevention of NP cells from herniating or leaking out of the disc [12]. Intervertebral disc degeneration (IDD) occurs with age due to a consequence of many factors, such as injury, genetics, microenvironment changes, inflammation and apoptosis, causing symptoms of pain, numbness, tingling in the arms or shoulders, and neck pain [13]. During the past several years, emerging evidence has demonstrated that many signaling pathways are involved in IDD. For instance, the transcription factor nuclear factor kappa β (NF-κB) and mitogen-activated protein kinase (MAPK) pathways can mediate proinflammatory genes, such as *tumor necrosis factor* alpha (TNF-α), interleukin 1 beta (IL-1β) and interleukin 6 (IL-6), to initiate and propagate IDD [14, 15]. The activation of the Wnt pathway with LiCl treatment in NP cells accelerates cellular senescence and leads to IDD [16]. However, as far as we know, there have been no reports that iron deficiency or iron overload is involved in the process of IDD.

Organisms require DNA polymerases to accurately and efficiently replicate the genome [17]. According to sequence homology, DNA polymerases can be further subdivided into seven different families: A, B, C, D, X, Y, and RT [18]. Eukaryotic organisms mainly utilize family B DNA polymerases (Polα, Polδ and Polε) for DNA replication [17, 18]. Polα is composed of four subunits: the catalytic subunit POLA1, the regulatory subunit POLA2, and two primase subunits, named PRIM1 and PRIM2 [17, 18]. Polδ also consists of four subunits: the catalytic subunit POLD1, and the regulatory subunits POLD2, POLD3, and POLD4 [17, 18]. Polε is composed of four subunits: PolE (catalytic subunit), PolE2, PolE3 and PolE4 [17, 18]. Of these three enzymes, Polα functions to initiate DNA replication, elongating the primer with ~20 nucleotides, Polε functions to extend the leading strand during replication, and Polδ primarily replicates the lagging strand [17, 18]. All three polymerases contain two cysteine-rich metal binding sites known as CysA and CysB in their C-terminal domain (CTD). CysA is a zinc-binding site, whereas CysB is an iron-sulfur cluster [4Fe-4S] [17, 18].

In the clinical examination of patients with IDD, we coincidentally identified a patient with serious degeneration accompanied by anemia. To test whether there is a correlation between anemia and IDD, we measured the iron concentration in blood serum from a large number of IDD patients. Our results showed that the degenerative severity of IDD is negatively correlated with iron level in patients. In order to investigate the underlying molecular mechanism between anemia and IDD, we performed microarray analysis using mRNAs from patients with different degenerative severities. We found that the expression of *PolE* was markedly decreased with the severity of IDD and was accompanied by activation of apoptosis. Similarly, we also observed a decrease in PolE level and apoptosis activation in NP cells with the addition of the iron chelator deferoxamine (DFO). These results indicated that the decrease in iron level might lead to the downregulation of PolE, which in turn affects the stability of Polε complex. Subsequently, we also observed a lower PolE level and apoptosis activation in NP cells knocking down *PolE*, *DMT1* or *TfR1*. Therefore, we concluded that iron deficiency might affect the stability of Polε complex, resulting in apoptosis and eventually causing IDD.

Materials and methods

Patients and serum sample collection

The blood serum from a total of 120 IDD patients who were under different Pfirrmann grades (from 1 to 5, 24 patients in each grade) was collected under the conduction of protocols approved by the ethical board of Kunming Medical University. The patients' basic information is shown in Supplementary Table 1. All patients were informed of the purpose of this examination and signed a consent form. Twenty-four volunteers without IDD were used as controls. All blood samples were collected

from veins, immediately stored at 4°C, and subjected to the required experiments within 8 hours.

Measurement of serum ions

The concentrations of serum iron, calcium and sodium were measured by using kits purchased from Abcam (Catalog #ab83366, #ab102505 and #ab211096, respectively), according to the manufacturer's instructions. Serum potassium level was measured by using Potassium Assay Kit (Crystal Chem Inc. USA, Catalog #80169) following the manufacturer's guideline.

Cell culture and transfection

The human NP cells were obtained from ScienCell Research Laboratories (Catalog #4800). Cells were maintained in Nucleus Pulposus Cell Medium (ScienCell, Catalog #4801) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Invitrogen, USA) and 1% penicillin/streptomycin. For small interfering RNA (siRNA) transfection, cells were grown at 37°C with 5% CO_2 and subjected to transfection using Lipofectamine 2000 (Invitrogen, USA) when the density reached approximately 80% confluence. After incubation at 37°C for 48 h, the transfected cells were subjected to the required experiments. For short hairpin RNA (shRNA) knockdown, the control vector (pLKO.1-ConshRNA) as well as three *PolE* shRNA vectors, including TRCN0000052973, TRCN0000052975 and TRCN0000052976, *DMT1* shRNA (TRCN0000043248) and *TfR1* shRNA (TRCN0000040161), were purchased from Sigma (USA). The lentiviruses containing shRNAs were transfected into NP cells following standard procedures. After puromycin (5 μg/mL) selection and PCR detection, the confirmed knockdown cells were subjected to the required experiments.

Microarray analysis

The microarray assay was performed as previously described [19]. Briefly, a total of 0.5 mg of RNA from IDD patients who underwent different Pfirrmann grades (from 0 to 5) was subjected to cRNA synthesis using a GeneChip 3' *In Vitro* Transcription (IVT) Express Kit (ThermoFisher Scientific, USA, Catalog #902789), following the manufacturer's instructions. The biotin-labeled cRNA was then fragmented and subjected to hybridization with a GeneChip Human Genome U133 Plus 2.0 Array System (ThermoFisher Scientific, USA, Catalog #902483). After incubation at 45°C for 16 h, chips were washed, stained with streptavidin-phycoerythrin (SAPE) at 35°C for 300 seconds, and scanned with a GeneChip™ Scanner 3000 7G system (ThermoFisher Scientific, USA, Catalog #000213).

Immunoblot analysis

The immunoblot analysis was carried out as previously described [20]. Briefly, the whole cell protein extracts were lysed and sonicated with RIPA buffer (ThermoFisher Scientific, USA, Catalog #89900) supplemented with 1 × complete protease cocktail inhibitor (Roche, USA, Catalog #11697498001). After denaturing at 95°C for 10 minutes, equal amounts of proteins from each sample were subjected to electrophoresis. The antibodies used in this study included anti-PolE (mouse, ThermoFisher Scientific, USA, Catalog #MA5-13616), anti-PolE2 (rabbit, Sigma, USA, Catalog #HPA027555), anti-PolE3 (rabbit, Sigma, USA, Catalog #SAB2-101839), anti-caspase-3 (rabbit, Abcam, USA, Catalog #ab90437), anti-caspase-8 (mouse, Cell Signaling Technology, USA, #9746), anti-PARP (Abcam, USA, Catalog #ab15496) and anti-GAPDH (rabbit, Abcam, USA, Catalog #ab128915). The enhanced chemiluminescence (ECL) signals were recorded using ChemiDoc MP (Bio-Rad, USA).

Quantitative RT-PCR (qRT-PCR) analysis

Total RNA was isolated using TRIzol™ Reagent (ThermoFisher Scientific, USA, Catalog #15596026). A total of 1 μg of RNA from each sample was used for reverse transcription with a High-Capacity cDNA Reverse Transcription Kit (ThermoFisher Scientific, USA, Catalog #4368814), according to the manufacturer's instructions. The resulting cDNAs were diluted 10-fold and then subjected to PCR amplification with the primers listed in Supplementary Table 2. The individual gene expression quantified based on the cycle threshold (Ct) values and *β-ACTIN* was used as an internal control.

Flow cytometry analysis

The flow cytometry analysis to detect cell apoptosis was carried out as previously described [21]. Briefly, a minimum of 10^4 cells were resus-

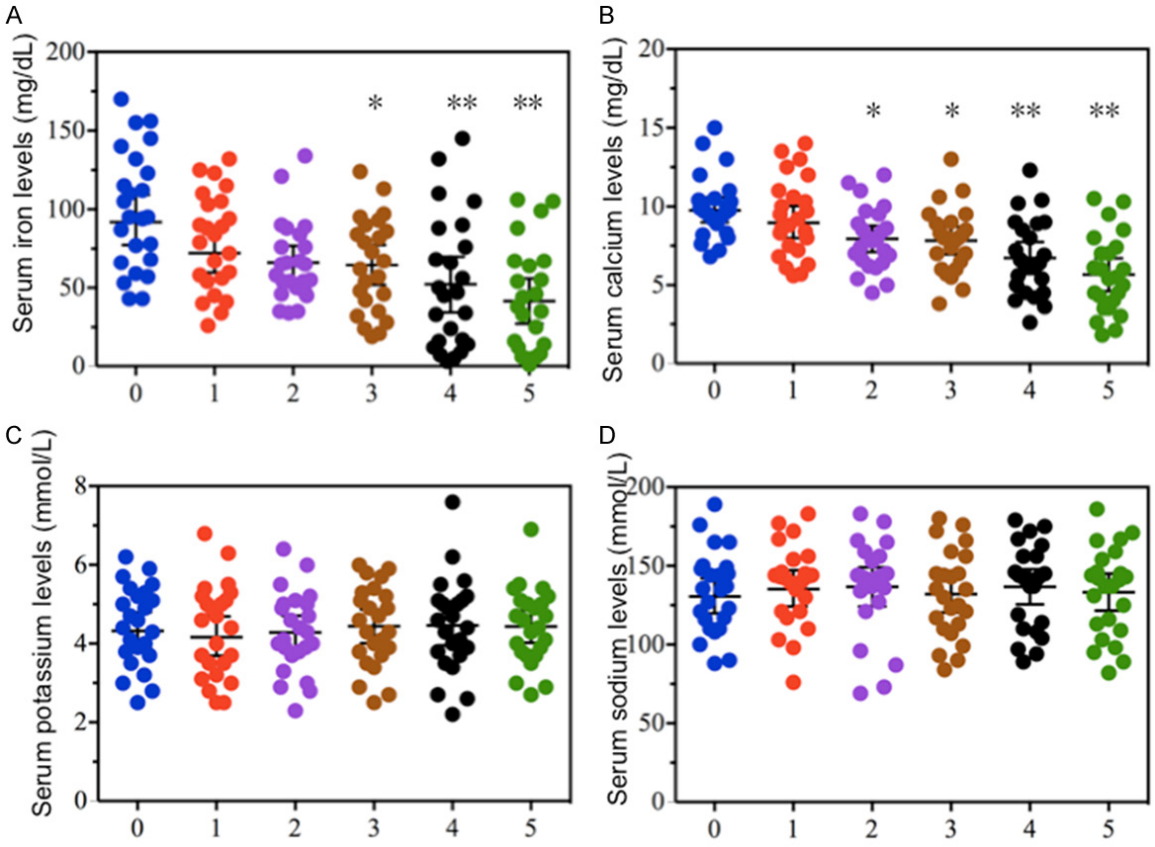

Figure 1. The serum iron concentrations are decreased in IDD patients. The venous blood samples were collected from IDD patients who underwent different Pfirrmann grades (from 0 to 5, 24 patients in each grade), and subjected to measure serum iron levels (A), serum calcium levels (B), serum potassium levels (C), and serum sodium levels (D), respectively. $*P < 0.05$, $**P < 0.001$.

pended in 100 μL of binding buffer, followed by the addition of 6 μl of Annexin V-PE and 6 μl of 7-AAD, respectively, and were allowed to react for 15 minutes at room temperature in the dark. The resulting cells were subjected to flow cytometry.

Statistical analysis

All experiments in this study were repeated independently three times or more. Student's *t*-test was applied for comparison of two groups, and differences with $P < 0.05$ were considered significant. Data were represented as the mean ± standard deviation (SD).

Results

Iron deficiency is associated with IDD

During long-term clinical examination and observation, we found that many patients with degenerative discs were accompanied by anemia. In order to systematically study whether there is a correlation between IDD and anemia, we conducted a large number of tests and statistical analyses. Briefly, we divided the obtained clinical samples into 5 grades according to the standard of Pfirrmann grades, with 24 patients in each grade, and recruited 24 volunteers without disc degeneration as controls. We measured the concentrations of iron, potassium, calcium, and sodium ions in the blood of all subjects. Interestingly, compared with healthy controls, we noticed a significant decrease in iron and calcium levels but not potassium and sodium concentrations in the blood of IDD patients (**Figure 1**). Both iron and calcium concentrations gradually decreased with the severity of IDD, that is, the concentration of both ions decreased most significantly in patients with Pfirrmann grades 4 and 5 (**Figure 1A** and **1B**). These results suggest that the deficiency

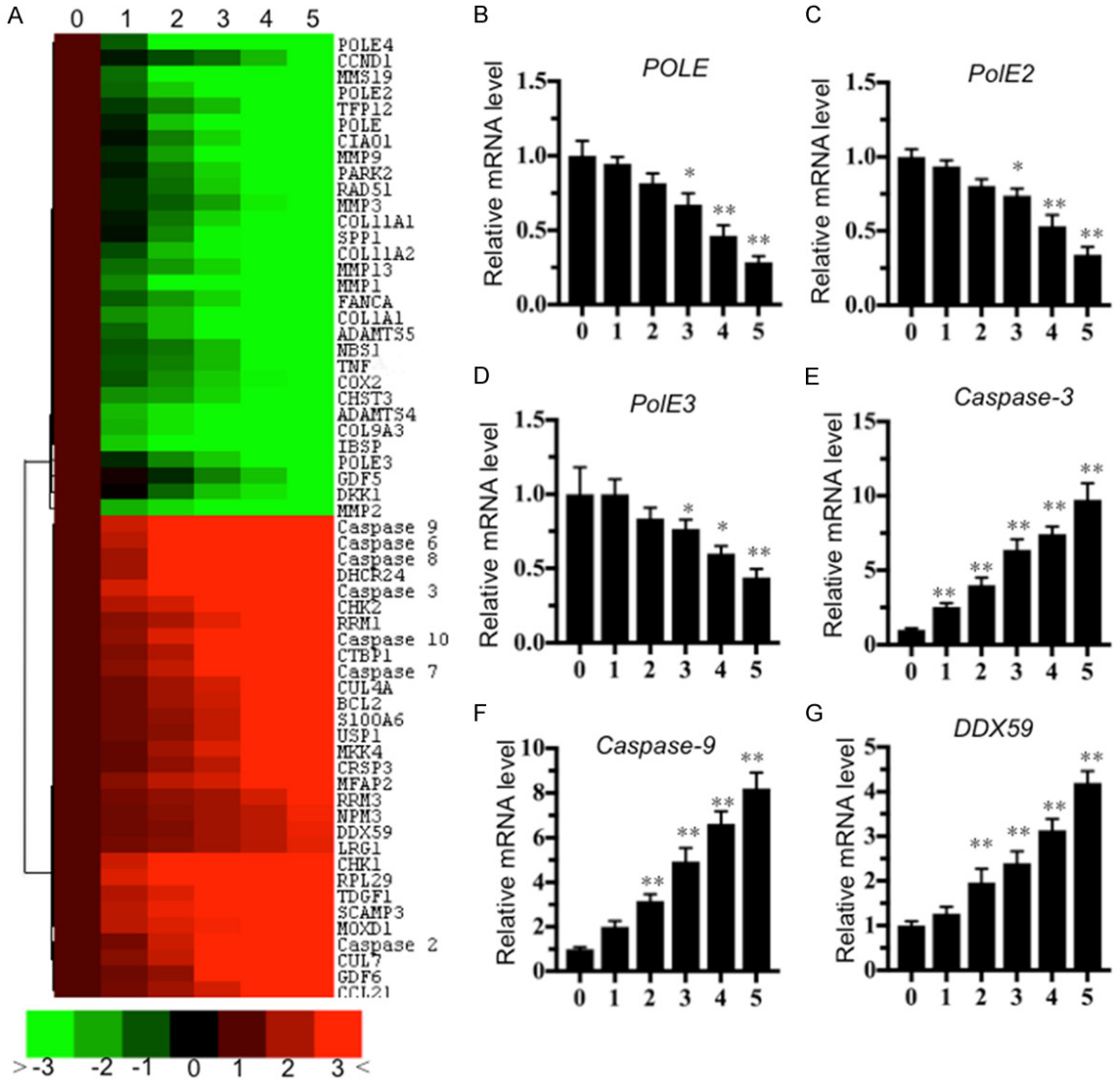

Figure 2. The aberrant gene expression profile in IDD patients. (A) The heat maps of the consistently downregulated and upregulated genes in different IVD degeneration patients. The mRNAs from IDD patients who underwent different Pfirrmann grades (from 0 to 5) were subjected to microarray analysis. The heat maps indicated high (red) or low (green) levels of gene expression. (B-G) Verification of mRNA levels in IDD patients. The qRT-PCR was performed to verify the expression of three downregulated genes, including *PolE* (B), *PolE2* (C), and *PolE3* (D), and three upregulated genes, including *Caspase-3* (E), *Caspase-8* (F), and *DDX59* (G). *$P < 0.05$, **$P < 0.001$.

of iron and calcium may be important pathogenic causes of IDD.

PolE is significantly downregulated in the process of IDD

Ion homeostasis is essential for the functional maintenance of iron-containing proteins. Since we have found that iron and calcium concentrations were significantly reduced in IDD patients, their deficiency should inevitably disrupt many gene expression levels or protein functions. For this purpose, we subjected the mRNAs from patients who were under different Pfirrmann grades (from 0-5) to microarray analysis. Overall, we found that the expression of a total of 327 genes was consistently increased or decreased in IDD patients with different Pfirrmann grades (data not shown). Of them, 119 genes were upregulated, while the other 208 genes were downregulated. As shown in the heat map, we selected 30 genes with the

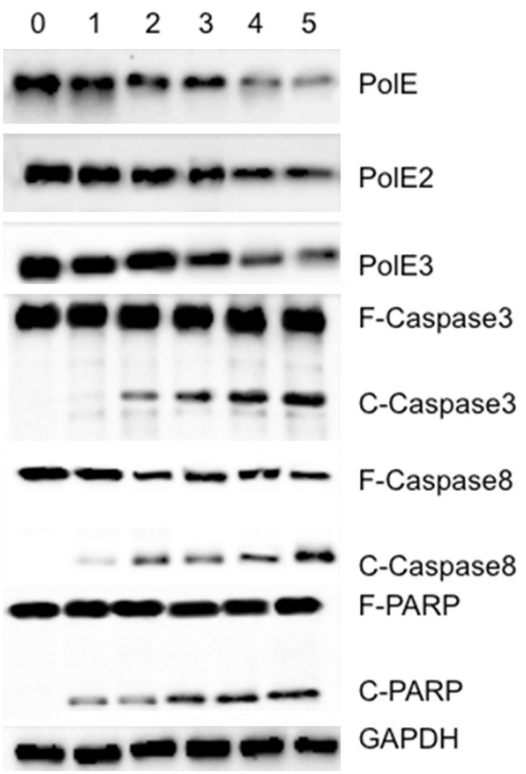

Figure 3. Iron supplementation restores PolE protein level and decreases cell apoptosis. The primary NP cells from IDD patients who underwent different Pfirrmann grades (0, 2 and 4) were treated with (+) or without (-) 100 μM $FeCl_3$. After 24 hr, cells were collected and lysed, followed by immunoblots to examine protein levels of PolE, PolE2, PolE3, Caspase-3, Caspase-8 and PARP. GAPDH was used as a loading control. F represents full length; C represents cleavage.

most significant upregulation and downregulation to display (**Figure 2A**). Interestingly, of these most dramatically downregulated genes, we found an iron-containing gene, *PolE*, as well as some genes that encode proteins interacting with PolE, including *PolE2*, *PolE3*, *PolE4*, nucleotide excision repair protein homolog *MMS19*, and cytosolic iron-sulfur assembly component 1 (*CIAO1)* (**Figure 2A**), which have been reported to form a complex that mediates the incorporation of iron-sulfur cluster into extramitochondrial Fe/S proteins. Of these most significantly upregulated genes, we found that a number of them are involved in apoptosis, including Caspase 3, -6, -7, -8, -9 and -10 (**Figure 2A**). In order to verify the expression of these genes involved in IDD, we selected 6 genes, 3 from the downregulated group (*PolE, PolE2,* and *PolE3*) and 3 from the upregulated group (*Caspase-3, -9* and *DDX59*), and examined their expression by qRT-PCR using the same RNA samples in microarray analysis. Consistent with the microarray results, the expression of *PolE, PolE2* and *PolE3* was gradually decreased with increasing severity of IDD (**Figure 2B-D**), whereas the expression levels of *Caspase-3, Caspase-9* and *DDX59* were reversed (**Figure 2E-G**). In addition, we also examined protein levels of these downregulated and upregulated genes in samples that were under different Pfirrmann grades. Similarly, our results confirmed that the protein levels of PolE, PolE2, and PolE3 were gradually decreased with an increase in Pfirrmann grades, while the protein levels of Caspase-3, -9, and PARP (Poly ADP-ribose polymerase 1) were gradually increased (Supplementary Figure 1). These results clearly indicated that iron deficiency in IDD patients was able to cause instability of PolE complex and the activation of apoptosis.

Iron supplementation reverses the defects of PolE instability in the primary NP cells from IDD patients

Since we have found that iron deficiency may be an important factor in accelerating the pathology of IDD, we next attempted to detect the PolE complex levels and apoptotic proteins in the primary NP cells from IDD patients by supplementation with iron. Briefly, we isolated the primary NP cells from patients who were under Pfirrmann grades of 0, 2 and 4 and then grew these cells by supplementation of 100 μM $FeCl_3$. After 24 hr of incubation, the protein levels of PolE, PolE2, PolE3, Caspase-3, Caspase-8, and PARP were detected. Interestingly, the addition of iron increased the protein levels of PolE, PolE2 and PolE3 while reducing apoptotic proteins in IVD degenerating cells (**Figure 3**). These results further demonstrated that iron concentration determined the status of PolE complex stability and apoptosis.

Iron chelator DFO causes the instability of PolE complex and induces apoptosis

To further confirm that iron deficiency can lead to the decrease of PolE complex level and cause apoptosis, we next sought to treat NP cells with the iron chelator DFO, which has been widely used in the clinical treatment of iron overload diseases. In short, the commercial NP cells were treated with or without different con-

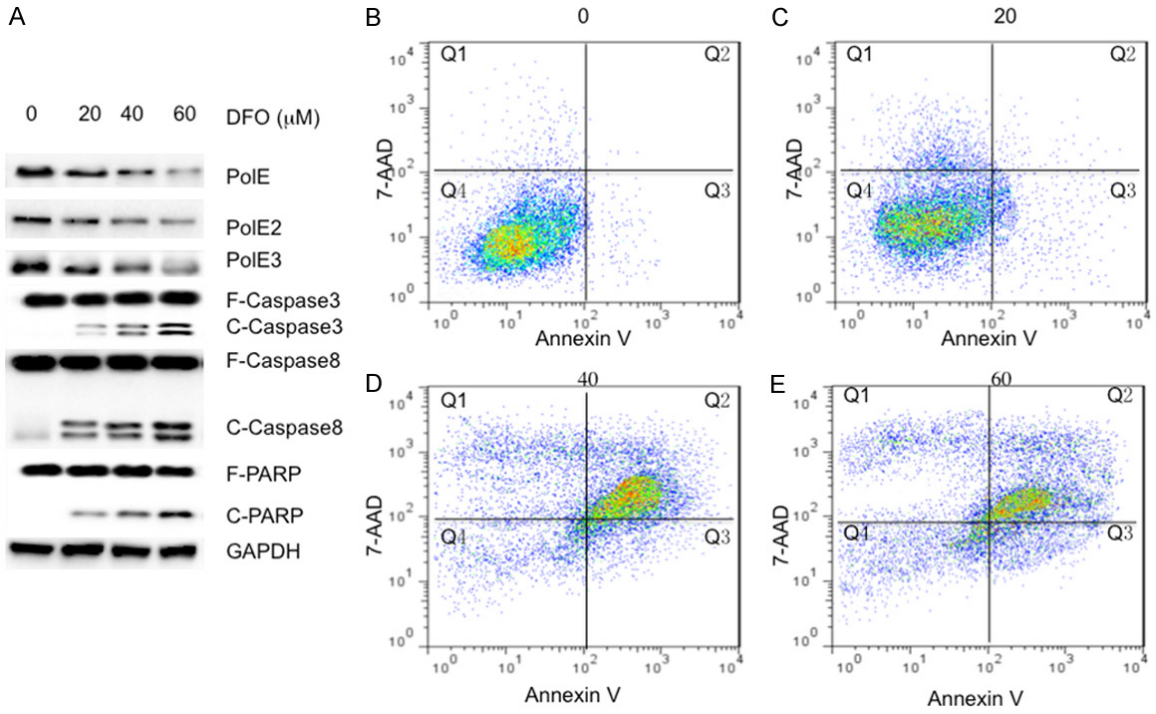

Figure 4. Iron depletion decreases PolE protein level and causes apoptosis. The human NP cells were treated with different concentrations (0, 20, 40 and 60 μM) of iron chelator DFO. After 24 hr, half of the DFO-treated cells were collected and lysed, followed by immunoblots to examine protein levels of PolE, PolE2, PolE3, Caspase-3, Caspase-8 and PARP. GAPDH was used as a loading control (A). The other half of DFO-treated cells were collected and stained with Annexin V-PE/7-AAD, followed by flow cytometry analysis (B-E). (B) No DFO treatment; (C) 20 μM; (D) 40 μM; and (E) 60 μM DFO treatment.

centrations of DFO (0, 20, 40 and 60 μM), followed by the detection of protein levels of PolE complex and apoptotic proteins. Our results showed that the protein levels of PolE, PolE2 and PolE3 were gradually decreased with an increase in DFO concentrations, and the levels of these three proteins were minimized in cells treated with 60 μM DFO (**Figure 4A**). In addition, we also detected cellular apoptosis status in different treatments using immunoblot and flow cytometry. The immunoblot results indicated that the cleavage of Caspase-3, Caspase-8 and PARP was gradually increased with an increase in DFO concentrations, and the activation of apoptosis was most apparent in 60 μM DFO-treated cells. By using Annexin V-PE and 7-AAD to stain cells that were treated with different concentrations of DFO, we found that the DFO treatment significantly increased cell population in Q2 (early apoptosis) and Q3 (late apoptosis) (**Figure 4** and Supplementary Figure 2), which suggested that the cellular iron deficiency caused by iron chelator treatment can result in apoptosis.

Knockdown of PolE in NP cells caused apoptosis

From the above results, we can see an interesting phenomenon: the decrease in the PolE protein level in IDD patients or in DFO-treated NP cells is accompanied by the occurrence of apoptosis. To verify whether apoptosis is dependent or independent on the amount of PolE protein, we next sought to detect cell apoptosis by directly knocking down PolE expression. Accordingly, we selected three shRNAs that specifically targeted PolE and then subjected them to lentiviruses package and transfection in NP cells. After screening in the puromycin-containing medium, we obtained a number of PolE knockdown cell lines and picked three of them (KD-1, -2 and -3) to examine protein levels of PolE complex and apoptotic proteins. The results showed that with the knockdown of PolE, the protein levels of PolE2 and PolE3 were also decreased, while the cleavage of Caspase-3, Caspase-8 and PARP was increased significantly (**Figure 5A**). In addi-

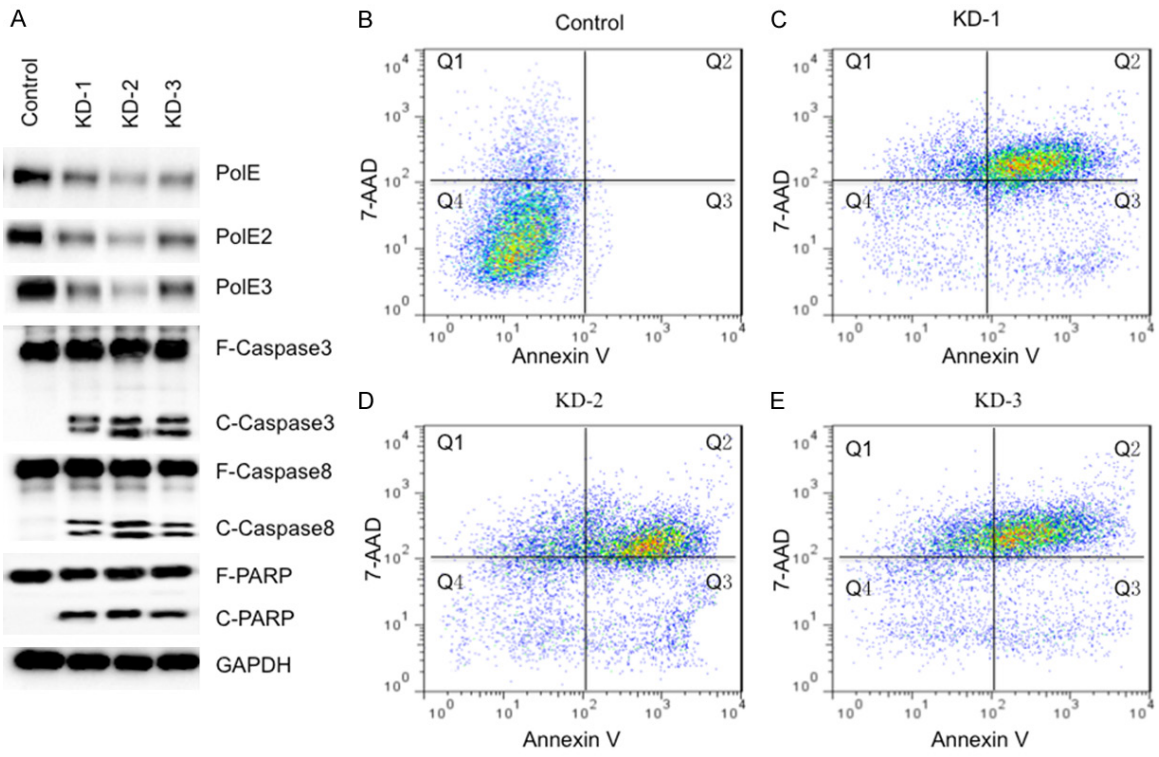

Figure 5. PolE knockdown induces apoptosis. The human NP cells were infected with lentiviruses containing either control shRNA or three PolE-specific shRNAs. After selection in puromycin-containing medium, the control shRNA transfected cells (control) and PolE knockdown cells (KD-1, -2 and -3) were subjected to immunoblots to examine protein levels of PolE, PolE2, PolE3, Caspase-3, Caspase-8 and PARP. GAPDH was used as a loading control (A). The same cells used in (A) were also collected and stained with Annexin V-PE/7-AAD, followed by flow cytometry analysis (B-E). (B) Control cells; (C) KD-1 cells; (D) KD-2 cells; and (E) KD-3 cells.

tion, we also stained cells using Annexin V-PE and 7-AAD to detect apoptosis in KD-1, -2 and -3 through flow cytometry. As shown in **Figure 5B-E** and Supplementary Figure 3, we observed a significantly increased cell population in Q2 and Q3 in all three PolE knockdown cell lines, which suggested that knockdown of PolE can cause apoptosis.

Knockdown of either DMT1 or TfR1 resulted in PolE complex instability and apoptosis

As previously mentioned, DMT1 and TfRs are the most critical proteins for iron absorption. We next investigated whether their downregulation could have an effect on the stability of PolE complex and cellular apoptotic status. Accordingly, we first used DMT1- and TfR1-specific shRNAs to transfect NP cells and obtained three cell lines (KD-1, -2 and -3) with lower expression of DMT1 and TfR1, respectively, to detect PolE level in these cell lines. After verifying the expression of DMT1 and TfR1 in their corresponding knockdown cell lines through qRT-PCR (**Figure 6A** and **6B**), we detected the mRNA and protein levels of PolE in these cell lines. Interestingly, we found that knockdown of DMT1 or TfR1 alone caused a dramatic decrease of PolE mRNA and protein levels (**Figure 6C-F**). Based on the similar expression level of PolE in the three DMT1 or TfR1 knockdown cell lines, we only used DMT1-KD1 and TfR1-KD1 cell lines for the following studies.

Since PolE expression was significantly reduced in cell lines with DMT1 and TfR1 knockdown, we next sought to see if overexpression of *PolE* in the DMT1-KD1 and TfR1-KD1 cell lines can restore the protein levels of PolE2 and PolE3 as well as reduce apoptosis. As shown in **Figure 7A**, the overexpression of *PolE* in DMT1-KD1 or TfR1-KD1 cells did not affect the protein levels of PolE2 and PolE3 nor did it alter the apoptosis status. The flow cytometry results also indicated that DMT1-KD1 and DMT1-KD1-PolE-OE cells, as well as TfR1-KD1 and TfR1-KD1-PolE-

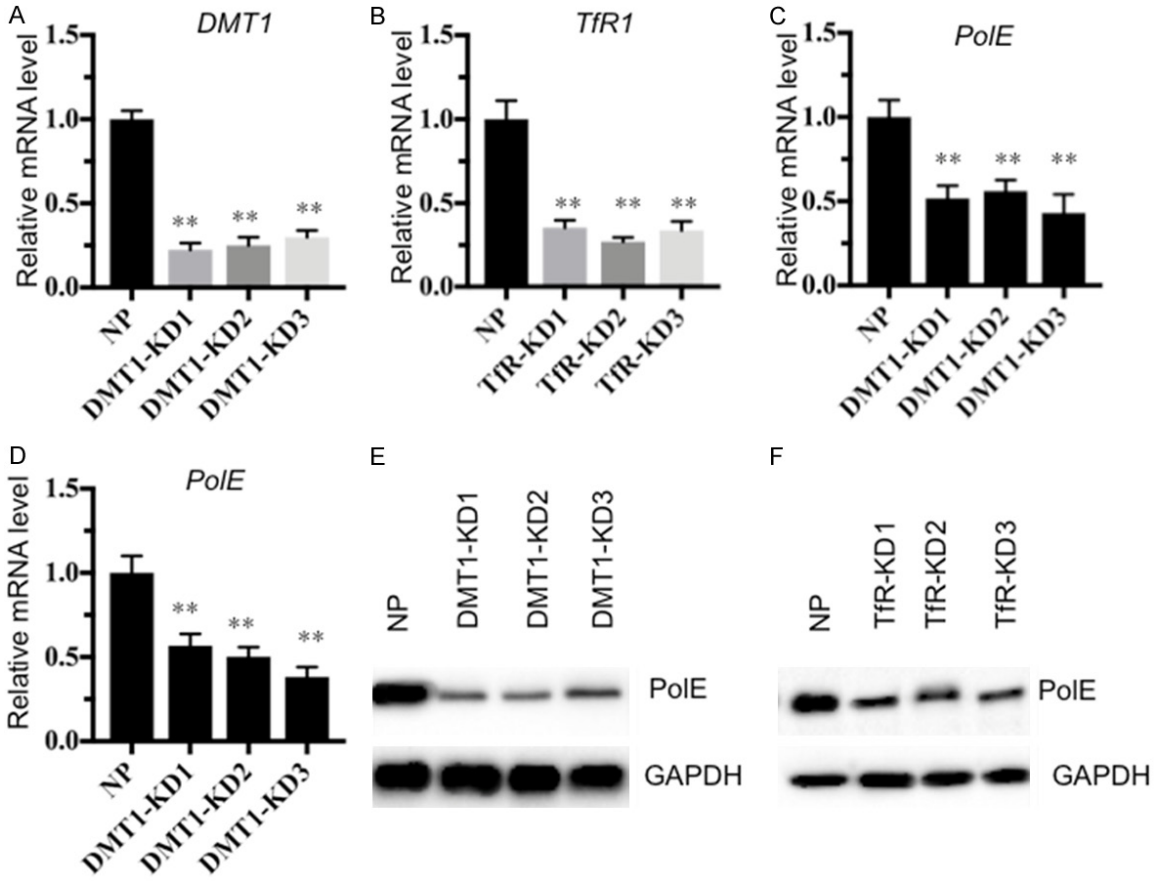

Figure 6. Knockdown of either DMT1 or TfR1 decreases PolE level. The human NP cells were infected with lentiviruses containing control shRNA, DMT1-specific shRNA or TfR1-specific shRNA. After selection in puromycin-containing medium, the control shRNA transfected cells (NP), DMT1 knockdown cells (DMT1-KD1, -2 and -3) and TfR1 knockdown cells (TfR1-KD1, -2 and -3) were subjected to examine mRNA and protein levels of PolE. (A) The relative mRNA level of DMT1 in NP, DMT1-KD1, -2 and -3 cells. (B) The relative mRNA level of TfR1 in NP, TfR1-KD1, -2 and -3 cells. (C-F) The relative mRNA and protein levels of PolE in NP, DMT1 knockdown cells (DMT1-KD1, -2 and -3) and TfR1 knockdown cells (TfR1-KD1, -2 and -3). $**P < 0.001$.

OE cells, had similar cell population distributions (**Figure 7B-F** and Supplementary Figure 4). These results suggested that the knockdown of DMT1 or TfR1 resulted in lower intracellular iron concentration, which disrupted the assembly of Fe-S in PolE while limiting PolE function even in the condition of *PolE* overexpression.

Discussion

Iron-containing proteins have been found to be involved in numerous biological processes, such as gene transcription, DNA damage repair, and cell cycle regulation [1, 2]. Systemic and intracellular iron balance is essential for maintaining the function of these iron-containing proteins [1, 2]. In this study, we examined the serum of IDD patients and found that there is a correlation between the decrease in iron concentration and IDD pathology. In normal NP cells, iron enters the cell mainly through the absorption of DMT1 and TfR1 and is then utilized by iron-containing proteins (**Figure 8A**). In NP cells of IDD patients, iron that can be absorbed by DMT1 and TfR1 is reduced, which inevitably affects the normal function of a series of proteins including PolE. Once PolE is unable to assemble into a functional protein without iron, its associated complex will fail to complete DNA replication and cause apoptosis (**Figure 8B**).

In this study, we first found that serum iron concentration in patients with severe IVD degeneration was also very low (**Figure 1**). Although we

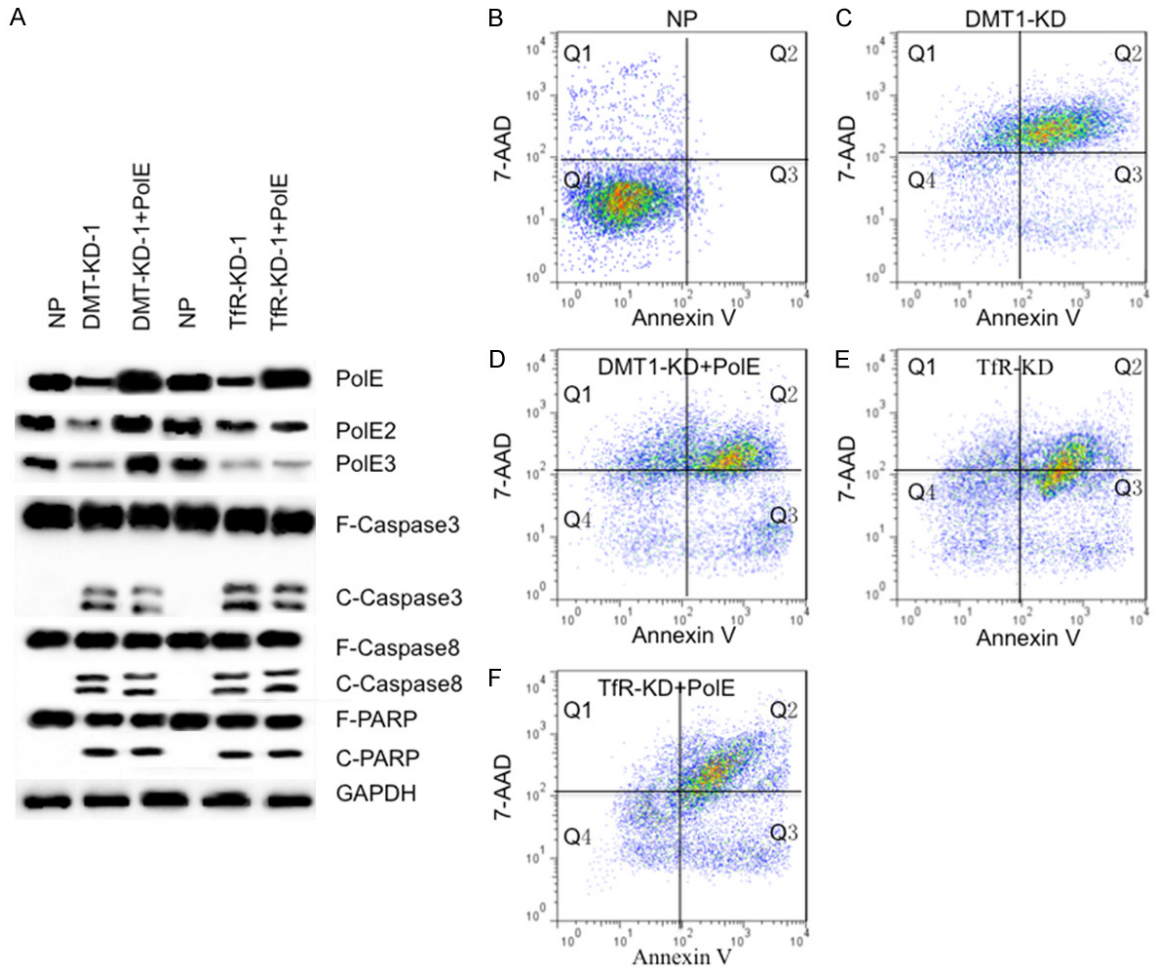

Figure 7. Overexpression of PolE in DMT1 or TfR1 knockdown cells is unable to reduce apoptosis. The DMT1-KD1 and TfR1-KD1 cells were transfected with pCDNA3-2xFlag or pCDNA3-PolE-2xFlag plasmid, respectively, to obtain *PolE* overexpression cell lines (DMT1-KD1+PolE and TfR1-KD1+PolE). Then, these two cell lines, together with NP, DMT1-KD1 and TfR1-KD1 cells were subjected to immunoblots to examine protein levels of PolE, PolE2, PolE3, Caspase-3, Caspase-8 and PARP. GAPDH was used as a loading control (A). The same cells used in (A) were also collected and stained with Annexin V-PE/7-AAD, followed by flow cytometry analysis (B-F). (B) NP cells; (C) DMT1-KD1 cells; (D) DMT1-KD1+PolE cells; (E) TfR1-KD1 cells; and (F) TfR1-KD1+PolE cells.

did not know the causal relationship between the two variables at the beginning, we speculated that the decrease in ion concentration would affect the functions of some of the iron-containing proteins involved in the IDD pathology. In order to clarify which iron-containing protein is affected, the easiest method we performed was detecting the gene expression in the IDD samples by microarray analysis and examining if there was the iron-containing gene in genes with aberrant expression. Fortunately, we found that *PolE*, an iron-containing gene, was significantly downregulated in severe IDD samples, and we also found some genes that could form complexes with PolE, such as *PolE2*, *PolE3*, *PolE4*, *CIAO1*, and *MMS19*. In contrast, the expression of some apoptotic genes, such as *Caspase-3*, *-8* and *-9,* was significantly increased (**Figure 2A**). By supplementation or depletion of iron, we also observed the opposite PolE protein level and apoptotic status (Supplementary Figure 1 and **Figure 3**), which clearly demonstrated that iron determined PolE function and IDD pathology. Although it is easy to expect that the decrease of ion concentration will affect functions of many iron-containing proteins, in this study, we did not detect whether the expression of other iron-containing genes was also affected, except for *PolE*. In future studies, we will examine in depth wheth-

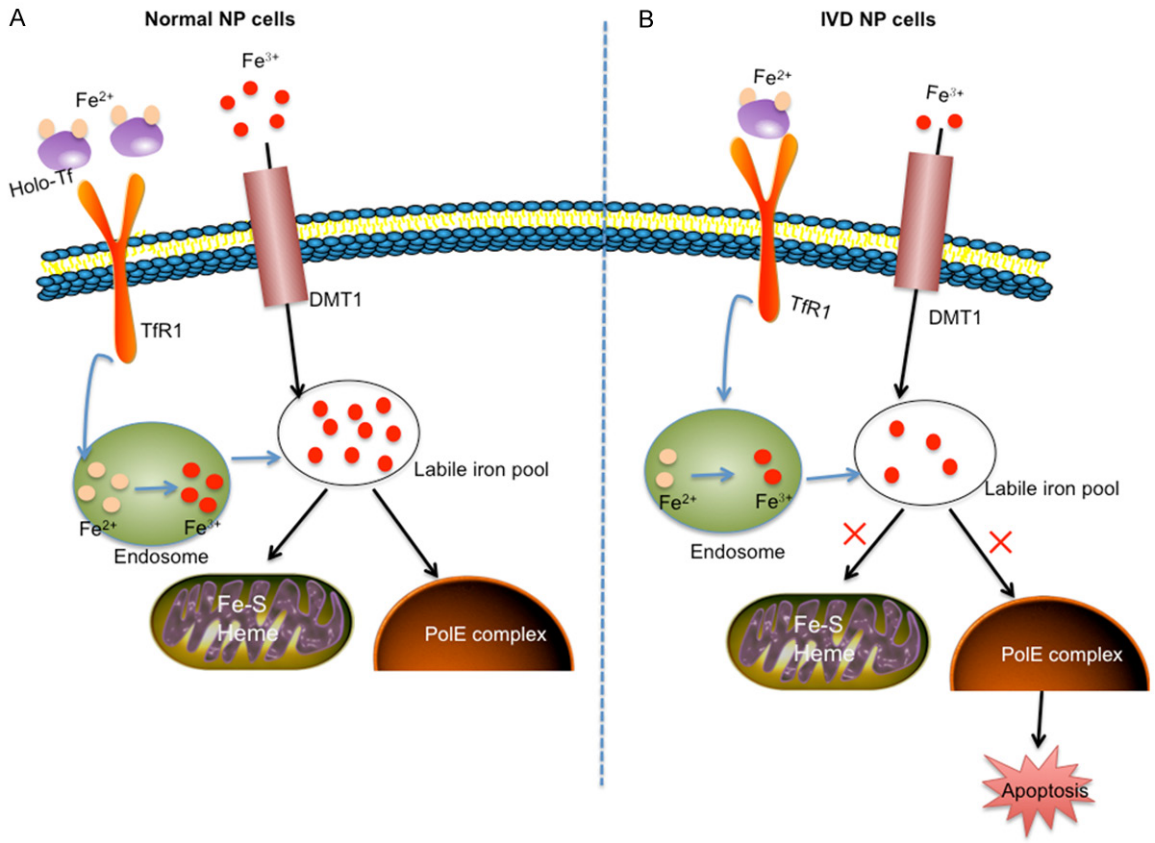

Figure 8. Schematic model of iron deficiency in the process of IDD. A. Iron absorption and utilization in normal cells. In normal cells, DMT1 and TfR1 transport ferric iron (Fe^{3+}) and ferrous iron (Fe^{2+}) into cell, respectively. Ferrous iron is further transported across the endosomal membrane to form Fe^{3+}. The newly acquired iron is stored into cytosolic "labile iron pool" (LIP). The LIP can be utilized by iron-containing proteins that localize in different cellular compartments. B. Iron absorption and utilization in degenerative NP cells. Iron deficiency makes DMT1 and TfR1 unable to transport enough iron into the cell, which limits the assembly of iron-containing proteins. Take PolE as an example, when iron level is low in the nucleus, PolE cannot complete DNA damage and repair, which leads to the occurrence of apoptosis.

er other iron-containing proteins are associated with IVD degeneration. In addition, we only found a dramatic decrease in PolE, but not POLA1 and POLD1 (data not shown), as well as two other catalytic subunits of B family DNA polymerases, which also need iron to sustain their functions. We currently do not know how cells only utilize PolE, but not POLA1 and POLD1, in the process of IDD. One possible mechanism is involved in telomere replication, in which PolE functions in the leading strand to replicate DNA continuously, while POLD1 plays a role in the lagging strand replicating DNA discontinuously.

Another very interesting phenomenon is that the decrease in the PolE protein level was accompanied by apoptosis. In order to verify this causal relationship, we knocked down PolE in NP cells and found that it could also cause apoptosis. Therefore, we conclude that down-regulation of PolE caused apoptosis and present the first case to reveal that PolE knockdown results in apoptosis. Although we do not yet know how apoptosis occurs in PolE-deficient cells, it has been reported that PolE is involved in DNA replication, recombination and repair synthesis, which may imply the underlying mechanism. By knocking down DMT1 or TfR1 in NP cells, we also observed a decrease in PolE protein levels and apoptosis. However, overexpression of PolE in these cells did not restore PolE2 and PolE3 protein levels or decrease apoptosis, which suggests that the intracellular iron concentration is the most important factor determining PolE function. In the future, studies should construct and screen PolE knockout cell lines and then transfect a PolE mutant that

has a deleted Fe-S binding domain to verify that overexpression of PolE (Fe-S)-deficient mutant cannot reduce apoptosis.

Conclusion

In this study, we found that iron deficiency is an important factor leading to the pathogenesis of IDD. Iron deficiency can lead to the disabling of PolE and disrupt the formation of DNA polymerase epsilon complex, which in turn cannot complete DNA damage repair, thus resulting in apoptosis. The supplementation of appropriate iron to IVD-degenerative patients may be beneficial in alleviating the degeneration process.

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Disclosure of conflict of interest

None.

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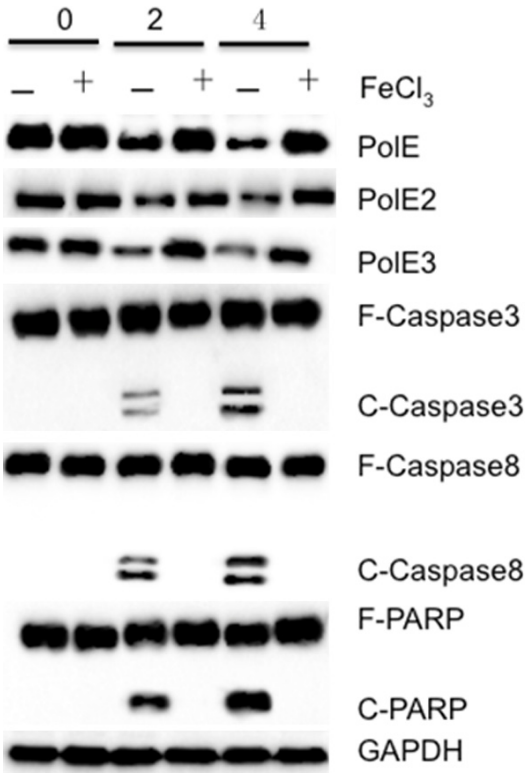
Supplementary Table 1. The clinicopathological futures of IVD patients

Patients	Age	Gender	Pfirrmann grade
1	44	F	0
2	36	M	0
3	42	M	0
4	40	F	0
5	46	M	0
6	50	F	0
7	42	F	0
8	33	M	0
9	38	M	0
10	36	M	0
11	38	F	0
12	39	M	0
13	42	F	0
14	42	F	0
15	41	M	0
16	43	F	0
17	46	M	0
18	47	M	0
19	48	M	0
20	50	F	0
21	37	F	0
22	51	M	0
23	37	F	0
24	45	F	0
25	35	M	1
26	37	M	1
27	43	M	1
28	44	M	1
29	39	M	1
30	40	M	1
31	41	F	1
32	45	F	1
33	47	F	1
34	50	F	1
35	43	M	1
36	44	F	1
37	45	M	1
38	51	M	1
39	43	M	1
40	32	F	1
41	37	F	1
42	36	F	1
43	39	F	1
44	40	F	1
45	45	F	1
46	51	M	1
47	53	M	1
48	38	M	1
49	45	M	2
50	46	M	2
51	43	M	2
52	46	M	2
53	47	F	2
54	49	F	2
55	43	M	2
56	51	F	2
57	43	F	2
58	56	F	2
59	53	F	2
60	42	F	2
61	45	F	2
62	57	M	2
63	54	M	2
64	53	M	2
65	55	M	2
66	43	M	2
67	54	M	2
68	43	F	2
69	42	F	2
70	47	F	2
71	48	F	2
72	55	F	2
73	57	F	3
74	60	M	3
75	43	M	3
76	47	M	3
77	55	M	3
78	57	M	3
79	65	M	3
80	44	F	3
81	37	F	3
82	39	F	3
83	37	F	3
84	45	F	3
85	43	F	3
86	40	F	3
87	48	F	3
88	54	F	3
89	53	F	3
90	42	F	3
91	60	F	3
92	55	M	3
93	43	M	3
94	47	M	3
95	42	M	3
96	41	M	3
97	39	M	4

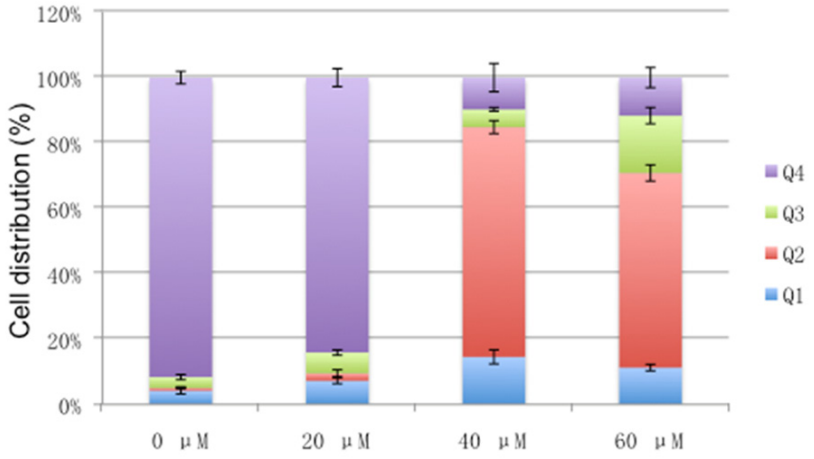
98	44	M	4	122	64	M	5
99	47	M	4	123	58	M	5
100	48	M	4	124	67	F	5
101	64	M	4	125	66	F	5
102	68	M	4	126	55	F	5
103	64	F	4	127	60	F	5
104	63	F	4	128	62	F	5
105	62	F	4	129	56	M	5
106	61	F	4	130	54	M	5
107	54	F	4	131	53	M	5
108	55	F	4	132	46	M	5
109	53	F	4	133	58	M	5
110	60	F	4	134	62	F	5
111	44	F	4	135	64	F	5
112	42	F	4	136	68	F	5
113	47	M	4	137	73	F	5
114	62	M	4	138	76	F	5
115	59	M	4	139	56	F	5
116	64	M	4	140	67	M	5
117	45	M	4	141	69	F	5
118	63	M	4	142	55	M	5
119	44	F	4	143	59	M	5
120	55	M	4	144	58	F	5
121	71	M	5				

Supplementary Table 2. Primers used for qRT-PCR analyzes

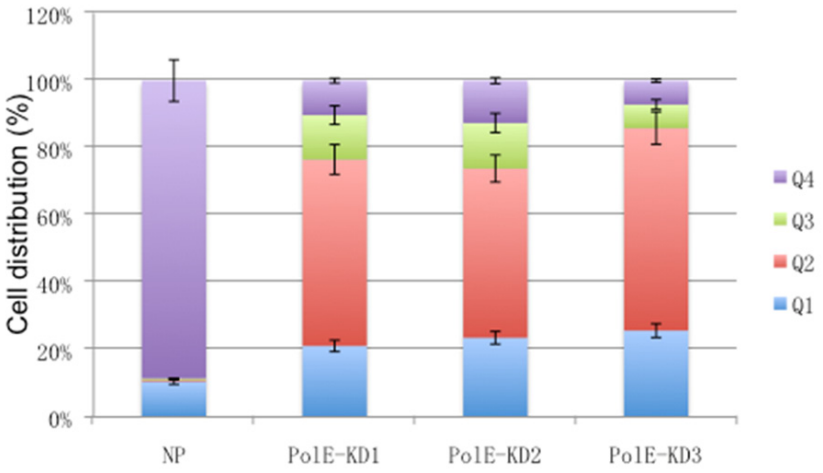
Gene	Forward primers	Reverse primers
PolE	5'-TGCCATCGAGATGACGCTGGT-3'	5'-CGTAGTGCTGGGCAATGTTCC-3'
PolE2	5'-GCAGCAGTCCAGGAATGCAG-3'	5'-TGCCTGTGGGTCCTCTGG-3'
PolE3	5'-GTGCTCTCAGCCATGGAAGA-3'	5'-TCTGTTCTTCTTCTTCCAGCC-3'
Caspase3	5'-TGCTACAATGCCCCTGGATCT-3'	5'-GCTCAGGCTCAAACCATCTACT-3'
Caspase8	5'-TGCAGGGGCTTTGACCACGA-3'	5'-TCAGTGCCATAGATGATGCCC-3'
DDX59	5'-CAGCTAGCAAGCCAGCTTCT-3'	5'-CCTGTGATTTTCTGAACGGCTT-3'
DMT1	5'-GGTCATAAAGGCACTCTGTG-3'	5'-TGCCATTTAATTGGAAGGAGT-3'
TfR1	5'-GGTCTGACACGTCTGCCTAC-3'	5'-CCCTAGGCTGTGCTCACTTC-3'
β-Actin	5'-GATGAGATTGGCATGGC-3'	5'-GACACATTCAAGTGAGGCTG-3'



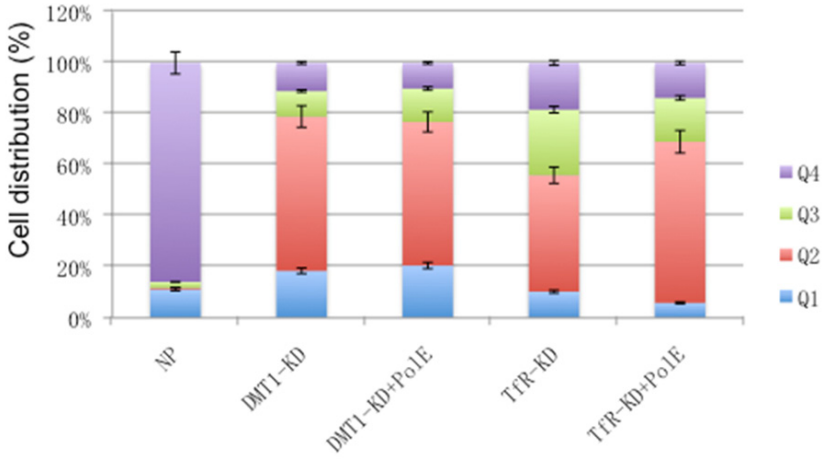
Supplementary Figure 1. PolE protein level is decreased in IDD patients. The total protein extracts from IDD patients who underwent different Pfirrmann grades (from 0 to 5) were subjected to immunoblots to examine protein levels of PolE, POLE2, POLE3, Caspase-3, Caspase-8 and PARP. GAPDH was used as a loading control.



Supplementary Figure 2. DFO treatment increases cell apoptosis. The cell distributions in **Figure 4B-E** were summarized.



Supplementary Figure 3. PolE knockdown increases cell apoptosis. The cell distributions in **Figure 5B-E** were summarized.



Supplementary Figure 4. PolE overexpression cannot reverse the defects caused by DMT1 or TfR1 knockdown. The cell distributions in **Figure 7B-F** were summarized.