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Molecular Therapy
Original Article

GRP94 is indispensable for definitive endoderm specification of human induced pluripotent stem cells

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Human induced pluripotent stem cell (hiPSC)-derived insulin-producing β cell therapy shows promise in treating type 1 diabetes and potentially type 2 diabetes. Understanding the genetic factors controlling hiPSC differentiation could optimize this therapy. In this study, we investigated the role of glucose-regulated protein 94 (GRP94) in human β cell development by generating *HSP90B1*/GRP94 knockout (KO) hiPSCs, re-expressing GRP94 in the mutants and inducing their β cell differentiation. Our results revealed that GRP94 depletion hindered β cell generation by promoting cell death induced by endoplasmic reticulum (ER) stress and other stressors during definitive endoderm (DE) differentiation. Moreover, GRP94 deletion resulted in decreased activation of WNT/β-catenin signaling, which is critical for DE specification. Re-expression of GRP94 in GRP94 KO iPSCs partially reversed DE differentiation deficiency and alleviated cell death. These findings highlight the previously unrecognized indispensable role of GRP94 in human DE formation and consequent β cell development from hiPSCs. GRP94 mitigates ER stress-induced cell death and regulates the WNT/β-catenin signaling pathway, which is both crucial for successful β cell differentiation. These results provide new insights into the molecular mechanisms underlying β cell differentiation from hiPSCs and suggest that targeting GRP94 pathways could enhance the efficiency of hiPSC-derived insulin-producing cell therapies for diabetes treatment.

INTRODUCTION

Destruction of pancreatic β cell survival and function are significant features of type 1 diabetes (T1D) and type 2 diabetes (T2D). Genetic variants or predispositions contribute to the pathogenesis of T1D and T2D by combining with environmental factors.[1,2] Genome-wide association studies have identified more than 700 risk loci for T2D[3] and more than 50 genetic variants associated with T1D.[4] Recent genomic research has revealed that most genes linked to heightened vulnerability to diabetes are intricately intertwined with the regulation of β cell growth and function throughout embryonic and fetal development, highlighting their role in pancreatic β cell development.[5] Understanding the high-risk genes that cause defects in pancreatic β cell development could pave the way for more targeted and effective treatments for diabetes.

Glucose-regulated protein 94 (GRP94), or GP96, is a chaperone protein of the heat shock protein 90 (HSP90) family, which is encoded by the *HSP90B1* gene in humans. It resides in the endoplasmic reticulum (ER) of a cell and is involved in regulating vital biological functions as an ER chaperone. GRP94 also plays an essential role in protein folding, quality control of secretory proteins, calcium binding, and other biological functions via interactions with either clients or co-factors.[6] Evidence from animal models indicates that GRP94 is essential for mouse embryonic development, β cell function, and insulin secretion. Deleting the *HSP90B1* gene in mice led to embryonic lethality by gestational day 7, before gastrulation and mesoderm formation.[7] Deficient GRP94 activity results in a substantial loss of intracellular proinsulin and reduced insulin secretion.[8,9] Our previous studies also demonstrated that mice in which GRP94 was deleted in pancreatic and duodenal homeobox 1 (PDX1)-expressing cells exhibited pancreatic hypoplasia at embryonic days E16.5 to E18.5 and had significantly reduced β cell mass at 4 weeks after birth.[10] These studies using mouse models provide insight into pancreatic development and β cell physiology. However, the exact role of GRP94 in human β cell development remains largely unknown, mainly because of the inherent limitations of animal models due to critical differences with humans at the genetic and physiological levels.[11,12]

Primary human islets isolated from the pancreases of cadaveric donors are essential sources for studying the effect of genetic variation on islet function.[13] However, the sources of human islets are scarce, and they often exhibit considerable variability after isolation.[14,15] Moreover, it is challenging to culture isolated human islets for extended periods, and this disadvantage limits the capabilities to

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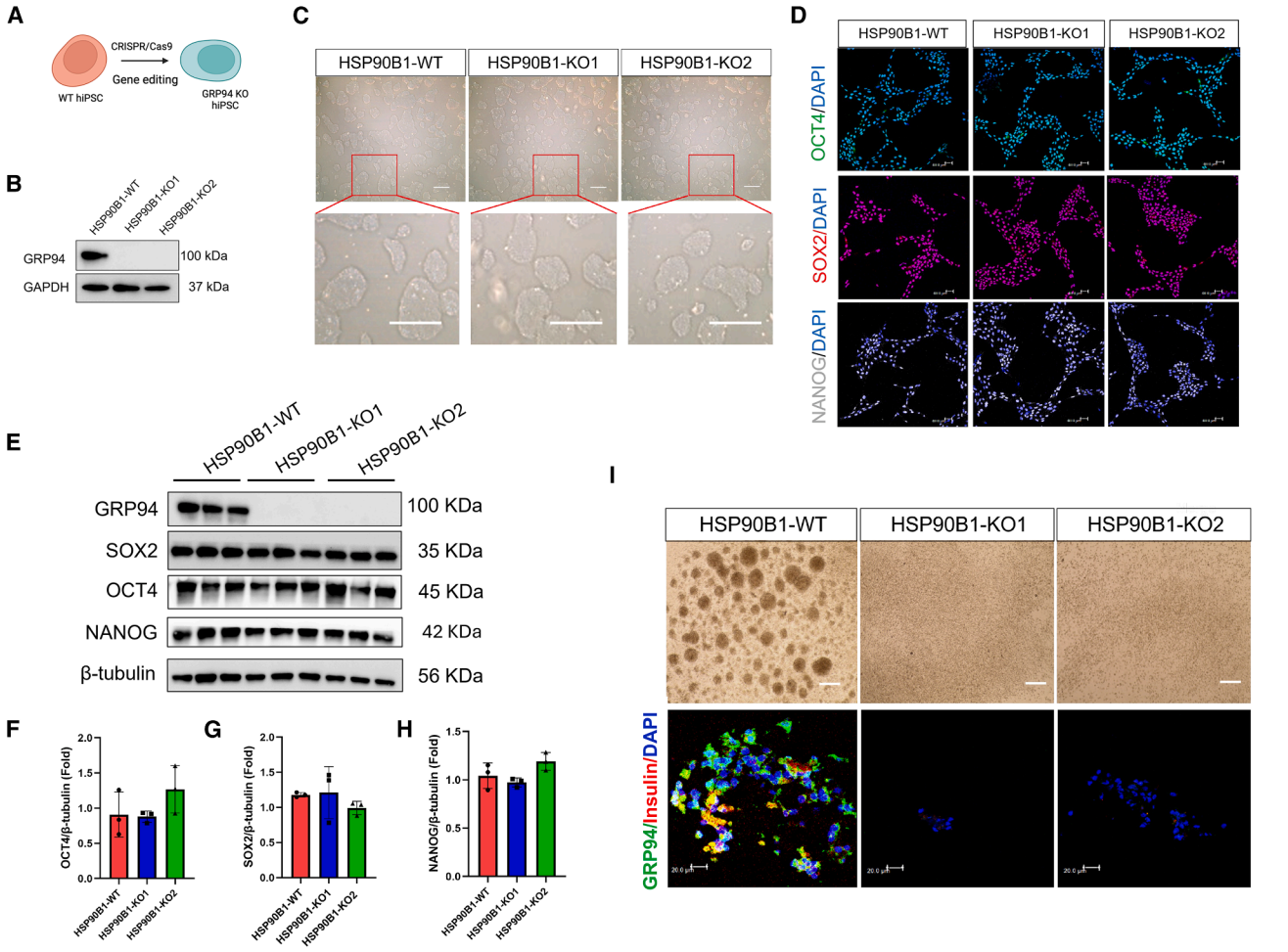

Figure 1. GRP94 KO hiPSCs failed to differentiate into insulin$^+$ β cells

(A) Schematic of *HSP90B1*/GRP94 KO cell generation by CRISPR-Cas9 gene editing. (B) Western blot analysis illustrating GRP94 expression levels in HSP90B1-KO1, HSP90B1-KO2, and HSP90B1-WT cells. (C) Morphology of HSP90B1-KO1, HSP90B1-KO2, and HSP90B1-WT cells observed under the light microscope. Scale bar, 200 μm. (D) Immunostaining analysis of Oct4, Sox2, and Nanog in HSP90B1-KO1, HSP90B1-KO2, and HSP90B1-WT iPSCs. Scale bar, 40 μm. (E–H) Western blot analysis and quantification (relative to β-tubulin) of the pluripotent markers in HSP90B1-KO1, HSP90B1-KO2, and HSP90B1-WT cells. (I) Morphology (top) and immunostaining (bottom) of GRP94 and insulin in iPSC-derived β cells after stage 7 in HSP90B1-KO1, HSP90B1-KO2, and HSP90B1-WT cells. Green, GRP94; red, insulin; blue, DAPI. Scale bars, top: 200 μm and bottom: 20 μm.

manipulate them to study the impact of genetic variants.[16] In contrast, human induced pluripotent stem cells (hiPSCs), provide *in vitro* models of inaccessible human cell types, yielding new insights into disease mechanisms. Generating pancreatic endocrine-like cells from iPSCs represents an approach to investigating genetic defects leading to impaired β cell development and function.[16,17]

In this study, we investigated the role of GRP94 in human β cell development by generating *HSP90B1* knockout (KO) hiPSCs. These cells facilitated a comparison of definitive endoderm (DE) and β cell differentiation between the KO and wild-type (WT) hiPSCs. The GRP94 KO cells with exogenous GRP94 expression were used to confirm the role of GRP94 during DE differentiation.

RESULTS

GRP94 deletion prevents β cell differentiation of human iPSCs

We previously reported that deletion of GRP94 in Pdx1$^+$ or insulin$^+$ cells in mice reduced β cell mass.[10] To decipher the potential role of GRP94 in human β cell development, we generated two *HSP90B1* KO hiPSCs, HSP90B1-KO1 and HSP90B1-KO2, using CRISPR-Cas9 gene editing in K3 iPSCs (Figure 1A). The HSP90B1-KO1 line carried a 21-bp deletion in one allele and a 1,034 bp deletion in the other, while the HSP90B1-KO2 line had a 13- and 14-bp deletions in each allele, respectively (Figures S1A and S1B). These deletions led to frameshift mutations and complete loss of GRP94 protein expression in both KO lines (Figure 1B). Despite the absence of GRP94, both HSP90B1-KO1 and HSP90B1-KO2 iPSC lines

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maintained typical hiPSC morphology (Figure 1C), with similar expressions of pluripotency markers, including OCT4, SOX2, and NANOG, as assessed by immunostaining analysis (Figure 1D) and western blot and quantification (Figures 1E–1H).

To assess the role of GRP94 in human β cell development, we compared β cell differentiation between HSP90B1-KO1 and HSP90B1-KO2, with HSP90B1-WT control iPSCs. The differentiation of β cells from HSP90B1-WT cells was induced using a revised protocol based on previous publications.[18,19] The process was characterized by the presence of transcription factors or markers specific for key stages during β cell differentiation: DE (FoxA2 and SOX17), pancreatic progenitor PDX1 and NKX6.1), and mature β cell (C-peptide) (Figure S2A). Further confirmation of β cell lineage was obtained through GFP expression in pGreenZeo differentiation reporter transfected iPSCs and positive insulin staining, both at stage 7 of differentiation (Figures S2B and S2C). In contrast, both HSP90B1-KO1 and HSP90B1-KO2 exhibited distinct morphological differences during differentiation, notably failing to form organoids from stage 5, compared with HSP90B1-WT cells (Figure 1I, top). Immunostaining also revealed a complete absence of insulin$^+$ β cells in the mutated lines at stage 7 (Figure 1I, bottom). These results suggest that deletion of GRP94 in hiPSCs impairs their ability to differentiate into β cells.

GRP94 deletion prevents DE formation of hiPSCs

DE differentiation is the first major checkpoint in the process of β cell differentiation of hiPSCs.[20] To investigate the underlying mechanism of a β cell differentiation defect in GRP94 KO cells, we first compared the DE differentiation potential of two mutants with HSP90B1-WT cells. qPCR analysis revealed a significant reduction in the mRNA expression of DE-associated genes, including *FOXA2*, *GATA4*, *FOXA1*, *CXCR4*, and *SOX7*, in both HSP90B1-KO1 and HSP90B1-KO2 at 72 h of DE differentiation, compared with WT controls (Figure 2A). However, we did not observe any difference in the mRNA expression of *GATA6*, another critical transcriptional factor involved in endoderm specification,[21] between the two mutants and WT cells. In addition, we measured the expression of SOX17, a master regulator that initiates and drives the early stage of DE differentiation from iPSC.[22] Flow cytometry and immunostaining analysis confirmed a significant reduction of SOX17$^+$ cells (Figures 2B and 2C) and FOXA2 (Figure 2E) in HSP90B1-KO1 and HSP90B1-KO2, compared with HSP90B1-WT cells. Together, these findings indicate that GRP94 deletion impairs DE deficiency in hiPSCs.

During gastrulation in embryogenesis, DE is generated alongside mesoderm and ectoderms from pluripotent epiblast cells.[20,23] To investigate whether the differentiation defect induced by GRP94 deletion is specific to DE differentiation, we compared mesoderm and ectoderm differentiation potentials of two mutants with HSP90B1-WT cells. Significant decreases of the mesoderm markers *TBXT* and *BMP2* were observed at both the mRNA and protein levels in the mutants compared with HSP90B1-WT cells after 48 h of differentiation (Figures 2A and 2F–2H). In contrast, no significant changes were detected in the mRNA levels of *BMP4*, *CDH2*, and *FLK1* (Figure 2A), despite their previously reported roles as important regulators in mesodermal differentiation of stem cells.[24–26] In contrast, no significant differences were observed in ectoderm mRNA expression, including *NEUROD1*, *MSI1*, and *OTX2* (Figure 2A), or percentage of OTX2-positive cells (Figures 2I–2K) between two mutants and HSP90B1-WT cells during ectoderm differentiation, suggesting that GRP94 is not essential for ectoderm differentiation of hiPSCs. Therefore, GRP94 preferentially regulates both DE and mesoderm differentiation of hiPSCs, with a more pronounced role in DE differentiation.

RNA sequencing analysis of gene expression regulated by GRP94 during DE differentiation

To further elucidate the mechanisms underlying the DE differentiation defects associated with GRP94 deletion, we conducted bulk RNA sequencing (RNA-seq) analysis to compare the global gene expression profiles between two HSP90B1-WT hiPSC lines (K3 and SV20) and two GRP94-deleted lines (HSP90B1-KO1 and HSP90B1-KO2). GRP94 deletion resulted in changes in gene expression (Figure 3A), with 976 genes exhibiting significantly altered expression ($p_{adjusted} < 0.05$) (Figure 3B), which were selected for further analysis.

Gene Ontology (GO) enrichment analysis of the 585 downregulated genes revealed significant involvement in various biological processes, cellular components, and molecular functions. Biological process analysis identified a prominent enrichment in genes associated with the response to ER stress and others (Figure 3C). Cellular component analysis indicated that most genes were associated with the ER lumen and others (Figure 3C). Molecular function analysis highlighted enrichment in scaffold protein binding, phosphatidylinositol bisphosphate kinase activity, and several other functions (Figure 3C). Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis of the downregulated genes identified enriched pathways related to protein processing in ER, endocrine resistance, calcium signaling, and signaling pathways regulating pluripotent of stem cells, as well as other related pathways (Figure 3D). For the 391 upregulated genes, GO analysis revealed enrichment in processes related to response to extracellular stimulus, components of the proteinaceous extracellular matrix, and the ER lumen, as well as extracellular binding (Figure 3E). KEGG analysis indicated that these genes are involved in mitogen-activated protein kinase signaling (MAPK), advanced glycation end-product (AGE) and recpetor of AGE (RAGE) signaling, and other relevant pathways (Figure 3F).

GRP94 deletion increases apoptosis and cell death upon ER stress during DE differentiation

Given the role of GRP94 in the cellular response to ER stress and protein processing, as indicated by bulk RNA-seq, we evaluated cell death, including apoptosis in HSP90B1-KO1, HSP90B1-KO2, and HSP90B1-WT cells at 72 h post DE differentiation. There was a significant increase in cell death in both mutant lines compared with

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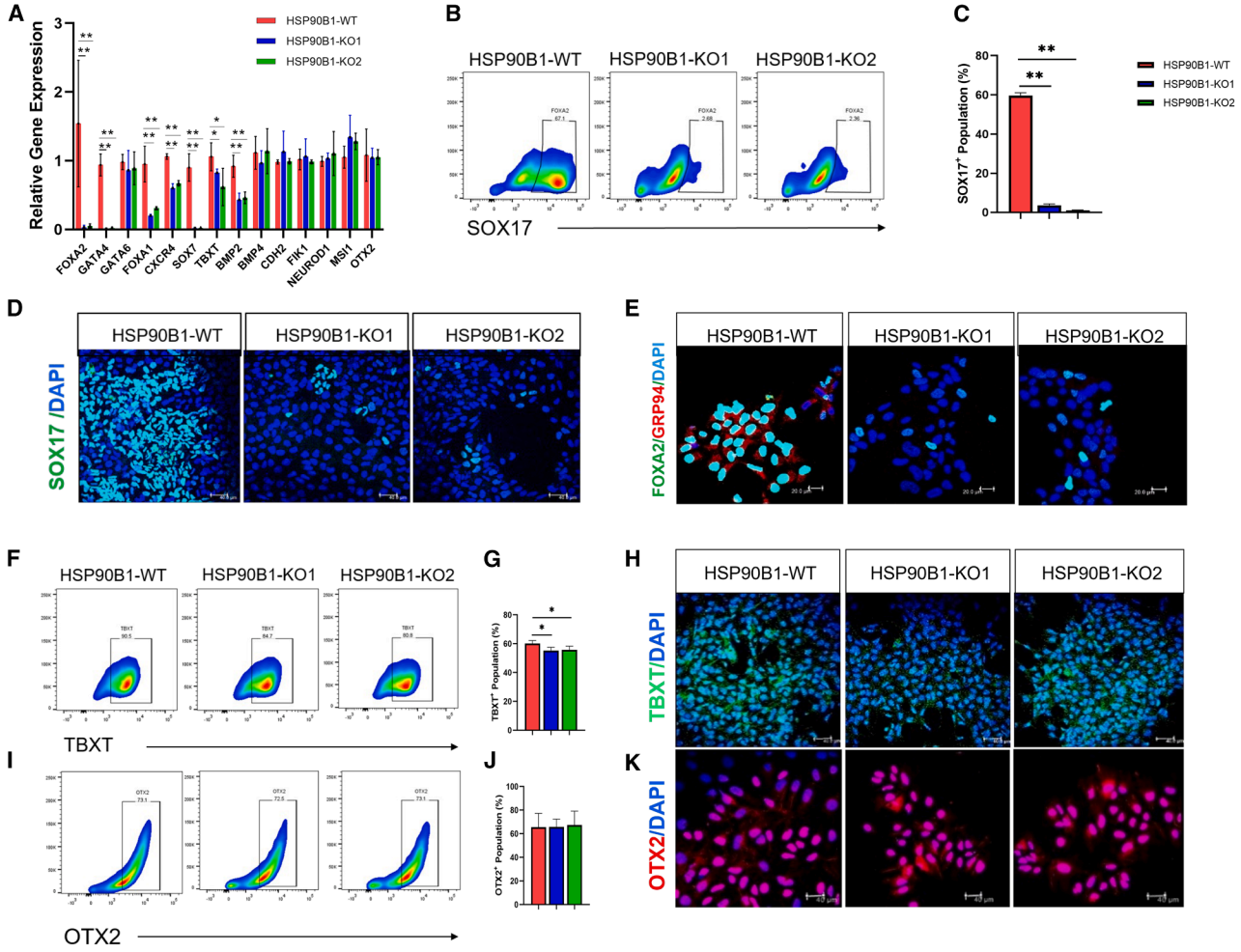

Figure 2. GRP94 deletion prevented DE differentiation of hiPSCs

(A) mRNA expressions of typical markers for DE, mesoderm, or ectoderm lineages were assessed after differentiation of HSP90B1-KO1, HSP90B1-KO2, and HSP90B1-WT cells toward DE, mesoderm, or ectoderm, respectively. Relative mRNA expression was normalized to 18S RNA. Data are presented as the mean ± SD of at least three independent experiments, each with two replicates. $^{**}p < 0.01$ and $^{*}p < 0.05$ versus HSP90B1-WT. (B and C) Flow cytometry analysis of marker for DE (SOX17) and quantification in WT and mutant cells. Immunostaining of SOX17 (D) and FOXA2 (E) in differentiated cells. Scale bar, 40 μm in D and 20 μm in (E). Flow cytometry analysis and immunostaining of mesoderm marker, TBXT (F–H), and ectoderm marker OTX2 (I–K) in WT and mutant cells. Scale bar, 40 μm. Data are presented as mean ± SD of at least three independent experiments. $^{**}p < 0.01$ and $^{*}p < 0.05$ versus HSP90B1-WT.

HSP90B1-WT (Figures 4A and 4B). Immunostaining at 24, 48, and 72 h after DE induction showed a progressive increase in cleaved caspase3 (c-Cas3) positive apoptotic cells in both mutants relative to the WT controls (Figure 4C). These results collectively suggest that GRP94 deletion promotes increased cell death, specifically apoptosis, during DE differentiation of hiPSCs.

iPSCs and DE cells are more susceptible to ER stress than other cell types, such as fibroblasts.[27] Based on RNA-seq analysis, which revealed a reduced expression of genes involved in the ER stress response (Figures 3B and 3C), we speculated that increased cell apoptosis and death observed in GRP94 deletion cells might result from an inability to maintain ER homeostasis under stress conditions. To investigate this, we measured the expression of ER stress-related proteins in undifferentiated HSP90B1-KO1, HSP90B1-KO2, and HSP90B1-WT cells. We found that critical ER stress sensors, including protein kinase RNA-like ER kinase (PERK) and activating transcription factor 6 (ATF6)[28,29] were significantly reduced in two mutant cell lines compared with HSP90B1-WT, while the expression of inositol-requiring enzyme-1α (IRE1α) and protein disulfide isomerase (PDI) was relatively unchanged (Figures 4D–4H). In DE cells derived from these undifferentiated iPSCs, we observed a marked decrease in PERK and IRE1α levels in HSP90B1-KO1, along with an increase in BiP

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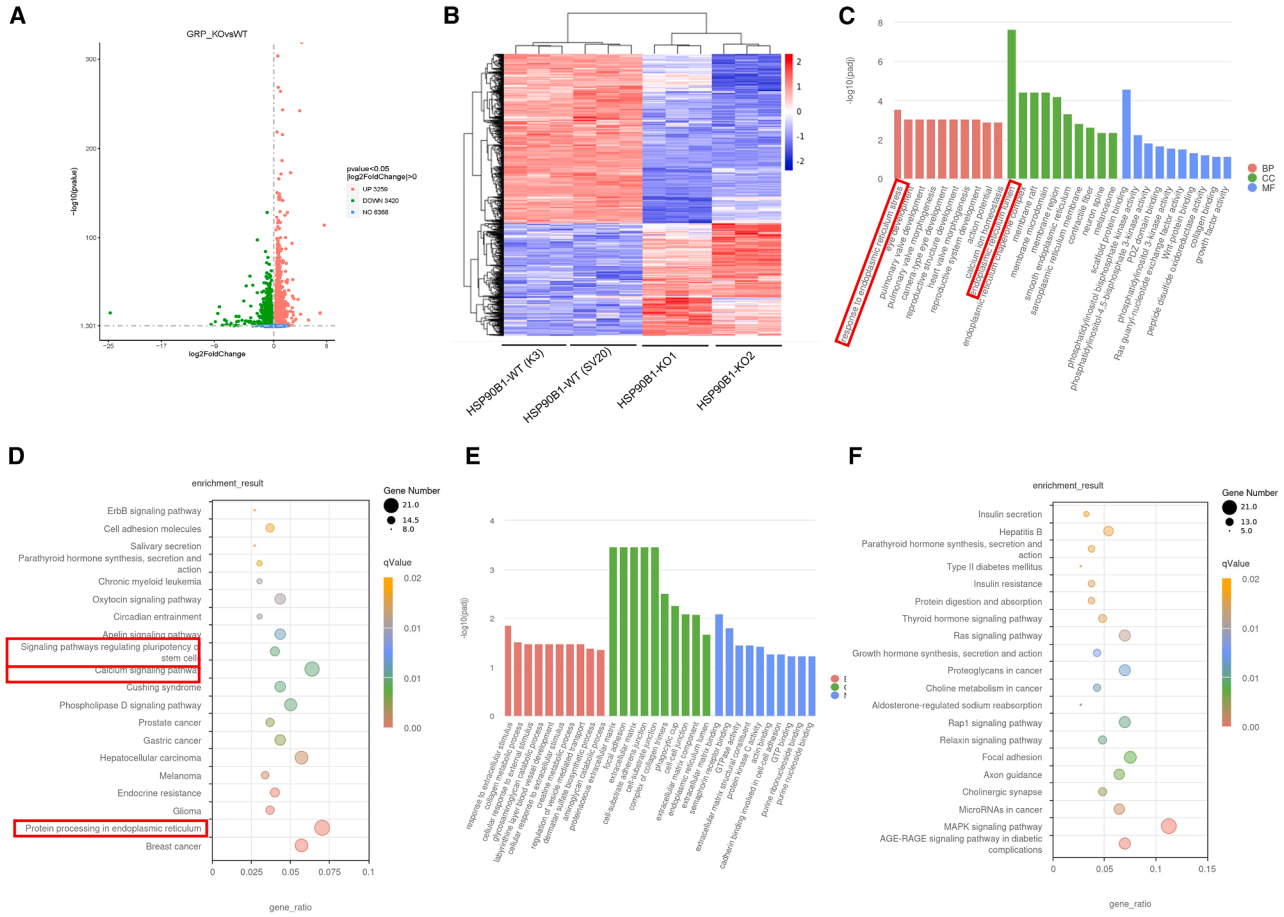

Figure 3. Bulk RNA-seq analysis showed differential gene expression between HSP90B1 KO and HSP90B1-WT iPSCs

(A) Volcano plot showing differential gene expression between HSP90B1-WT versus KO cells. (B) Heatmap of 976 significantly differentially expressed genes ($p_{adjusted} \leq 0.05$) between HSP90B1-WT and KO cells. (C) GO analysis of the 585 significantly downregulated genes in HSP90B1-WT compared with KO cells. (D) KEGG analysis of the downregulated genes. (E) GO analysis of the 391 significantly upregulated genes in HSP90B1-WT compared with KO cells. (F) KEGG analysis of the upregulated genes.

(GRP78) in comparison with HSP90B1-WT and no change in PDI expression (Figures 4I–4M). The unfolded protein response (UPR) is activated to restore ER homeostasis under stress.[30,31] Because PERK, IRE1, and ATF6 are crucial for UPR activation, these findings suggested that UPR is compromised in GRP94 KO cells. Therefore, GRP94 deletion impaired the UPR and cellular response to ER stress, contributing to increased cell death.

GRP94 deletion reduces the activation of Wnt/β-catenin signaling

To further investigate the mechanisms underlying the differentiation defect in DE cells following GRP94 deletion, we measured key regulators involved in driving DE differentiation of hiPSCs, including Smads and β-catenin, which are critical components of the transforming growth factor (TGF)-β and WNT/β-catenin signaling pathways.[13,32,33] We first examined the expression of genes integral to TGF-β signaling pathway, such as *TGFB1, TGFB2, TGFB3* and *TGFB4* receptors and *SMAD2*, *SMAD3*, *SMAD4*. However, qPCR analysis indicated no significant change in the expression of these genes (Figure 5A). Additionally, western blot and immunostaining analysis showed no marked difference in the expression or activation of *p*-Smad2 and Smad2/3 between two mutants and the HSP90B1-WT (Figures 5B, 5D, 5E, and 5H), suggesting that GRP94 deletion does not impact TGF-β signaling pathway.

We next examined the expression levels of key genes involved in the WNT pathway including *CTNNB1*, *LRP6*, and *GSK3B* in the WT and mutant cells. CTNNB1, which encodes β-catenin, is a central component of the Wnt/β-catenin signaling pathway.[34] LRP6 stabilizes β-catenin by inhibiting its phosphorylation, independent of Axin degradation.[35] GSK3β, in contrast, regulates β-catenin levels.[36,37] However, qPCR analysis indicated no noticeable change in *CTNNB1*, *LRP6*, and *GSK3B* in HSP90B1-KO1 and HSP90B1-KO2 compared with HSP90B1-WT cells by qPCR analysis (Figure 5A). Moreover, western blot analysis also showed no discernible difference in β-catenin expression between the two

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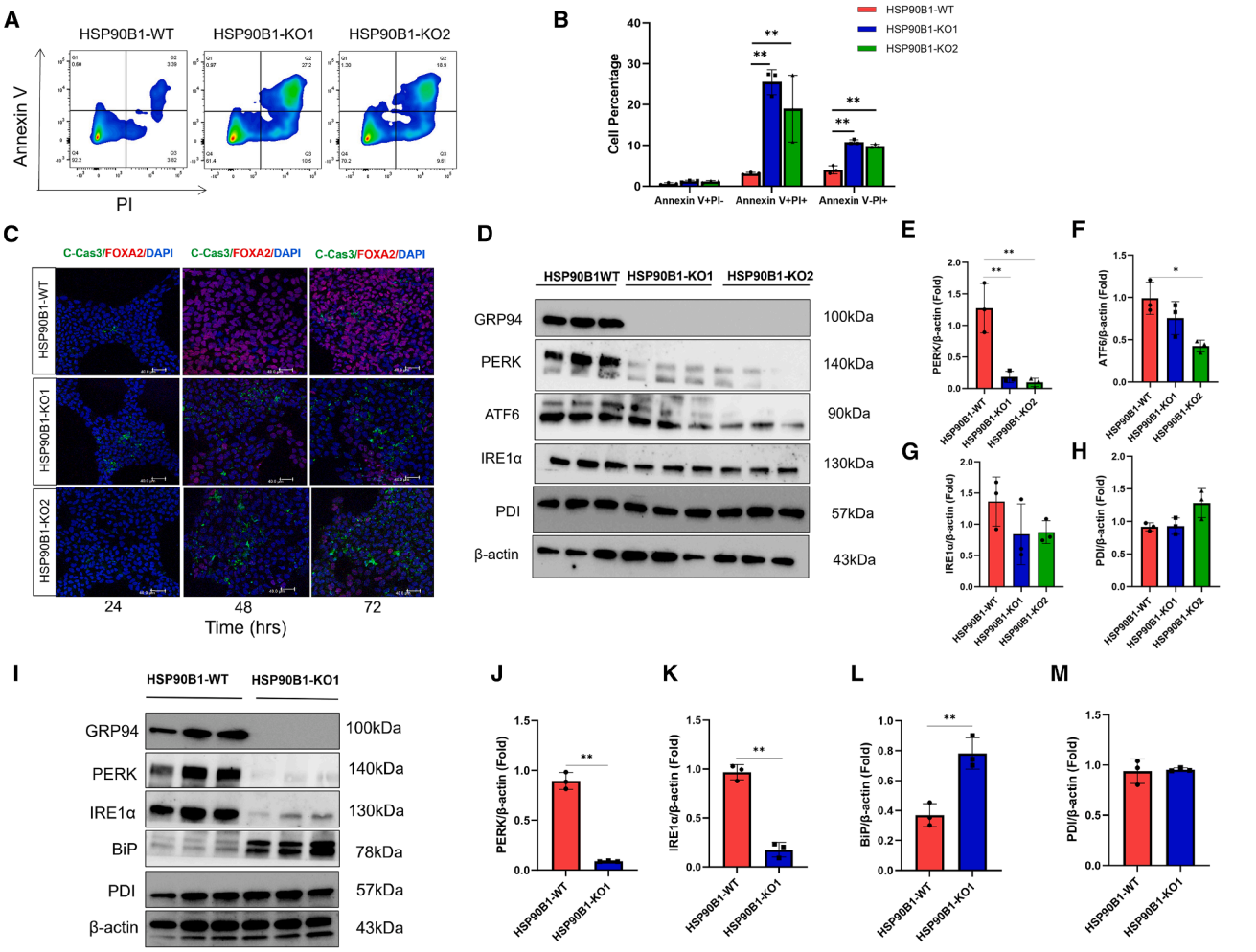

Figure 4. GRP94 deletion reduced cell response to ER stress in hiPSCs and during DE differentiation of hiPSCs

(A and B) DE differentiation of HSP90B1-KO1, KO2, and WT cells was induced, and the cell death was analyzed at 72 h of differentiation by flow cytometry. Data are presented as mean ± SD of at least three independent experiments. (C) Cells were fixed at 24, 48, and 72 h of DE differentiation, and the cleaved caspase 3 (c-Cas-3) was measured by immunostaining. Scale bar, 40 μm. Blue, DAPI; green, GRP94; red, FOXA2. (D–H) The ER proteins PERK, IRE1α, ATF6, and PD1 were measured in undifferentiated HSP90B1-KO1, HSP90B1-KO2, and HSP90B1-WT iPSCs by Western blot, quantified using β-actin as endogenous control. (I) Western blot analysis showed expression of ER-stress-related proteins at 72 h of DE differentiation of HSP90B1-KO1, and HSP90B1-WT cells. (J–M) Quantification of protein expression using β-actin as endogenous control. Triplicates have been shown.

$^{**}p < 0.01$ and $^{*}p < 0.05$ versus HSP90B1-WT.

mutants and WT (Figures 5B and 5C). However, the translocation of β-catenin from the cytosol to the nucleus was significantly decreased in HSP90B1-KO2 compared with HSP90B1-WT cells (Figures 5E–5G). The decrease in nuclear translocation of β-catenin induced by GRP94 deletion was further confirmed by immunostaining, which showed a higher amount of β-catenin in the cytosol but not in the nucleus of HSP90B1-KO1 and HSP90B1-KO2 compared with HSP90B1-WT cells before and after DE differentiation (Figures 5H and 5I). These findings suggest that GRP94 deletion inhibits the activation of the Wnt/β-catenin signaling pathway by impairing the nuclear translocation of β-catenin.

Exogenous expression of GRP94 in the GRP94 KO hiPSCs partially restored their potential for DE differentiation and rescued DE cell death

To further validate the role of GRP94 in DE differentiation, we induced exogenous GRP94 expression in HSP90B1-KO1 and HSP90B1-KO2 cells by electroporation and designated them as HSP90B1-KOR1 and HSP90B1-KOR2 (Figure 6A). GRP94 expression in these cells was confirmed by western blot and immunostaining (Figures 6B and 6C). Gene expression comparisons among HSP90B1-WT, HSP90B1-KO1, HSP90B1-KO2, HSP90B1-KOR1, and HSP90B1-KOR2 were further analyzed using bulk RNAs-eq data, including those shown in Figure 3B. Exogenous GRP94

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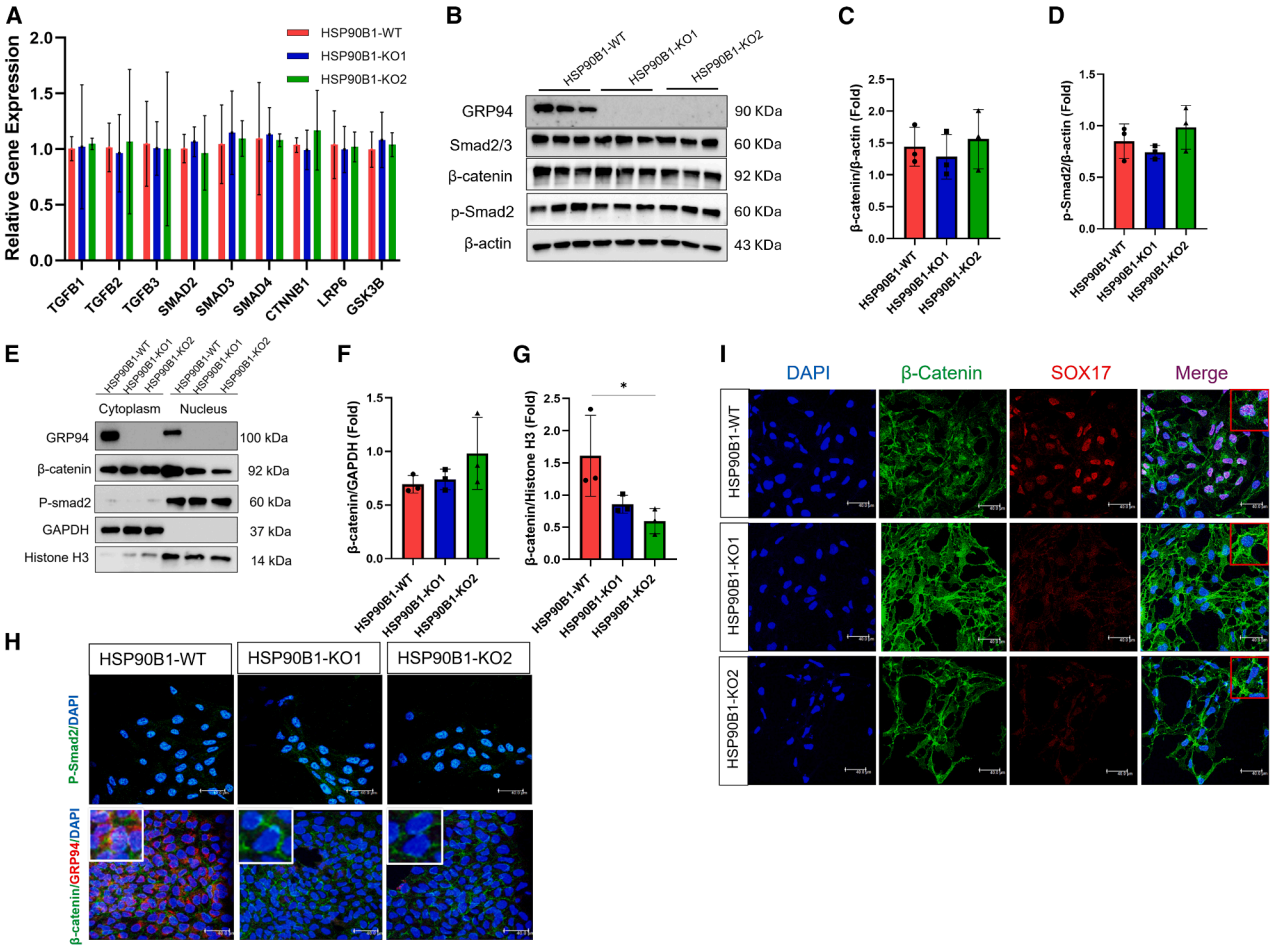

Figure 5. GRP94 deletion prevented Wnt/β-catenin signaling activation but did not affect TGF-β signaling

(A) Total RNA was extracted from HSP90B1-KO1, KO2, and WT cells, and the expression of genes involved in the TGF-β and Wnt/β-catenin signaling pathwas were analyzed by qPCR. Gene expression was normalized to 18S. Data are presented as mean ± SD at least three independent experiments and two replicates in each experiment. (B–D) Proteins involved in the TGF-β and Wnt/β-catenin signaling pathways were analyzed by Western blot quantification. (E–G) Cytoplasmic (identified by GAPDH expression) and nuclear (identified by histone H3) protein fractions of HSP90B1-KO1, KO2, and WT cells were isolated, and the *p*-Smad2 and β-catenin proteins were measured by western blot. (H) The distribution of *p*-Smad2 and β-catenin in HSP90B1-KO1, HSP90B1-KO2, and HSP90B1-WT cells was measured by immunostaining. Scale bar, 40 μm. (I) The distribution of β-catenin at 72 h of DE differentiation was detected in HSP90B1-KO1, HSP90B1-KO2, and HSP90B1-WT cells. Scale bar, 40 μm. Blue, DAPI; green, β-catenin; red, SOX17.

re-expression in both HSP90B1 KO cells partially reversed expression changes of genes induced by GRP94 deletion, which include 107 significantly downregulated and 41 upregulated genes (Figure 6D). Metascape analysis of the downregulated genes induced by GRP94 deletion, which were rescued by exogenous GRP94, revealed their involvement in processes such as the response to ER stress, intracellular protein transport, activation of chaperons by IRE1α, protein secretion, chaperon-mediated protein folding, and other processes (Figure 6E). Moreover, exogenous GRP94 expression in the KO cells led to the upregulation of genes associated with processes like DNA replication and regulation of protein polymerization, among other functions impacted by GRP94 deletion (Figure 6F).

Flow cytometry analysis showed a significant reduction in dead or apoptotic cells in HSP90B1-KO1R and HSP90B1-KO2R compared with HSP90B1-KO1 and HSP90B1-KO2 during DE differentiation (Figures 6G and 6H). These results indicate that the re-expression of GRP94 partially rescued cell death induced by GRP94 deletion. In addition, the numbers of SOX17- and FOXA2-positive cells were increased in HSP90B1-KO1R and HSP90B1-KO2R compared with HSP90B1-KO1 and HSP90B1-KO2 during their DE differentiation (Figures 6I and 6J), indicating that exogenous GRP94 re-expression partly restored DE differentiation potential induced by GRP94 deletion. Together, these findings support the essential role of GRP94 in DE differentiation of hiPSCs, likely through the regulation of cell responses to ER stress.

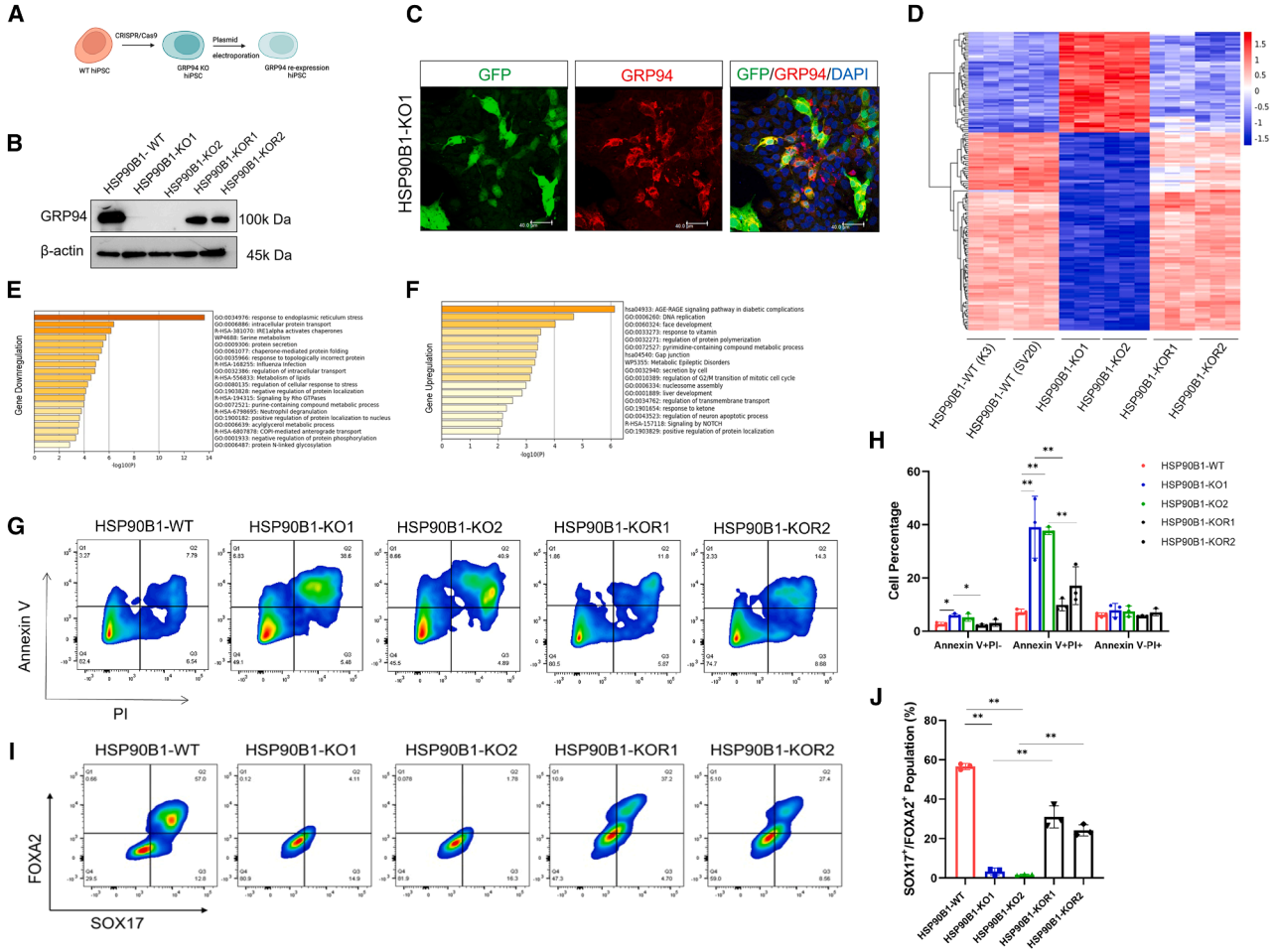

Figure 6. Exogenous expression of GRP94 decreased cell apoptosis/death and rescued DE differentiation deficiency induced by GRP94 deletion

(A) Schematic outlining the generation of GRP94 KO RE (exogenous GRP94 re-expression) cells, in GRP94 KO cells . (B) Western blot analysis of GRP94 expression in GRP94 KO cells (HSP90B1-KO1 and HSP90B1-KO2), exogenous re-expression of GRP94 cells (HSP90B1-KOR1 and R2), and wild type cells (HSP90B1-WT). (C) Immunostaining analysis of GFP and GRP94 expression in HSP90B1-KO1 cells. Scale bar, 40 μm. Blue, DAPI; green, GFP; red, GRP94. (D) Heatmap of significantly differentially expressed genes ($p_{adjusted} \leq 0.05$) in HSP90B1-WT versus GRP94 KO, and GRP94 KO versus GRP94 KO RE. WT and KO data were from Figure 3B. The top 107 downregulation and top 44 upregulation genes are represented. Triplicates are listed in each cell line. (E) Metascape functional enrichment of the 107 downregulated genes in HSP90B1-WT, GRP94 KO, and GRP94 KO RE cells. (F) Metascape analysis of the 44 upregulated genes in HSP90B1-WT, GRP94 KO, and GRP94 KO RE cells. (G and H) Cell apoptosis and death were analyzed at 72 h of DE differentiation by Annexin V and PI staining using flow cytometry. Data are presented as mean ± SD of at least three experiments. $^{**}p < 0.01$ and $^{*}p < 0.05$. (I and J) DE differentiation potential of HSP90B1-WT, GRP94 KO, and GRP94 KO RE cells was quantified by flow cytometry using FOXA2 and SOX17 analysis. Data are presented as mean ± SD of at least three experiments. $^{**}p < 0.01$ and $^{*}p < 0.05$.

DISCUSSION

While the involvement of GRP94 in mesoderm induction and muscle differentiation has been documented,[7,38,39] its role in human DE and β cell specification, as well as its underlying mechanism, remain poorly understood. Because human gastrulation and early lineage commitment cannot be studied *in vivo*, and GRP94 deletion is embryonically lethal in mouse models,[7,40] we turned to hiPSC models to investigate the critical role of GRP94 in human β cell development. Our findings demonstrate that GRP94 is indispensable for DE specification and differentiating β cells from hiPSCs. This role was further validated through the exogenous expression of GRP94, which rescued the phenotype defects associated with GRP94 deletion. These results offer valuable insights into the molecular mechanisms of human DE specification and β cell development.

The ER plays a critical role in the transport and processing of secretory, lysosomal, and transmembrane proteins, as well as in regulating various physiological processes, including cell differentiation. Disruptions in protein processing can compromise ER homeostasis, leading to ER stress.[41] In response, the cells activate an adaptive UPR to restore ER homeostasis by initiating ER stress sensors, including PREK, ATF6, and IRE1.[42] While physiological levels of

ER stresses are essential for proper cell differentiation, prolonged or excessive ER stress can inhibit differentiation and may trigger cell death.[43,44] Our study revealed that GRP94 deletion led to reduced expression of key genes involved in the UPR and ER stress pathways, including ATF6, IRE1, and PERK. This disruption of UPR caused by the absence of GRP94 contributed to increased cell death and impaired DE differentiation, likely due to the inability to maintain ER homeostasis. This impairment in DE differentiation seems to stem from disturbed UPR and ER signaling, a finding that aligns with previous research underscoring the critical role of UPR in the DE specification of mouse embryonic stem cells.[45,46]

While the regulatory role of GRP94 in Wnt/β-catenin signaling has been documented,[47] the precise mechanisms remain unclear. In this study, we assessed the impact of GRP94 on the WNT/β-catenin and TGF-β signaling pathways, both of which are required to trigger DE differentiation of hiPSCs. Although we did not observe significant changes in the expression levels of key genes involved in the WNT/β-catenin signaling pathway between WT and mutant cells, our analysis revealed a notable decrease in the translocation of β-catenin from the cytoplasm to the nucleus in the mutants. This suggests a downregulation of β-catenin activity despite the lack of significant changes in gene expression. Our RNA-seq analysis suggests that GRP94 regulates the expression of genes associated with intracellular protein transport. Consistent with this, GRP94 deletion impaired the nuclear translation of β-catenin from the cytoplasm, a defect that reversed upon re-expression of GRP94. These findings suggest that inhibiting WNT/β-catenin signaling due to GRP94 deletion is a key factor in the observed deficiency in DE differentiation of hiPSCs. It is worth noting that the activation of WNT/β-catenin signaling is also critical for mesoderm differentiation, as demonstrated in our study and previous work.[48,49] This explains the reduced mesoderm differentiation efficiency in GRP94 KO cells observed here. In addition to Wnt/β-catenin signaling, TGF-β signaling is also required for DE differentiation. However, we did not observe a significant impact of GRP94 on the TGF-β signaling during differentiation. The specific regulatory role of GRP94 in the DE differentiation stage warrants further investigation.

In previous mouse models, we observed that GRP94 deletion in Pdx-1$^+$ or insulin$^+$ cells led to pancreas atrophy and a reduction in β cell mass but did not significantly impact β cell maturation, suggesting that GRP94 is required before the endocrine progenitor stage during embryonic development.[10] However, investigating GRP94 KO during early embryonic development *in vivo* is challenging, as GRP94 KO mice embryos die by day 7 of gestation.[7] This limitation led us to investigate the role of GRP94 further using an *in vitro* human iPSC differentiation model. In the current study, we further demonstrate that GRP94 plays a critical role in survival and differentiation during DE differentiation of hiPSCs, reinforcing its indispensability in the early stage of human β cell development. Therefore, the data obtained from our *in vitro* model complement our previous *in vivo* findings, providing insights into the critical role of GRP94 across the entire β cell development process, specifically related to human DE development. These combined data also help to elucidate the potential mechanisms underlying GRP94 deficiency, which could inform strategies for targeted therapeutic interventions.

We found that the impaired DE differentiation and increase in cell death caused by GRP94 deletion were partially rescued by exogenous re-expression of GRP94 in the mutant iPSCs. This highlights the critical regulatory role of GRP94 in these processes. Additionally, RNA-seq analysis revealed that GRP94 regulates a broader range of gene expressions beyond those associated with ER stress response and protein transport. It is worth noting that re-expression of GRP94 leads to upregulations of genes involved in DNA replication and protein polymerization. The transition from pluripotency to differentiation of iPSCs is governed by complex molecular mechanisms that regulate the cell cycle and DNA replication during differentiation.[50] Specifically, it has been shown that human embryonic stem cells initiate differentiation in the early G1 phase and commit to specific lineages in the G2 phase.[51] Additionally, protein polymerization plays a critical role in cell differentiation by influencing cell shape and structure, mainly through filaments such as actin and microtubules.[52] While, to the best of our knowledge, there is no direct evidence linking DNA replication and protein polymerization to DE or β cell differentiation, we believe that investigating whether GRP94 regulates these processes during DE differentiation and β cell development is an interesting topic for future research.

GRP94 and its client proteins can serve as promising targets for improving the efficacy of cell therapy in diabetes treatment through various strategies. First, as informed by current evidence, boosting GRP94 expression and activity during stem cell differentiation into functional β cells could improve the effectiveness of stem cell-based therapies for diabetes. The second approach can focus on enhancing the function of pancreatic β cells, as GRP94 plays a critical role in proinsulin handling,[8] as well as β cell survival and function.[10] Moreover, defects in β cell proinsulin handling in GRP94 KO cells activates inflammatory pathways and sensitizes β cells to immune attack.[53] By modulating GRP94 expression in these cells, either through gene editing or small molecule modulators, insulin secretion, sensitivity, and β cell survival could be enhanced, potentially benefiting both T1D and T2D patients.[53,54] These approaches, alone or in combination, could improve the efficacy of cell therapy in treating diabetes.

Taken together, our results indicate that GRP94 is indispensable for β cell differentiation of hiPSCs by regulating DE specification. The impaired cellular response to ER stress and reduced β-catenin activation caused by GRP94 deletion are key mechanisms underlying the DE differentiation deficiency observed in GRP94 KO iPSCs.

MATERIALS AND METHODS

Cell culture and gene modification of hiPSCs

hiPSC lines K3 and SV20 were provided by Dr. Stephen Duncan.[55,56] The study was approved by the MUSC Stem Cell Research Oversight

Committee. No human subjects were involved. The cells were routinely cultured on Matrigel (BD Biosciences) in Stemflex medium (Thermo Fisher Scientific) at 37°C with 5% CO_2 and were passaged when approximately 80% confluent using Versene (Thermo Fisher Scientific). For the generation of *HSP90B1* KO in the K3 cells, a sgRNA targeting exon 2 of *HSP90B1* was used. The guide sequence AGCTGACGATGAAGTTGATG was cloned into the pSpCas9 (BB)-2A-puro vector (pX495 V2.0, Addgene), which was then introduced into K3 cells by transfection with Lipofectamine 3000 (Invitrogen). Twenty-four hours after transfection, transfected cells were selected by incubation with 1 μg/mL puromycin (Thermo Fisher Scientific) in mTeSR1 cGMP PSC Maintenance Medium for 48 h. Surviving cells were grown until clones could be collected. Genomic DNA was extracted from the clones using QuickExtract DNA extraction solution (Epicenter), and the targeted regions were amplified using Herculase Fusion Polymerase (Agilent). The primers specific to the target area include the forward primer (5′TGCACTCTTTCATCCCCACC3′) and the reverse primer (5′CTCTACTTTCCATTTAAGAATGGCT3′). Amplicons were subjected to restriction digest screening to identify clones with insertion-deletion events (INDELS) and sequenced to confirm the identity of the INDELS.

Viral infection of iPSCs to express the pGreenZeo human insulin differentiation reporter

The lentivirus carrying the pGreenZeo human insulin differentiation reporter (System Bioscience,) was packaged in 293 T cells with packaging vectors psPAX2 and pMD2.G in the Opti-MEM medium (Thermo Fisher Scientific). Sixteen hours after transfection, the medium was changed to StemFlex medium (Thermo Fisher Scientific), and cells were continuously cultured for another 24 h. The supernatant was then collected and used to infect the hiPSC cells. The infection was repeated three times.

Exogenous expression of GRP94 in *HSP90B1* KO hiPSCs by electroporation

Two *HSP90B1* deletion iPSCs, HSP90B1-KO1 and HSP90B1-KO2, were dissociated with Versene (Thermo Fisher Scientific). Then 1×10^6 cells were resuspended in 100 μL buffer R, mixed with 20 μg of human *HSP90B1* expressing plasmid (VectorBuilder, vector ID: VB900115-8943aap), and then transfected by electroporation with two 30-s pulses at 1,050 V (Neon Transfection System, Thermo Fisher Scientific). The transfected cells were then plated in the StemFlex medium containing 10 μM Y-27632 ROCK inhibitor (Tocris Bioscience). The expression of GRP94 was confirmed by western blot and immunostaining.

Tri-lineage differentiation of hiPSCs

Tri-lineage differentiation of iPSCs was performed following previously described methods with some modifications.[18,57] For DE differentiation, hiPSCs were dissociated with Accutase (STEMCELL Technologies) and treated with 3μM CHIR99021 (Selleck Chemicals) and 100 ng/mL GDF8/myostatin (PeproTech) in MCDB131 medium supplemented with 0.5% BSA, 1× glutamate, 10 mM glucose, and 1.5 g/L sodium bicarbonate for 24 h, followed by treatment with 100 ng/mL GDF8/myostatin for another 48 h. For early mesoderm differentiation, the dissociated hiPSCs were cultured in RPMI1640 containing B27 minus insulin (Gibco) and 6 μM CHIR99021 (Selleckchem) for 48 h. The StemXVivo Ectoderm Kit (R&D Systems) was used to induce hiPSCs differentiation according to the manufacturer's instructions for ectoderm differentiation. Differentiated cells were then collected for flow cytometry analysis, RNA isolation, or fixed with 4% paraformaldehyde (PFA) for immunocytochemical analysis.

β cell differentiation from hiPSCs

β cell differentiation was performed as described previously with some modifications.[18,19] In brief, the dissociated hiPSCs were seeded at a density of 0.6×10^6 cells/well in 12-well plates with Stemflex medium containing 10 μM ROCK inhibitor, and differentiation was induced the following day. In stage 1, cells were induced for DE differentiation, as described above. In stage 2, cells were cultured in MCDB131 medium supplemented with 0.5% BSA, 1.5 g/L sodium bicarbonate 1× glutamate, 10 mM glucose, and 50 ng/mL recombinant human FGF7 (Peprotech) for 2 days. In stage 3, cells were cultured in MCDB131 medium supplemented with 2% BSA, 2.5 g/L sodium bicarbonate 1× glutamate, 10 mM glucose, 50 ng/mL FGF7, 0.25 mM vitamin C (STEMCELL Technology), 0.25 μM SANT1(Sigma), 1 μM retinoic acid (RA; Sigma), 100 nM LDN193189 (LDN; Stemgent), 1:100 ITS-X (Life Technology), and 200 nM TPB (EMD Millipore) for 2 days. In stage 4, cells were cultured in MCDB131 supplemented with 2% BSA, 2.5 g/L sodium bicarbonate, 1× glutamate, 10 mM glucose, 2 ng/mL FGF7, 0.25 mM vitamin C, 0.25 μM SANT1, 0.1 μM RA, 200 nM LDN, 1:100 ITS-X, and 100 nM TPB for 3 days. In stage 5, spheroids were cultured in MCDB131 supplemented with 2% BSA, 1.5 g/L sodium bicarbonate, 1× glutamate, 20 mM glucose, 0.25 μM SANT1, 0.05 μM RA, 100 nM LDN, 1:100 ITS-X, 1 μM 3,3′,5-triiodo-l-thyronine sodium salt (T3; Sigma), 5 μM ALK5 inhibitor II (Enzo Life Sciences), 5 μM zinc sulfate heptahydrate (Sigma), and 10 μg/mL heparin for 2 days. In stage 6, spheroids were cultured in MCDB131 supplemented with 2% BSA, 1.5 g/L sodium bicarbonate, 1× glutamate, 20 mM glucose, 100 nM LDN, 1:100 ITS-X, 1 μM T3, 10 μM ALK5 inhibitor II, 100 nM γ-secretase inhibitor (Millipore), 10 μM zinc sulfate heptahydrate, and 10 μg/mL heparin for 6 days. In stage 7, spheroids were cultured in MCDB131 supplemented with 2% BSA, 1.5 g/L sodium bicarbonate, 1× glutamate, 20 mM glucose, 100 nM LDN, 1:100 ITS-X, 1 μM T3, 10 μM ALK5 inhibitor II, 10 μM zinc sulfate heptahydrate, 1 mM *N*-acetyl cysteine (N-Cys, Sigma), 10 μM Trolox (EMD), 2 μM R428 (SelleckChem), and10 μg/mL heparin for 6 days.

Immunofluorescence staining and confocal microscopy analysis

For immunofluorescent staining of cells cultured in 2D system, cells were fixed with 4% PFA for 10 min, permeabilized with 0.3% Triton X- for 10 min, and then blocked with 2% BSA in PBS for 1 h. Cells were then incubated with primary antibodies against OCT4 (see Table S1 for detailed information), SOX2, NANOG, FOXA2,

β-catenin, SOX17, TBXT, OTX2, and GRP94, at 4°C overnight. Samples were then incubated with fluorescent conjugated secondary antibodies (Invitrogen) for 1 h at room temperature. Cells were washed and mounted onto glass slides with Fluoroshield mounting medium (Sigma-Aldrich). Confocal images were captured with a Leica SP5 confocal microscope. For cells cultured in 3D, clusters were embedded in optimal cutting temperature compound (OCT) freezing media and frozen at −80°C. Serial sections (5 μm thick each) were collected from each cluster at 25 μm apart. The slides were fixed with 4% PFA for 10 min, treated with 3% H_2O_2 for 10 min, and then incubated with primary and secondary antibodies as described above.

Bulk RNA-seq analysis

Total RNA was extracted using the RNeasy Micro Kit (Qiagen) and stored at −80°C. The concentration and purity of all samples were measured. Once initial quality testing was passed, samples were sent for library preparation, gene expression analysis, and quantification by Novogene. Significantly changed genes were analyzed by Metascape (Metascape data: https://metascape.org/gp/index.html#/main/step1), GO, and the KEGG analyses.

Flow cytometry analysis

Cells were dissociated using Accutase, then fixation and permeabilization with eBioscience Intracellular Fixation & Permeabilization Buffer Set (Thermo Fisher Scientific) for 30 min on ice. Samples were incubated with phycoerythrin (PE)-conjugated anti-human FOXA2 (BD Biosciences, Table S1), PerCP-Cy 5.5-conjugated anti-human SOX17 (BD Biosciences), PE-conjugated anti-Brachyury rabbit mAb (Cell Signaling Technology), and PE-conjugated anti-human OTX2 (Novus Biologicals) antibodies in eBioscience Permeabilization Buffer (Thermo Fisher Scientific) at room temperature for 25 min. Flow cytometry was analyzed on a BD LSRFortessa Cell Analyzer (BD Biosciences).

Cell apoptosis analysis

Cell death was analyzed using the APC Annexin V Apoptosis Detection Kit with propidium iodide (PI, BioLegend). Briefly, 1×10^6 dissociated cells were washed and then incubated with 5 μL of Annexin V and 10 μL of PI in 100 μL of Annexin V binding buffer for 15 min at room temperature in the dark. Then, 400 μL of binding buffer was added to each tube for flow cytometry analysis.

RNA isolation and PCR analysis

Total RNA was isolated from cells using TRIzol reagent (Invitrogen), followed by reverse transcription using M-MLV reverse transcriptase and olig (dT)18 primers (Thermo Fisher Scientific). Quantitative PCR was conducted using SYBR Green I (Thermo Fisher Scientific) on a CFX96 Real-Time PCR Detection System (Bio-Rad). The thermal profile for qPCR included an initial denaturation step at 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 15 s and annealing/extension at 60°C for 1 min. Relative quantitation was performed by normalizing the results to 18S rRNA expression. PCR primer sequences are listed in Table S2.

Protein extraction and western blot analysis

Cell pellets stored at −80°C were thawed and re-suspended in lysis buffer (Thermo Fisher Scientific). Protein concentration was measured using a BCA assay (Thermo Fisher Scientific). Twenty micrograms of protein was separated by SDS-PAGE, transferred to a PVDF membrane, and incubated with antibodies against OCT4 (see Table S1 for antibody information), SOX2, NANOG, GRP94, GAPDH, β-catenin, IRE1α, PERK, *p*-smad 2, smad2/3, β-tubulin, and β-actin separately, followed by incubation with horseradish peroxidase-conjugated secondary antibodies (Cell Signaling Technology). Signals were visualized using an ECL detection kit (Thermo Fisher Scientific).

Statistical analysis

Data are presented as mean ± SD. Comparisons between the two groups were performed using Student's t test. Comparisons between multiple groups were performed using one-way ANOVA with post-hoc correction. Statistical significance was defined as a p value of less than 0.05.

DATA AVAILABILITY

The datasets generated and/or analyzed in this study can be available from the corresponding author after writing a request.

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AUTHOR CONTRIBUTIONS

H. Wei designed and performed the experiment, analyzed data, wrote the manuscript. C. K. produced iPSCs. E.G., H.J., and T.Y. performed some experiments. H. Wang designed the experiments, participated in data analysis, and wrote the manuscript.

DECLARATION OF INTERESTS

The authors declare no conflict of interest.

SUPPLEMENTAL INFORMATION

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