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Activation of the c-Jun N-Terminal Kinase Pathway Aggravates Proteotoxicity of Hepatic Mutant Z Alpha1-Antitrypsin

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Alpha1-antitrypsin deficiency is a genetic disease that can affect both the lung and the liver. The vast majority of patients harbor a mutation in the serine protease inhibitor 1A (*SERPINA1*) gene leading to a single amino acid substitution that results in an unfolded protein that is prone to polymerization. Alpha1-antitrypsin defciency-related liver disease is therefore caused by a gain-of-function mechanism due to accumulation of the mutant Z alpha1-antitrypsin (ATZ) and is a key example of an disease mechanism induced by protein toxicity. Intracellular retention of ATZ triggers a complex injury cascade including apoptosis and other mechanisms, although several aspects of the disease pathogenesis are still unclear. We show that ATZ induces activation of c-Jun N-terminal kinase (JNK) and c-Jun and that genetic ablation of JNK1 or JNK2 decreased ATZ levels *in vivo* by reducing c-Jun–mediated *SERPINA1* gene expression. JNK activation was confirmed in livers of patients homozygous for the Z allele, with severe liver disease requiring hepatic transplantation. Treatment of patient-derived induced pluripotent stem cell-hepatic cells with a JNK inhibitor reduced accumulation of ATZ. *Conclusion:* These data reveal that JNK is a key pathway in the disease pathogenesis and add new therapeutic entry points for liver disease caused by ATZ. (HEPATOLOGY 2017;65:1865-1874).

Alpha1-antitrypsin (AAT) deficiency occurs in approximately 1 in 2,000-5,000 births in North American and European populations and can cause both liver and lung disease.[1] The hepatic disease has been increasingly recognized in both children and adults with liver failure requiring hepatic transplantation. The vast majority of patients carry the Z mutation in the serine protease inhibitor 1A (*SERPINA1*) gene, which results in a single glutamic acid to lysine substitution at amino acid position 342 (p.Glu342Lys). This mutation changes the protein structure leading to misfolding, polymerization, and accumulation of Z alpha1-antitrypsin (ATZ) in the endoplasmic reticulum (ER).[2,3] In protease inhibitor type ZZ (PIZZ) individuals (homozygous for the Z mutation), intrahepatic retention of ATZ results in low circulating levels of AAT and inadequate antiprotease protection in the lower respiratory tract, which

Abbreviations: AAT, alpha1-antitrypsin; AP-1, activator protein 1; ATZ, Z alpha1-antitrypsin; ChIP, chromatin immunoprecipitation; C_t, cycle threshold; ELISA, enzyme-linked immunosorbent assay; ER, endoplasmic reticulum; HCC, hepatocellular carcinoma; IgG, immunoglobulin G; iPSC, induced pluripotent stem cell; JNK, c-Jun N-terminal kinase; PAS, periodic acid–Schiff; PIZZ, protease inhibitor type ZZ; SERPIN1A, serine protease inhibitor 1A; 5′-UTR, 5′ untranslated region.

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can lead to progressive lung emphysema.[4] PIZZ individuals are also at risk of developing liver disease due to intracellular retention and accumulation of aberrantly folded ATZ, which can lead to hepatitis, cirrhosis, liver failure, and hepatocellular carcinoma (HCC).[5,6] Transgenic PiZ mice genetically engineered to express the human *SERPINA1* gene with the Z mutation under the control of the human promoter region have been a valuable experimental model of liver disease due to AAT deficiency.[7] In these mice, ATZ protein accumulates in hepatocytes in a nearly identical manner to livers of humans homozygous for the Z mutation. These mice have been instrumental to understanding the complex cascade of events leading to liver injury, including activation of nuclear factor κB and autophagy.[8-11] Nevertheless, the precise mechanisms leading to the activation of these important pathways are still unclear. Here, we show that c-Jun N-terminal kinase 1 (JNK1) and 2 (JNK2) and c-Jun play important roles in the pathogenesis of the liver disease by affecting the degree of ATZ accumulation. Genetic ablation of JNK1 or JNK2 resulted in marked reduction of ATZ accumulation and *SERPINA1* expression *in vivo*. Moreover, treatment of patient-derived induced pluripotent stem cell (iPSC)-hepatic cells with JNK inhibitors reduced ATZ accumulation. Therefore, our findings suggest that the JNK signaling pathway is an important target for the correction of liver disease due to ATZ expression and could be investigated for the development of therapeutic approaches.

Materials and Methods

MOUSE STUDIES

Animal procedures were performed in accordance with regulations of the Italian Ministry of Health. PiZ transgenic mice[12] were maintained on a C57/BL6 background. JNK1$^{-/-}$ and JNK2$^{-/-}$ mice were purchased from Jackson Laboratory and bred with PiZ mice.

HUMAN LIVER SAMPLES

Human liver samples were collected and anonymized according to applicable human study approvals from subjects homozygous for the p.Glu342Lys mutation in the *SERPINA1* gene and age-matched control subjects homozygous for the wild-type allele of *SERPINA1*.

LIVER ANALYSES

Gene expression analysis was performed on 6-week-old male PiZ and C57/BL6 wild-type mice (n = 3/group). All samples were processed on Affymetrix Mouse 430A 2.0 arrays using GeneChip 3′-IVT Plus and Hybridization Wash and Stain kits by means of Affymetrix's standard protocols. Raw intensity values of the six arrays were processed and normalized by the Robust Multi-Array Average Method[13] using the Bioconductor[14] R package Affy.[15] Differentially expressed genes between conditions (PiZ versus control) were identified using a Bayesian *t* test.[16] For each *P* value, the Benjamini-Hochberg procedure was used to calculate the false discovery rate to avoid the problem of multiple testing. The selected gene lists were obtained using the following thresholds: false discovery rate <0.05 and abs(ratio) >1.5. Expression array data are deposited in GEO with the accession number GSE93115.

Liver protein extracts and serum samples were analyzed by periodic acid–Schiff (PAS), immunofluorescence (against both total and polymer ATZ), and enzyme-linked immunosorbent assay (ELISA) for ATZ, as described.[9] Stained liver sections were examined under a Zeiss Axiocam MRm microscope. Nuclear and cytoplasmic protein extracts were

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prepared using the CelLytic NuCLEAR Extraction (NXTRACT) kit (Sigma-Aldrich) according to the manufacturer's instructions.

For western blotting, liver samples homogenized in radio immunoprecipitation assay buffer with protease inhibitor cocktail (Roche) were incubated for 20 minutes at 4°C and centrifuged at 16,800 g for 10 minutes. Ten to twenty micrograms of lysates were electrophoresed by 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis. After transfer to nitrocellulose membrane, blots were blocked in tris(hydroxymethyl)aminomethane-buffered saline-Tween20 with 5% nonfat milk for 1 hour at room temperature, and the primary antibody was applied overnight at 4°C. Antirabbit immunoglobulin G (IgG) or antimouse IgG conjugated with horseradish peroxidase (GE Healthcare) and enhanced chemiluminescence (Pierce) were used for detection. Primary antibodies used for immunoblots are listed in Supporting Table S1.

Total RNA extracted in TRIzol reagent (Invitrogen) using the RNeasy kit (Qiagen) was reverse-transcribed using a first-strand complementary DNA kit with random primers according to the manufacturer's protocol (Applied Biosystems). PCR was performed using the SYBR Green Master Mix (Roche). PCR conditions were as follows: preheating, 5 minutes at 95°C; cycling, 40 cycles of 15 seconds at 95°C, 15 seconds at 60°C, and 25 seconds at 72°C. Results were expressed in terms of cycle threshold (C_t). C_t values were averaged for each duplicate. β2-Microglobulin or hypoxanthine phosphoribosyltransferase 1 was used as an endogenous control gene. Differences between mean C_t values of tested genes and those of the reference gene were calculated as $\Delta C_{tgene} = C_{tgene} - C_{treference}$. The relative fold increase in expression levels was determined as $2^{-\Delta\Delta Ct}$. Primers for real-time PCR are listed in Supporting Table S2.

CHROMATIN IMMUNOPRECIPITATION

Livers from PiZ mice were homogenized and cross-linked by the addition of formaldehyde to a final concentration of 1% for 10 minutes. The cross-linking reaction was stopped by the addition of glycine at a final concentration of 0.125 mM for 5 minutes at room temperature. Liver homogenates were lysed in cell lysis buffer (piperazine-1,4-bis-2-ethanesulfonic acid 5 mM [pH 8.0], Igepal 0.5%, KCl 85 mM) for 15 minutes. Nuclei were lysed in lysis buffer (Tris HCl [pH 8.0] 50 mM, ethylene diamine tetraacetic acid 10 mM, sodium dodecyl sulfate 0.8%) for 30 minutes. Chromatin was sonicated to yield DNA fragments approximately 200-1,000 bp. DNA was coimmunoprecipitated using the chromatin immunoprecipitation (ChIP)–grade phospho-c-Jun (Ser73) antibody (Cell Signaling; CST-#3270) overnight at 4°C. No antibody was used as a negative control of the immunoprecipitation. Purified immunoprecipitated DNA samples and inputs were amplified by quantitative PCR with primers specific for the activator protein 1 (AP-1) binding sites of the 5′ untranslated region (5′-UTR) of the *SERPINA1* gene (Supporting Table S3).

LUCIFERASE ASSAY

For generation of the plasmid with the luciferase gene, human *SERPINA1* regulatory regions were amplified by PCR from genomic DNA of PiZ mice. PCR product was cloned into a pGL3-Basic vector (Promega) upstream of the firefly luciferase. AP-1 consensus sequences were mutagenized by QuickChange site-directed mutagenesis as described.[17] The complementary DNA of human *c-Jun* was amplified by RT-PCR from HuH7 cells and cloned into p3xFLAG-CMV-14 vector. Oligonucleotides used for generation of the constructs are shown in Supporting Table S4.

HeLa cells were cultured in Dulbecco's modified Eagle's medium in 10% fetal bovine serum and 5% penicillin/streptomycin and cotransfected with the plasmid containing the wild-type or mutated AP-1 consensus sequences and with a plasmid expressing human c-Jun. Each well was cotransfected with the pRL-TK plasmid (Promega) expressing renilla luciferase as control. Cells were harvested 48 hours after transfection and assayed for luciferase activity using the Dual-Luciferase Reporter Assay System (Promega). Data were expressed relative to renilla luciferase activity to normalize for transfection efficiency. Transfections were repeated at least three times.

GENERATION OF iPSCs AND HEPATIC DIFFERENTIATION

Normal human dermal fibroblast–derived iPSCs were generated by reprogramming with a single-integrated excisable copy of floxed hSTEMCCA lentiviral reprogramming vector,[18] followed by excision with transient Cre recombinase exposure to create the BMC1 and PiZZ100 lines, as published.[18,19] Recruitment of human subjects and all iPSC studies were approved by the Boston University institutional review board (BUMC IRB H-27636).

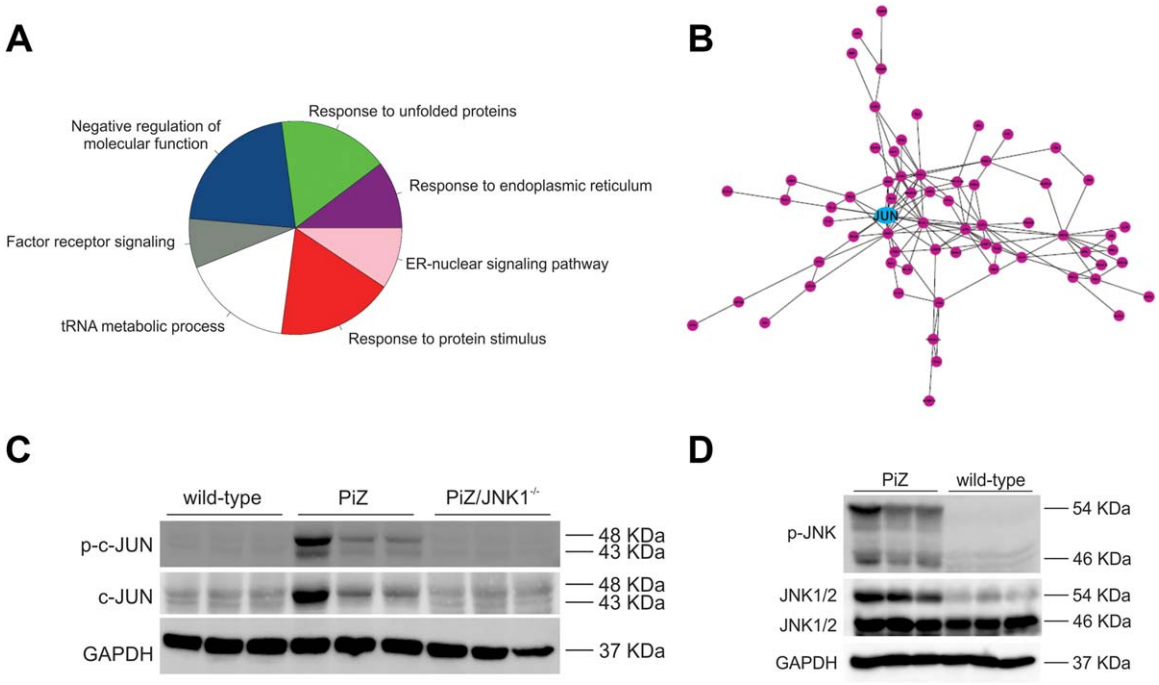

FIG. 1. The JNK pathway is activated in PiZ livers. (A) Genes up-regulated in PiZ mice were associated with the response to unfolded proteins, response to ER, ER nuclear signaling pathway, and response to protein stimulus. Livers of 6-week-old PiZ mice (n = 3) and age-matched and gender-matched controls (n = 3) were used for expression array profiling. (B) Search Tool for the Retrieval of Interacting Genes analysis showed enrichment on genes of cellular response to stress including c-Jun. (C) Western blotting of phosphorylated and total c-Jun in 6-week-old PiZ mouse livers compared to wild-type controls and PiZ/JNK1$^{-/-}$ (each lane corresponds to an independent mouse). (D) Western blotting of phosphorylated and total JNK in 6-week-old PiZ mouse livers compared to wild-type controls (each lane corresponds to an independent mouse). Abbreviations: GAPDH, glyceraldehyde 3-phosphate dehydrogenase; p-, phosphorylated; tRNA, transfer RNA.

The iPSCs were differentiated as monolayer cultures in serum-free conditions by sequential growth factor stimulation using a described protocol[20] for directed differentiation of pluripotent cells into definitive endoderm followed by early hepatocyte-like lineages. For flow cytometric analysis of differentiated iPSC-hepatic cells and staining of intracellular antigens, cells were fixed in 1.6% paraformaldehyde for 20 minutes at 37°C and permeabilized in saponin buffer (Biolegend). Fixed cells were stained with antibodies against human FOXA1 and AAT followed by goat antimouse IgG2a-DyLight488 (Jackson ImmunoResearch) or IgG1-DyLight649 (Jackson ImmunoResearch) antibodies. For all flow cytometric experiments, gating was based on isotype-stained controls. Staining was quantified using a FACSCantos II flow cytometer (BD Biosciences) and analyzed with FlowJo software (Tree Star Inc.). Cells were incubated with dimethyl sulfoxide or SP600125 (5 or 12.5 μM) for 24 hours before analysis.

STATISTICAL ANALYSES

Data are expressed as averages ± standard deviation. Statistical significance was computed using the Student two-tailed t test. $P < 0.05$ was considered statistically significant.

Results

ATZ ACTIVATES THE JNK PATHWAY, WHICH UP-REGULATES *SERPINA1* EXPRESSION

To investigate the pathogenesis of liver damage induced by ATZ, we analyzed livers of 6-week-old PiZ mice and wild-type controls by expression array profiling. Analysis of "biological function" terms indicated that most genes up-regulated in PiZ livers were associated with response to unfolded proteins, response

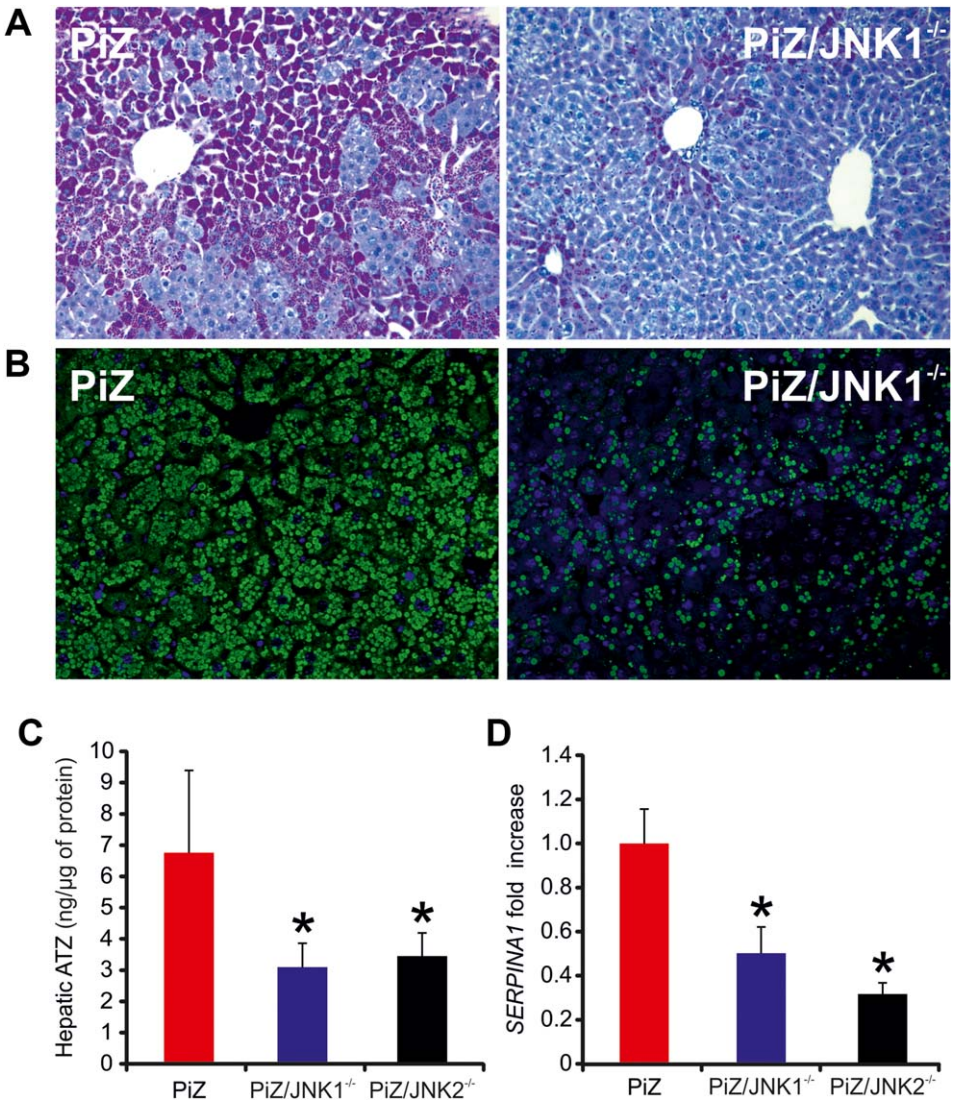

FIG. 2. PiZ/JNK1$^{-/-}$ and PiZ/JNK2$^{-/-}$ mouse livers show reduced ATZ accumulation and expression. (A,B) Representative PAS staining and immunofluorescence for ATZ on livers of PiZ and PiZ/JNK1$^{-/-}$ mice. (C) ATZ ELISA on livers of PiZ, PiZ/JNK1$^{-/-}$ and PiZ/JNK2$^{-/-}$ mice (at least n = 5/group). (D) *SERPINA1* expression in PiZ, PiZ/JNK1$^{-/-}$, PiZ/JNK2$^{-/-}$ mice (at least n = 5/group). β2-Microglobulin was used for normalization. Averages ± standard deviations are shown. *$P < 0.05$.

to ER, ER nuclear signaling pathway, and response to protein stimulus (Fig. 1A). Search Tool for the Retrieval of Interacting Genes analysis, which takes into consideration gene–gene and protein–protein interactions, revealed enrichment of genes induced by cellular response to stress, including c-Jun (Fig. 1B; Supporting Table S5), which has not been previously found to be affected in livers expressing ATZ. We confirmed c-Jun activation by the increased levels of phospho-c-Jun in PiZ mouse livers compared to wild-type controls (Fig. 1C). c-Jun is one of the main cellular substrates activated by JNK through phosphorylation,[21] and indeed a significant increase of phosphorylated JNK was observed in PiZ mouse livers (Fig. 1D). JNK and c-Jun showed variability among livers of PiZ mice as expected based on heterogeneous ATZ accumulation within the liver.[22] Nevertheless, the analysis of a larger set of liver samples confirmed the increased phosphorylated c-Jun and phosphorylated JNK in PiZ mouse livers compared to wild-type controls (Supporting Fig. S1).

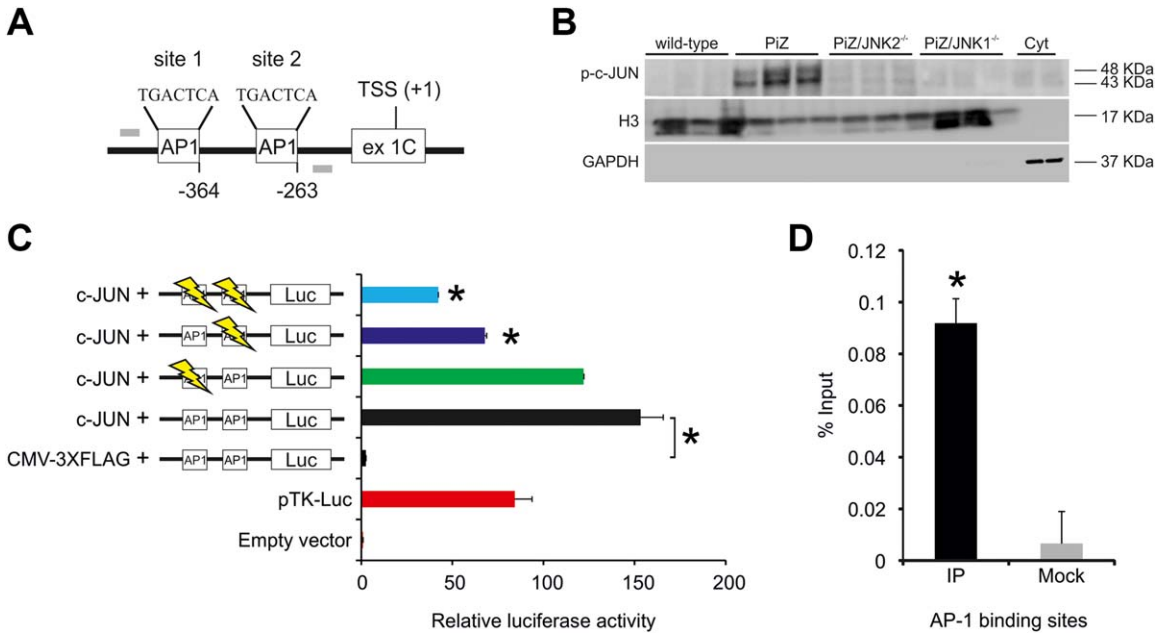

FIG. 3. c-Jun is increased in nuclear extracts of PiZ mouse livers and regulates expression of *SERPINA1*. (A) Schematic representation of the two putative AP-1 binding sites (consensus sequence 5′-TGACTCA-3′) in the 5′-UTR of the *SERPINA1* gene (not in scale). The hepatocyte regulatory region is located at positions –364 (site 1) and –263 (site 2) from the hepatic transcription starting site (TSS)[30] (genomic position site 1, chr14:94388968-94388974; site 2, chr14, 94388867-94388873, GRCh38/hg38 assembly). Gray bars show the position of the primers used for real-time PCR amplification of the ChIP shown in (D). (B) Western blot for phospho-c-Jun on nuclear extracts of livers of wild-type, PiZ, PiZ/JNK2$^{-/-}$, and PiZ/JNK1$^{-/-}$ mouse livers. Nuclear extracts were positive for the histone H3 but negative for the cytosolic glyceraldehyde 3-phosphate dehydrogenase (GAPDH) protein. The cytosolic fraction was negative for H3 and positive for GAPDH. (C) Luciferase expression in HeLa cells cotransfected with a plasmid containing the human *SERPINA1* enhancer regions with two AP-1 putative binding sites upstream of the firefly luciferase coding region and plasmid expressing the human c-Jun. Luciferase expression was significantly reduced when the two binding sites for c-Jun were mutagenized. Experiments were performed in duplicate, and averages ± standard deviations are shown. Mutagenized constructs cotransfected with c-Jun plasmid were compared to non-mutagenized contruct cotransfected with c-Jun. *$P < 0.05$. (D) ChIP with anti-phospho-c-Jun antibody on PiZ mouse livers showed enrichment of the 5′-UTR region containing the two AP-1 binding sites of the *SERPINA1* gene. The ChIP results were obtained by three independent experiments and are shown as percentage of input. Averages ± standard deviations are shown. *$P < 0.05$. Abbreviations: Cyt, cytosolic fraction; ex 1C, exon 1C; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; IP, immunoprecipitated DNA; p-, phosphorylated; TSS, transcription start site.

To investigate the role of JNK *in vivo*, we crossed PiZ mice with JNK1 or JNK2 knockout mice.[23,24] Being that c-Jun is a target of JNK, as expected, PiZ/JNK1$^{-/-}$ mice showed reduced hepatic phospho-c-Jun/total c-Jun compared to PiZ mice (Fig. 1C). Compared to PiZ, livers of PiZ/JNK1$^{-/-}$ mice showed reduced ATZ by PAS staining, immunofluorescence with both total anti-AAT and polymeric anti-ATZ, and ELISA (Fig. 2A-C; Supporting Fig. S2). *SERPINA1* mRNA levels in livers of PiZ/JNK1$^{-/-}$ mice were also significantly reduced compared to PiZ controls (Fig. 2D), suggesting that JNK induces ATZ accumulation by increasing *SERPINA1* transcription. Like PiZ/JNK1$^{-/-}$ mice, PiZ/JNK2$^{-/-}$ mice showed reduced hepatic phospho-c-Jun/total c-Jun compared to PiZ mice (Supporting Fig. S3). Similar reductions of ATZ by PAS staining (Supporting Fig. S4), ELISA (Fig. 2C), and real-time PCR (Fig. 2D) were observed in PiZ/JNK2$^{-/-}$ mice.

SERPINA1 is transcriptionally regulated at least by two tissue-specific regulatory regions located in the 5′-UTR: the liver-specific regulatory region located upstream of the noncoding exon 1C and a second regulatory region upstream of exon 1A that controls expression in monocytes.[25] The liver-specific regulatory region at the 5′-UTR contains two putative binding sites for AP-1[26] (Fig. 3C) that is composed of c-Jun and c-FOS.[27] Therefore, we hypothesized that expression and accumulation of ATZ are dependent upon increased JNK-mediated activation of c-Jun and

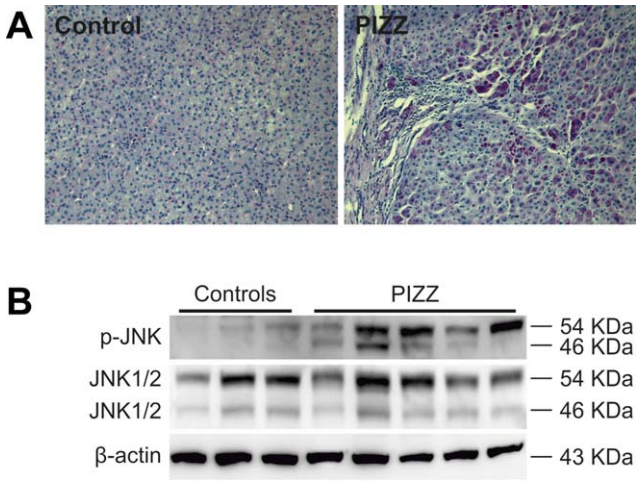

FIG. 4. JNK activation in human PIZZ livers. (A) Representative PAS staining from a PIZZ patient with cirrhosis who underwent liver transplantation and a control liver from a patient with liver failure due to an unrelated disease. (B) Western blotting of phosphorylated and total JNK in livers of five PIZZ patients and three controls from patients with liver failure due to unrelated diseases. Abbreviation: p-, phosphorylated.

subsequent *SERPINA1* transcriptional up-regulation. Consistent with this hypothesis, we found increased phospho-c-Jun in the nuclear extracts of PiZ mouse livers compared to control wild-type mice and compared to either PiZ/JNK1$^{-/-}$ or PiZ/JNK2$^{-/-}$ mouse livers (Fig. 3B). Next, we constructed a plasmid including the firefly luciferase gene under the control of the hepatic regulatory region at the 5′-UTR of the human *SERPINA1* gene including the two putative AP-1 binding sites (Fig. 3C). HeLa cells cotransfected with this plasmid and a plasmid expressing c-Jun showed increased levels of luciferase compared to cells cotransfected with the control plasmid (Fig. 3C). Luciferase expression was reduced when the one or two binding sites for c-Jun were mutagenized (Fig. 3C). Finally, we found an enrichment of the AP-1 binding sites in chromatin immunoprecipitates with anti-phospho-c-Jun antibody on PiZ mouse livers (Fig. 3D). Taken together, these results show that c-Jun up-regulates *SERPINA1* expression through binding and transactivation of the AP-1 sites at the 5′-UTR of *SERPINA1*.

JNK ACTIVATION IN HUMAN LIVERS

To interrogate the clinical relevance of our findings, we determined whether JNK is also activated in human livers from PIZZ patients. Liver samples were obtained from five PIZZ patients under 18 years of age homozygous for the p.Glu342Lys mutation in *SERPINA1* who underwent hepatic transplantation for

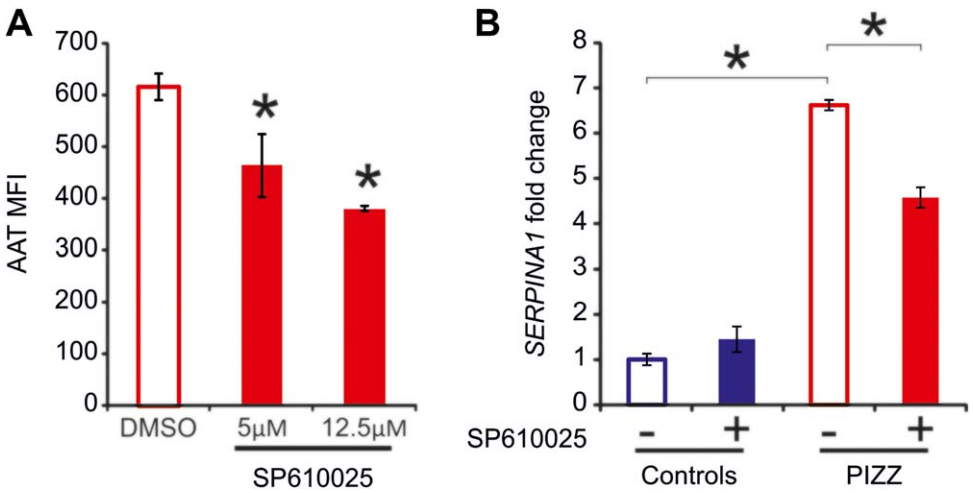

FIG. 5. JNK inhibitor SP600125 decreased ATZ levels in iPSC-derived hepatic cells. (A) Mean fluorescence intensity for ATZ by cytofluorimetry in iPSC-derived hepatic cells incubated with 5 μM or 12.5 μM of SP600125 compared to vehicle (dimethyl sulfoxide) (n = 3/group). (B) Real-time PCR for *SERPINA1* in wild-type and PIZZ iPSC-derived hepatic cells incubated with SP600125 or vehicle. The hypoxanthine phosphoribosyltransferase 1 gene was used for normalization. Averages ± standard deviations are shown. *$P < 0.05$. Abbreviations: DMSO, dimethyl sulfoxide; MFI, mean fluorescence intensity.

liver failure. Three control liver specimens were obtained from age-matched patients homozygous for the wild-type allele of the *SERPINA1* gene who also underwent liver transplantation. As expected, in addition to cirrhosis, livers of PIZZ patients showed accumulation of PAS-positive material that was not detected in control livers (Fig. 4A). Livers of PIZZ patients showed greater activation of JNK compared to controls, as shown by increased phosphorylated JNK (Fig. 4B), thus confirming JNK activation in livers of patients homozygous for the Z mutation.

JNK ACTIVATION IN PATIENT-DERIVED iPSC-HEPATIC CELLS

We investigated whether the JNK pathway was also affected in patient-derived iPSC-hepatic cells generated from PIZZ patients.[20] We differentiated iPSCs to the hepatic lineage using a directed differentiation protocol[20] and confirmed differentiation efficiency by intracellular staining for the hepatic marker FOXA1 (Supporting Fig. S5). Incubation of PIZZ iPSC-hepatic cells with the JNK inhibitor SP600125 showed a dose-dependent reduction of intracellular ATZ protein (Fig. 5A) and a reduction of ATZ mRNA levels (Fig. 5B). Interestingly, PIZZ iPSC-hepatic cells showed reduced *SERPINA1* expression compared to wild-type control cells (Fig. 5B). In summary, these results show that the JNK inhibitor mediates a reduction of ATZ in clinically relevant and patient-specific iPSC-hepatic cells.

Discussion

The main finding in this study is that JNK and c-Jun play an important role in liver disease due to toxic accumulation of ATZ by promoting *SERPINA1* transcriptional up-regulation. We showed that JNKs are activated in mouse and human livers and that in turn they activate their direct target c-Jun, which up-regulates *SERPINA1* expression. The lack of either JNK1 or JNK2 resulted in decreased c-Jun activation and *SERPINA1* expression as well as reduced hepatic ATZ accumulation. Finally, we showed that the JNK inhibitor SP600125 reduced levels of toxic ATZ in clinically relevant PIZZ iPSC-hepatic cells.

JNKs are important signaling molecules in multiple pathways in liver physiology and diseases. JNK activation affects liver regeneration, but it can also be detrimental, such as in carcinogenesis.[28] The liver expresses two JNK isoforms, JNK1 and JNK2, whereas JNK3 is mainly expressed in brain, heart, and testis.[29] c-Jun is a direct target of JNK and was found to be activated in PiZ livers. The *SERPINA1* gene has been found to contain two putative binding sites for AP-1,[30] composed of c-FOS and c-Jun.[27] The luciferase assay in HeLa cells cotransfected with human c-Jun and with the AP-1 consensus sequences of *SERPINA1* and the ChIP study indeed confirmed c-Jun binding and transactivation to the AP-1 sites on the 5′-UTR of *SERPINA1*.

Most patients homozygous for the Z mutation never develop clinically relevant liver disease. An analysis of autopsy data showed cirrhosis in 43% and HCC in 28% of patients with AAT deficiency.[31] The development of liver injury is thought to require environmental or genetic factors that in addition to the Z mutation can reach the threshold for intracellular accumulation and toxicity. AAT is an acute-phase protein, and its blood levels increase by 2-fold to 4-fold as a result of inflammation.[32] Moreover, hepatic *SERPINA1* expression is controlled by several cytokines, such as interleukin-6, interleukin-1β, tumor necrosis factor-α, oncostatin M, and bacterial lipopolysaccharide.[30,33] Interestingly, c-Jun up-regulates interleukin-6 and tumor necrosis factor-α expression,[34,35] and thus, it can also indirectly increase *SERPINA1* expression by increasing cytokine levels.

In summary, we propose a model in which hepatocytes expressing ATZ have increased activation of JNK and c-Jun and, as a consequence of a feedback positive loop, further increase *SERPINA1* transcription, aggravating ATZ accumulation. Based on the results of this study, factors acting on the JNK pathway such as concomitant inflammatory states might further increase *SERPINA1* transcription to reach the threshold of toxicity for the development of liver disease. Additionally, genetic modifiers, including those that modulate protein degradation or those that could be directly injurious to the liver, might be involved in achieving the toxicity threshold.

Although its involvement in livers expressing ATZ is controversial,[36-39] the unfolded protein response may be responsible for JNK activation.[40,41] Nevertheless, the factor(s) activating JNK remains to be investigated.

Liver disease caused by AAT deficiency predisposes to HCC.[6] Several clinical and experimental studies have implicated stress-activated mitogen-activated protein kinase cascades that converge on JNK and p38 as key regulators of hepatocarcinogenesis.[42,43] Hepatitis C and nonalcoholic steatohepatitis, which are

predisposing factors for HCC, both involve JNK activity. JNK1 and JNK2 are phosphorylated in samples from patients with primary HCC.[44] JNK1 down-regulates transcription of *p21* through c-MYC,[44] and c-Jun promotes hepatocarcinogenesis by antagonizing p53 activity.[45] Moreover, *c-Jun* deletion in mouse hepatocytes reduces the frequency of HCC.[44] Therefore, our findings of activated JNK and c-Jun in livers expressing ATZ are relevant to understanding cancer progression in AAT deficiency patients harboring the Z mutation.

From a therapeutic perspective, it will be of great importance to understand how the JNK pathway could be pharmacologically manipulated to improve liver disease caused by AAT deficiency. Based on the study of PIZZ patient-derived iPSC-hepatic cells, we found that inhibition of JNK might have therapeutic potential. However, JNK molecules play important roles in a large number of cellular processes, and their inhibition could result in significant side effects. Nevertheless, understanding crosstalk among JNK and other pathways activated in livers expressing ATZ as well as how these activities are linked with inflammation should provide new treatment and prevention strategies. In conclusion, our findings identified an important pathway involved in the pathogenesis of liver disease in patients homozygous for the Z mutation and highlight novel therapeutic targets.

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