



# High glucose-ROS conditions enhance the progression in cholangiocarcinoma via upregulation of MAN2A2 and CHD8

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## Abstract

Diabetes is a major risk factor in the development and progression of several cancers including cholangiocarcinoma (CCA). However, the molecular mechanism by which hyperglycemia potentiates progression of CCA is not clearly understood. Here, we showed that a high glucose condition significantly increased reactive oxygen species (ROS) production and promoted aggressive phenotypes of CCA cells, including proliferation and migration activities. Mannosidase alpha class 2a member 2 (MAN2A2), was upregulated at both mRNA and protein levels in a high glucose- and ROS-dependent manner. In addition, cell proliferation and migration were significantly reduced by MAN2A2 knockdown. Based on our proteome and in silico analyses, we further found that chromodomain helicase DNA-binding protein 8 (CHD8) was induced by ROS signaling and regulated MAN2A2 expression. Overexpression of CHD8 increased MAN2A2 expression, while CHD8 knockdown dramatically reduced proliferation and migration as well as MAN2A2 expression in CCA cells. Moreover, both MAN2A2 and CHD8 were highly expressed with positive correlation in CCA tumor tissues. Collectively, these data suggested that high glucose conditions promote CCA progression through ROS-mediated upregulation of MAN2A2 and CHD8. Thus, glucose metabolism is a promising therapeutic target to control tumor progression in patients with CCA and diabetes.

## KEYWORDS

CHD8, cholangiocarcinoma, high glucose, MAN2A2, reactive oxygen species

**Abbreviations:** CCA, cholangiocarcinoma; CHD8, chromodomain helicase DNA-binding protein 8; MAN2A2, mannosidase alpha class 2a member 2; NAC, N-acetyl-L-cysteine; NRF2, nuclear factor erythroid 2-related factor 2; ROS, reactive oxygen species.

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## 1 | INTRODUCTION

Cholangiocarcinoma (CCA) is a cancer that develops in bile duct epithelial cells. The incidence and mortality rate of this disease is increasing worldwide.<sup>1</sup> The highest incidence of CCA has been reported in northeastern Thailand.<sup>2</sup> Most CCA patients are usually diagnosed in late stages of the disease, resulting in poor patient outcomes<sup>3</sup> with cancer recurrence and high metastatic rates as major causes of death in CCA patients.<sup>4</sup> Liver fluke (*Opisthorchis viverrini*, OV) infection is a well defined major cause of CCA development in northeastern Thailand, an endemic region for OV.<sup>5,6</sup>

Diabetes mellitus (DM) describes a group of abnormal metabolic disorders caused by high levels of glucose in the blood circulation.<sup>7</sup> The prevalence of diabetes is increasing globally<sup>8</sup> Diabetic patients are susceptible to serious complications such as cardiovascular diseases and cancers.<sup>9,10</sup> Epidemiologic evidence indicates that high glucose is linked to increased risk for several cancers such as liver, pancreatic, colorectal, lung, and CCA.<sup>11,12</sup> Previous research has shown that CCA patients with diabetes in northeastern Thailand showed higher mortality rate when compared with liver cancer patients without diabetes.<sup>13</sup> More importantly, two-thirds of patients with CCA also have diabetes (Charupong Saengboonmee, 2015, unpublished data). This means that high glucose conditions may increase the risk of developing CCA. Although previous reports have shown that high glucose levels enhanced CCA progression by activating STAT3, NF- $\kappa$ B, and aberrant glycosylation,<sup>14,15</sup> the molecular mechanisms for high glucose promotion of the development and progression of CCA remain largely unknown.

ROS are powerful cellular signaling molecules that play essential roles in many physiologic and pathologic processes.<sup>16</sup> Tight regulation of the ROS balance is necessary for maintaining cellular survival, proliferation, and differentiation. Increased ROS levels are predominantly detected in various cancers,<sup>17-19</sup> resulting in promotion of tumorigenic signaling pathways. However, excessive ROS levels over the cytotoxic threshold can initiate apoptotic signaling in cancer cells.<sup>20,21</sup> Thus, the role of ROS in cancer is still controversial. It has long been accepted that increased ROS is a key feature in the development of diabetic complications. Several studies have revealed that high glucose levels induce ROS and the resultant ROS activates many signaling pathways.<sup>22</sup> However, there have been few reports showing that high glucose increased cancer progression by ROS production in pancreatic cancer,<sup>23,24</sup> breast cancer,<sup>25</sup> and prostate cancer.<sup>26</sup> Furthermore, it remains totally unknown whether high glucose levels can induce ROS signaling or ROS mediation in CCA cell progression.

The present study was designed to investigate whether CCA progression by high glucose levels is dependent on ROS and also attempted to reveal the molecular mechanisms for how high glucose-ROS signaling contributes to the progression of CCA cells.

## 2 | MATERIALS AND METHODS

### 2.1 | Cell culture

Human CCA cell lines, KKU-213A, KKU-213B,<sup>27</sup> and KKU-055 were obtained from the Japanese Collection of Research Bioresources (JCRB) Cell Bank, Osaka, Japan. Cell culture conditions have been described previously.<sup>15</sup> This study used NAC, which is a ROS inhibitor, to investigate the effect of high glucose associated with ROS signaling.

### 2.2 | Chemicals

NAC (A0918) was obtained from Sigma-Aldrich. Cell Counting Kit-8 (CCK-8 assay) was purchased from the Dojindo company. TRIZOL Reagent (#15596026) was purchased from Invitrogen, and Bio-Rad protein assay (#500-0006) was obtained from Bio-Rad.

### 2.3 | siRNA, plasmid, and antibodies

The levels of *MAN2A2*, chromodomain helicase DNA-binding protein 8 (*CHD8*), CCCTC-binding factor (*CTCF*), and *SMARCA4* were modulated by siRNA. These siRNAs were synthesized by Nippon Gene (Tokyo, Japan). siRNA sequence information is listed in Table S1. siGL3 was used as a negative control for knockdown experiments. Recombinant plasmid-pcDNA3.0-Flag-CHD8, and anti-CHD8 antibody were used.<sup>28</sup> Transfection experiments for siRNA and plasmids were conducted using Lipofectamine RNAiMAX<sup>®</sup> and FuGENE HD transfection reagents, respectively, following manufacturer's instructions. Primary antibody against HDAC1 (#SC-7872) was purchased from Santa Cruz Biotechnology. Anti-rabbit IgG antibody (#NA934V) was obtained from GE Healthcare.

### 2.4 | Measurement of ROS production

Cells were incubated in serum-free DMEM with 2.5  $\mu$ mol/L CM-H2DCFDA (Invitrogen Life Technologies) for 30 min at 37°C in 5% CO<sub>2</sub> in air. Cells were seeded into 12-well plates and cultured overnight in normal glucose culture medium. For high glucose treatment, culture medium was replaced with high glucose medium and cells were incubated for 2, 6, 12, or 24 h. Cells were washed twice with ice-cold PBS, trypsinized, and centrifuged to obtain a cell pellet. The pellets were resuspended in PBS. Fluorescence intensity was analyzed by flow cytometry.

### 2.5 | Sulforhodamine B (SRB) assays

Cell proliferation was determined by SRB assay as previously described.<sup>29</sup> CCA cells at a density of  $1.5 \times 10^3$  cells per well were

seeded into 96-well plates. Subsequently, cells were treated with high glucose with or without 5 mmol/L NAC for 24 h. After 24 h incubation, SRB assay was performed.

## 2.6 | Clonogenic assay

Cell proliferation was also assessed using clonogenic assay as previously described.<sup>29</sup> The cells were seeded at a density of 80 cells per well into 12-well plates and incubated for 24 h. After that cells were exposed to high glucose medium with or without NAC for 7 d. During incubation the medium was changed every 2 d. Colonies were stained and then counted under a microscopy. The stained colonies were solubilized with 10% acetic acid for 5 min. Absorbance was read at 540 nm using a microplate reader.

## 2.7 | Migration assay

For the Boyden chamber migration assay (Corning), after high glucose and NAC treatment for 24 h, prepared CCA cells at a density  $4 \times 10^4$  cells in serum-free medium were re-seeded into the upper chamber. Then complete medium with 10% fetal bovine serum was added to the lower chamber as a chemo-attractant. Next, migrated cells were fixed in 4% PFA for 15 min, and stained with 0.4 w/v SRB in 1% acetic acid (Sigma-Aldrich) for 30 min. The numbers of migrated cells were counted under a light microscope ( $\times 100$  magnification) assaying 9 randomly selected fields per treatment. Each experiment was performed in triplicate.

## 2.8 | RNA extraction and quantitative RT-PCR

Total RNA was isolated from CCA cells using TRIzol and following the manufacturer's instructions. RNA concentrations were determined using a NanoDrop™ 2000 spectrophotometer. Reverse transcription was performed using the ReverTra Ace qPCR RT Master Mix with gDNA Remover (TOYOBO Life Science). qRT-PCR was run with SYBR green fluorescence on a Step One Plus system (Applied Biosystems). Relative gene expression was obtained by normalization to the  $\beta$ -actin gene (*ACTB*). Experiments were performed in triplicate for each sample. The primer sets used in the present study are listed in Table S2.

## 2.9 | Western blot analyses

The nuclear extracts from cells were obtained using the NE-PER™ Nuclear and Cytoplasmic Extraction Reagent Kit following the manufacturer's instructions. Protein concentration of the cell lysate was quantified using the Bio-Rad protein assay. Nuclear extracts were separated by 15% SDS-polyacrylamide gel electrophoresis and transferred to PVDF membranes (GE Healthcare, Buckinghamshire,

UK). Membranes were then blocked with 5% skimmed milk at room temperature for 1 h and probed with primary antibody at 4°C overnight. After washing with PBST, the membranes were incubated with secondary antibody for 1 h at room temperature. Protein signals were detected using a chemiluminescence kit and an ImageQuant™ Imager LAS 4000 mini system (GE Healthcare). Protein band intensity was quantified by applying Image Quant™ Imager software.

## 2.10 | Proteomics analysis

### Sample preparation

Cells were washed twice with ice-cold PBS, trypsinized, and centrifuged to collect a cell pellet. Pellets were lysed in Tris-lysis buffer (20 mmol/L Tris-HCl, pH 7.5; 150 mmol/L NaCl; 10 mmol/L NaF; 1% [v/v] Triton-X). Protein concentration was measured by Bradford assay,<sup>30</sup> using bovine serum albumin as the standard. Protein samples were subjected to in-solution digestion. Samples were completely dissolved in 10 mmol/L ammonium bicarbonate (AMBIC), disulfide bonds were reduced by incubation in 5 mmol/L dithiothreitol (DTT) in 10 mmol/L AMBIC at 60°C for 1 h and sulfhydryl groups were alkylated using 15 mmol/L iodoacetamide (IAA) in 10 mmol/L AMBIC at room temperature for 45 min in the dark. For digestion, samples were mixed with 50 ng/ $\mu$ L sequencing grade trypsin (1:20 ratio) (Promega, Germany) and incubated at 37°C overnight. Prior to LC-MS/MS analysis, the digested samples were dried and protonated with 0.1% formic acid before injection into a LC-MS/MS system.

### Proteomics data analysis

To quantify the proteins in individual samples we used the MaxQuant 1.6.5.0 package. Label-free quantitation with MaxQuant's standard settings was performed: max. missed cleavages = 1, mass tolerance of 0.6 Da, trypsin as digesting enzyme, carbamidomethylation of cysteine as fixed modifications, and the oxidation of methionine as variable modifications. The false discovery rate value was fixed at 1% and estimated using the reversed search sequences. The maximal number of modifications per peptide was set to 5. As a search FASTA file, the 173 260 proteins present in the *Homo sapiens* proteome were downloaded from the UniProt Knowledge Base (UniProtKB) on September 9, 2019. The MaxQuant ProteinGroups.txt file was loaded into Perseus version 1.6.5.0 software. Maximum intensities were calculated in log<sub>2</sub>-transformed and pairwise comparisons between conditions using *t* tests. Venn diagrams were used to present the number of candidate proteins found in the experimental groups.

### 2.11 | Statistical analysis

All data analyses were performed using GraphPad Prism v.7 software. Descriptive data were presented as means  $\pm$  standard

deviation (SD). For data comparison between 2 groups, two-tailed Student *t* test was conducted to assess statistical significance. The association between *MAN2A2* and *CHD8* expression and its clinical relevance was assessed by boxplot analysis (*P*-value cutoff: .01). The association between *MAN2A2* and *CHD8* expression was calculated using Pearson's rank correlation coefficient. A *P*-value < .05 was considered to be statistically significant.

### 3 | RESULTS

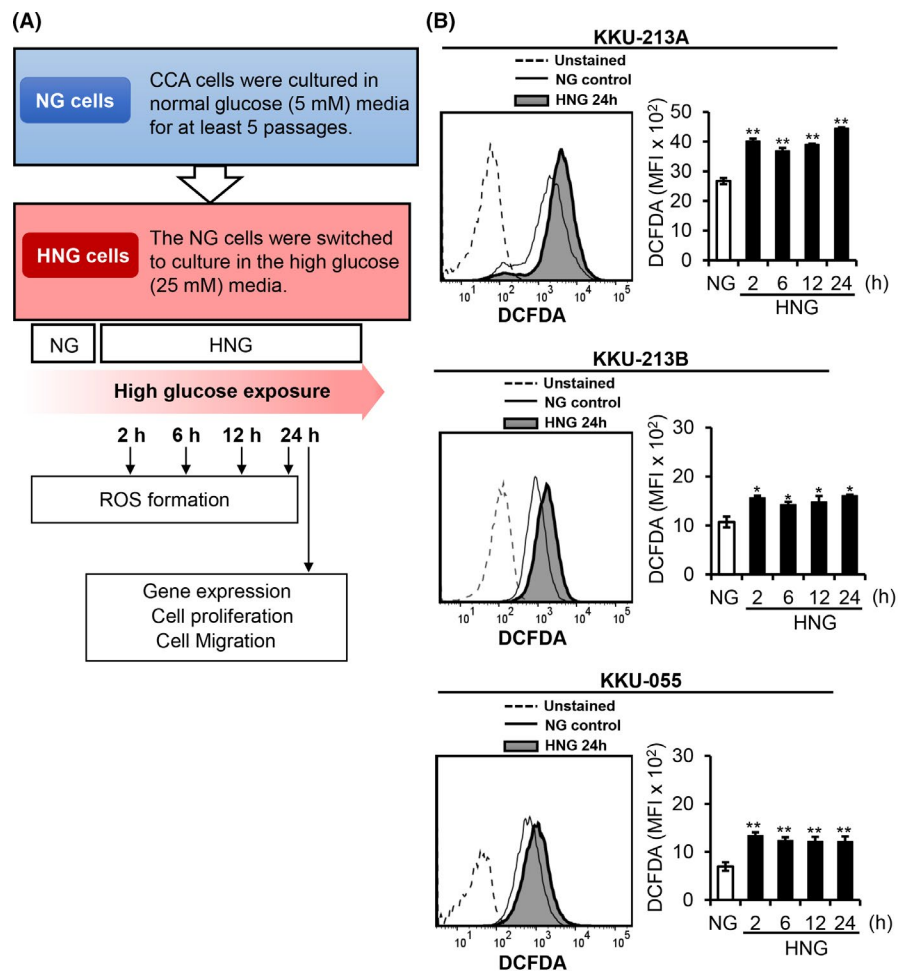
#### 3.1 | High glucose promoted ROS production, proliferation, and migration of CCA cells

To investigate whether high glucose influenced ROS production in CCA cells, all cell lines were cultured under normal or high glucose conditions. CCA cells were initially maintained in high glucose medium (25 mmol/L glucose concentration). We then established normal glucose cells by sequentially reducing the glucose concentration from high to normal glucose (5.5 mmol/L glucose concentration) in the medium. At least 5 passages of culture for all cell lines in normal glucose medium elicited an adaptive response in the cells to their conditions before use, as described (Figure 1A). CCA cells cultured in normal glucose medium were called normal glucose (NG) cells while

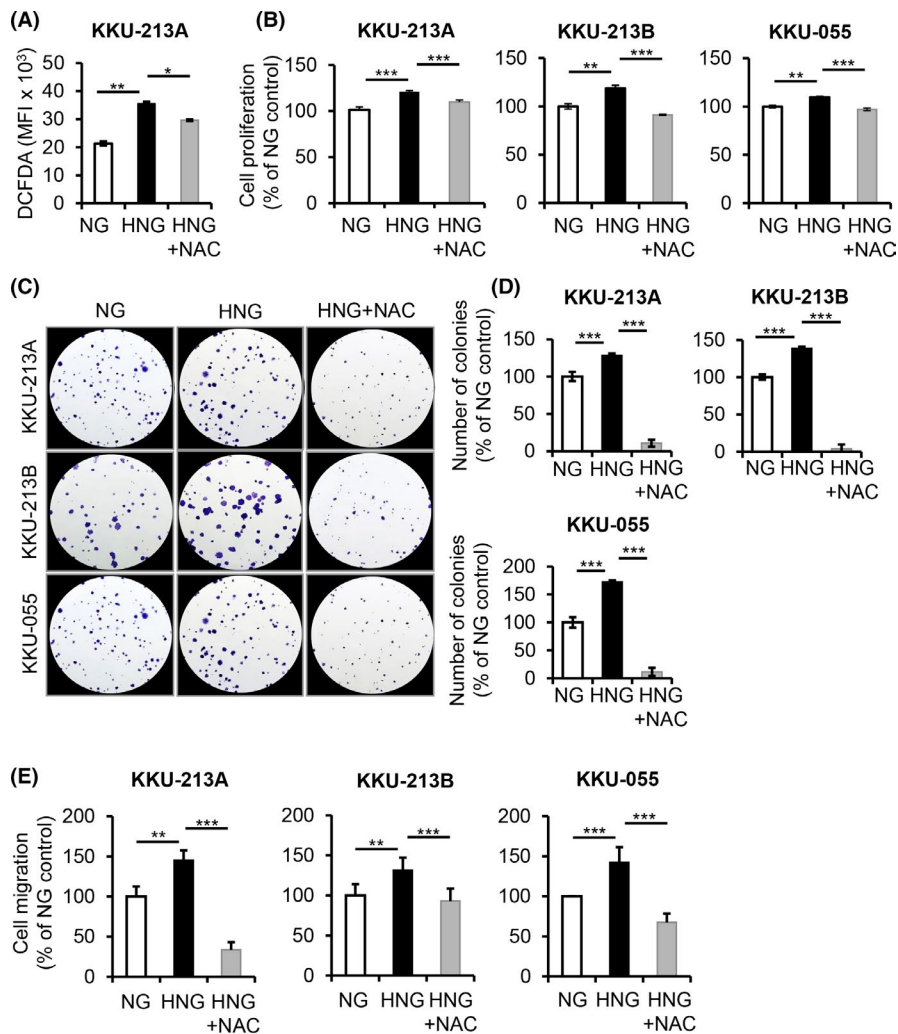
NG cells switched to be cultured in high glucose medium were called human neuronal-gliol (HNG) cells. We first determined the levels of ROS in CCA cells cultured with high glucose for 2, 6, 12, or 24 h. ROS production was significantly increased at 2-24 h after high glucose treatment compared with control NG cells in 3 CCA cell lines, including KKU-213A, KKU-213B, and KKU-055 (Figure 1B). These data suggested that high glucose could affect redox homeostasis in CCA cells. We used 24 h high glucose treatment for further studies.

#### 3.2 | High glucose enhanced the proliferation and migration ability through ROS signaling

To investigate whether high glucose-accelerated ROS production was required for progression of CCA cells, we added a ROS scavenger, namely NAC, into the system. At first, we confirmed that NAC treatment significantly reduced ROS levels in CCA cells (Figure 2A). We next examined the role of ROS in proliferation of CCA cells using SRB and colony formation assays. As shown in Figure 2B, HNG cells had a significantly higher proliferation rate compared with the NG cells. In contrast, induction of cell proliferation was clearly reduced by NAC treatment compared with HNG cells (Figure 2B). Consistent with the proliferation results from the SRB assay, the colony formation assay showed a reduced phenotype



**FIGURE 1** High glucose induces reactive oxygen species (ROS) in cholangiocarcinoma (CCA) cells. A, A schematic diagram depicting the establishment of high glucose cells. B, Levels of ROS in CCA cells treated with high glucose (HNG cells) compared with those cultured in normal glucose (NG) cells as indicated by DCFH-DA using flow cytometry. Data represent mean fluorescence intensity (MFI) ± SD of 3 replicates. Student *t* test was used to assess statistical differences (*P*-values of \**P* < .05, \*\**P* < .01 were considered statistically significant.)



**FIGURE 2** High glucose potentiates aggressive phenotypes of cholangiocarcinoma (CCA) cells via ROS. A, B, The effect of HG-ROS signaling on the proliferation of CCA cells using SRB assay. C, D, Representative colony formation ability of CCA cells using clonogenic assay and the number of colonies are shown. E, Cell migration revealed by Boyden chamber migration assay. Statistical differences were assessed using Student *t* test (\**P* < .05, \*\**P* < .01, \*\*\**P* < .001)

following NAC treatment (Figure 2C,D). Apart from proliferative effects, high glucose also promoted the progression of CCA cell migration, as assessed by Boyden chamber assay (Figure 2E). The relative migration rate increased significantly in HNG cells and induction was blocked by NAC treatment (Figure 2E). In addition, to examine whether ROS themselves activated proliferation of CCA cells, we performed the proliferation assay with H<sub>2</sub>O<sub>2</sub> treatment in CCA cells as shown in Figure S1. Our data showed that H<sub>2</sub>O<sub>2</sub> induced CCA cell proliferation to a similar extent following high glucose treatment when compared with untreated control. These findings suggested that high glucose-dependent ROS production was necessary for proliferation and migration ability of CCA cells.

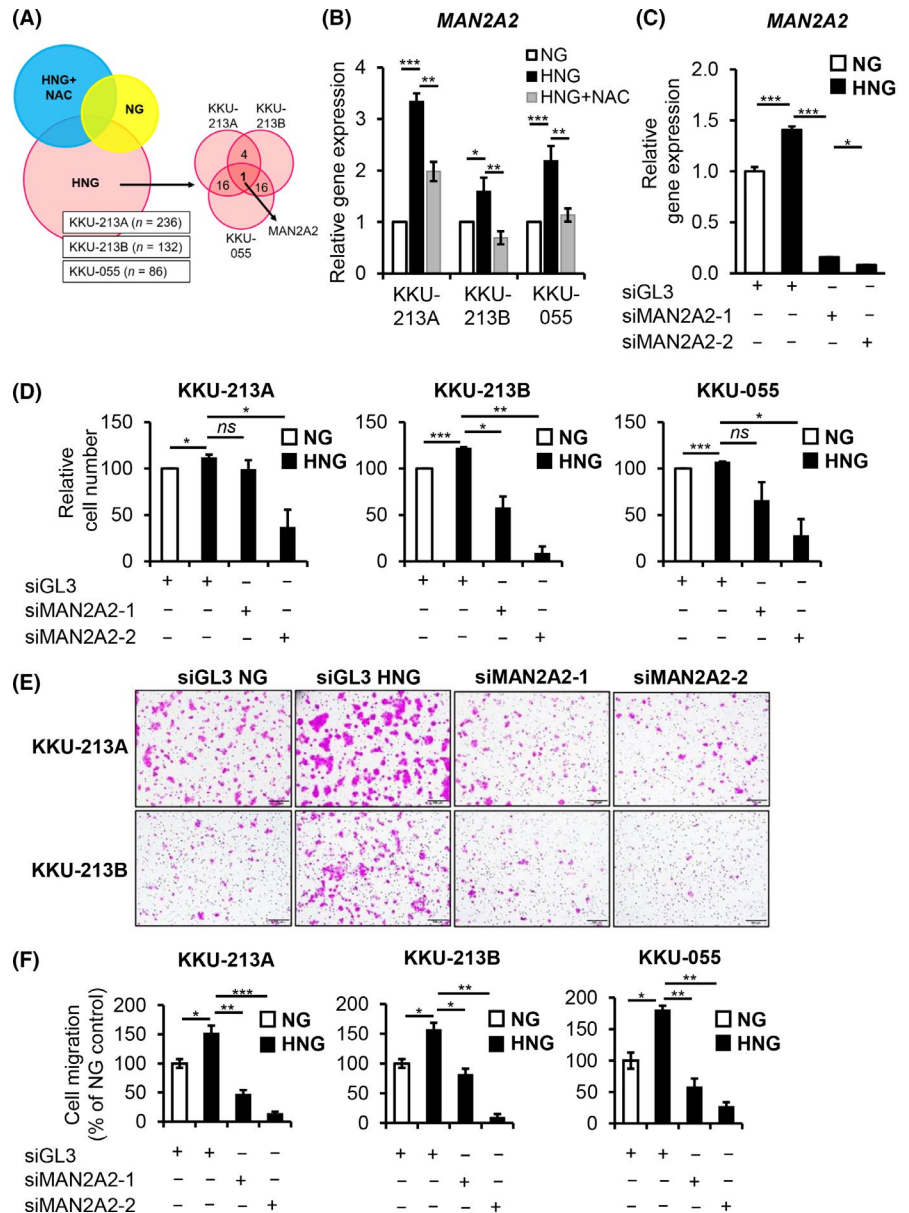
### 3.3 | High glucose-ROS signaling promotes progression of CCA cells by upregulation of MAN2A2

To understand the molecular mechanisms of how the high glucose-ROS axis potentiates the progression of CCA cells, we used unbiased high-throughput proteomic analysis to identify common proteins among 3 CCA cell lines detected only under HNG conditions but not under NG or HNG + NAC conditions. The Venn diagram represents

the strategy of how we evaluated the candidate proteins in the experimental setting (Figure 3A). The candidate proteins are listed in Table S3. Based on the analysis, mannosidase alpha class 2a member 2 (MAN2A2) was identified as a candidate protein (Figure 3A). We thus hypothesized that MAN2A2 might be an essential molecule for promoting aggressive phenotypes of CCA cells in a high glucose-ROS-dependent manner. Next, we determined the expression of MAN2A2 mRNA in a high glucose-ROS-dependent manner using qRT-PCR. Importantly, MAN2A2 mRNA levels were significantly upregulated in 3 CCA cell lines of HNG cells compared with control NG cells (Figure 3B). In addition, MAN2A2 expression was dramatically reduced in HNG cells treated with NAC, indicating that MAN2A2 expression was dependent on the high glucose-ROS axis in CCA cells.

We subsequently determined the role of MAN2A2 in the progression of CCA under high glucose conditions. MAN2A2 mRNA levels were clearly suppressed by 2 kinds of MAN2A2 siRNA (Figure 3C). Importantly, significant reduction in cell proliferation rate was observed in MAN2A2 knockdown in 3 CCA cell lines (Figure 3D). More obviously, knockdown of MAN2A2 suppressed the high glucose-dependent acceleration of migration in 3 CCA cell lines (Figure 3E,F). These data suggested that MAN2A2 is crucial for CCA cell proliferation and migration in a high glucose environment.

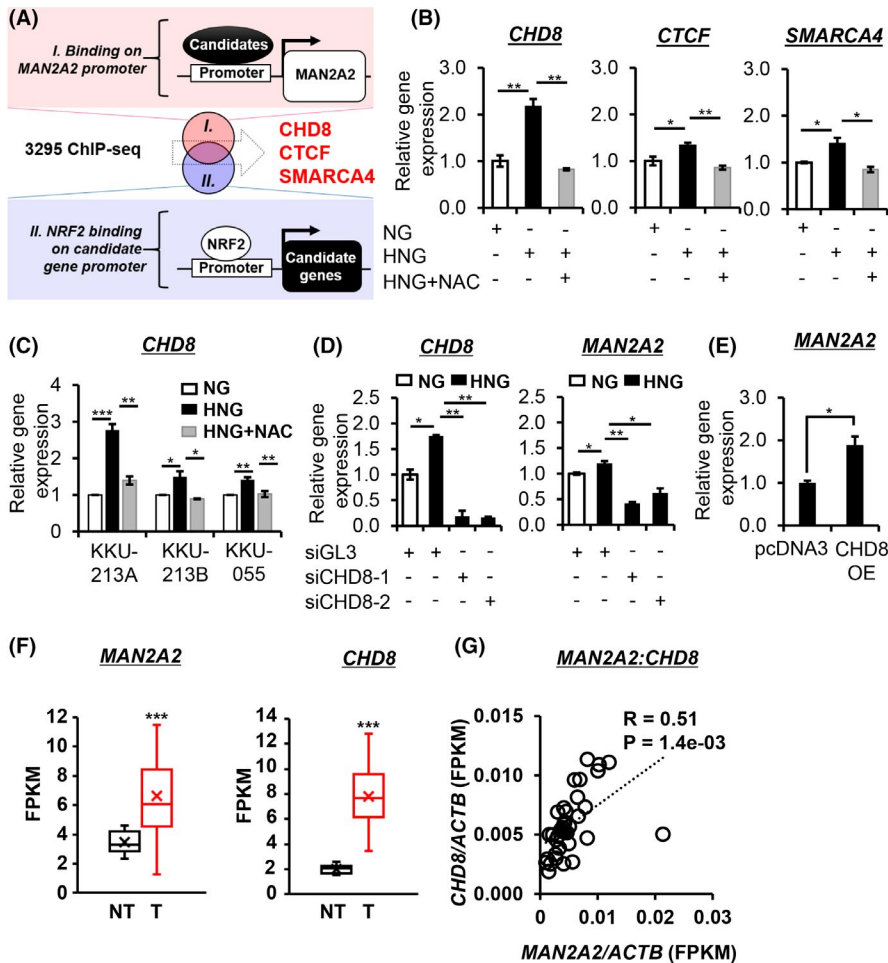
**FIGURE 3** MAN2A2 expression is associated with progressive phenotypes of cholangiocarcinoma (CCA) cells. A, Schematic diagram of how to identify candidate proteins involved in high glucose-ROS signaling by proteomics analyses. B, The relative expression of MAN2A2 mRNA was determined using qRT-PCR. C, Detection of MAN2A2 mRNA in siMAN2A2 by qRT-PCR. D, Cell proliferation inhibition in MAN2A2 knockdown using CCK-8 assay. E, Migrative image using the microscope ( $\times 100$  magnification). F, The number migrated cells were counted using 9 fields and normalized by NG control cells. Data represent mean  $\pm$  SD of migrated cells from 3 replicates. Scale bar: 200  $\mu$ m. \* $P < .05$ , \*\* $P < .01$ , \*\*\* $P < .001$ , ns represents not statistically significant ( $P > .05$ )



### 3.4 | Induction of MAN2A2 expression is regulated by CHD8 under high glucose-ROS signaling

To understand the molecular mechanisms of how MAN2A2 was increased under the high glucose-ROS signaling axis, we screened proteins that bound to MAN2A2 promoter. Because nuclear factor erythroid 2-related factor 2 (NRF2) transcription factor is commonly activated and leads to upregulation of target genes under the oxidative stress conditions, we first checked NRF2 binding to the MAN2A2 promoter. However, we failed to detect NRF2 binding to the MAN2A2 promoter (Figure S2A). This result led us to hypothesize that other factors were involved in MAN2A2 upregulation under high glucose-ROS signaling. As a first screening using in silico analysis of 3295 ChIP-seq data (<https://chip-atlas.org/>) and 107 transcription factors and transcription-related factors, we found 16 factors that could bind to the MAN2A2 promoter. As a second screening, we analyzed

NRF2 binding to their promoter. As a result, there were 3 genes, including CHD8, CTCF, and SMARCA4, that were candidates (Figure 4A). Data processing (Figure 4A) and promoter binding site mapping are shown (Figure S2A,B). Based on these data, we further evaluated the mRNA expression profiles of CHD8, CTCF, and SMARCA4 under high glucose conditions in the KKKU-213A cell line. As shown in Figure 4B, these 3 genes were significantly induced by high glucose treatment, and loss of expression was observed following NAC treatment. Because strongest induction was observed in CHD8 (Figure 4B) and proteomics studies also showed CHD8 induction in some groups (Table S3), we next focused on CHD8 to study the possible relationship to MAN2A2 regulation. Significant induction of CHD8 in a high glucose-ROS-dependent manner was also confirmed in 3 CCA cell lines (Figure 4C). Importantly, CHD8 knockdown significantly reduced CHD8 and MAN2A2 expression (Figure 4D). Conversely, CHD8 overexpression increased MAN2A2 mRNA levels (Figure 4E).



**FIGURE 4** The relationship of *MAN2A2* and *CHD8* in cholangiocarcinoma (CCA) cells and tissues. A, Schematic diagram of comprehensive analysis by using ChIP-seq data (3295 ChIP-seq data) to screen the protein binding on *MAN2A2* promoter and among them also related to *NRF2* binding on their promoter. B, Influence of high glucose on the relative mRNA expression of *CHD8*, *CTCF*, and *SMARCA4* in KKU-213A. C, Relative mRNA expression of *CHD8* in high glucose-ROS signaling in 3 CCA cell lines. D, *MAN2A2* mRNA level in *CHD8* knockdown. E, *MAN2A2* mRNA level in *CHD8* overexpressing. The relative gene expression was analyzed using qRT-PCR. F, The *MAN2A2* and *CHD8* mRNA in CCA tissues (T, n = 36) compared with adjacent normal tissues (NT; n = 9). G, Correlation between *MAN2A2* and *CHD8* was analyzed using the data from TCGA database and Spearman's rank correlation coefficient. mRNA levels represent fragments per kilobase of transcript per million mapped reads (FPKM) on individual patient. The values represent mean  $\pm$  SD of 3 replicates. \* $P < .05$ , \*\* $P < .01$ , \*\*\* $P < .001$

Although the induction ratio was weaker compared with *CHD8* (Figure 4B), a previous study had shown that *CHD8* directly interacted with chromatin insulator binding protein *CTCF*,<sup>28</sup> and both *CTCF* and *SMARCA4* were localized with *CHD8* on the *MAN2A2* promoter (Figure S2). We further investigated whether *MAN2A2* expression was influenced by other factors such as *CTCF* and *SMARCA4*. siRNA efficiently suppressed the expression of *CTCF* and *SMARCA4* (Figure S3A,B). The results showed that *CTCF* and *SMARCA4* knockdown significantly suppressed *MAN2A2* mRNA expression (Figure S3A,B). Based on these data, we further hypothesized that high glucose-ROS signaling could activate *CHD8*-*CTCF*-*SMARCA4* complex formation. Although siRNA for *CHD8*, *CTCF*, and *SMARCA4* suppressed the expression of *MAN2A2*, interaction of *CHD8* with either *CTCF* or *BRG1* was not observed in the immunoprecipitation assay, as shown in Figure S3C. These results suggested that high glucose-ROS signaling activated *CHD8*, *CTCF*, and *SMARCA4*, and that those proteins might be independently involved in the positive regulation of *MAN2A2* gene in CCA cells.

To further investigate whether high glucose-ROS-induced *MAN2A2* and *CHD8* expression might be regulated by the *NRF2* transducing pathway, we checked *NRF2* expression in KKU-213A and KKU-214B cells (Figure S4A,B). *NRF2* was stabilized by high glucose treatment in both cells, which was cancelled by NAC, a ROS

inhibitor. In addition, induction of *CHD8* and *MAN2A2* mRNA by high glucose was reduced by siRNA for *NFE2L2* (*NRF2*), as shown in Figure S4C *NFE2L2*, and a well known target gene, *NQO1*, was clearly suppressed by siRNA-*NFE2L2*. These data suggested that *CHD8* up-regulated *MAN2A2* mRNA expression under the high glucose-ROS-*NRF2* axis. To support our data that ROS is important for *CHD8* and *MAN2A2* upregulation in vivo, correlation between *NRF2* (*NFE2L2*) and *CHD8*/*MAN2A2* expression levels in human CCA tissue was analyzed using TCGA database. There was a significant linear correlation between *NRF2* and *CHD8*, or *NRF2* and *MAN2A2* expression in human CCA tissues, as shown in Figure S5.

### 3.5 | Comprehensive analyses of *MAN2A2* and *CHD8* expression in CCA patient tissues

To determine the clinical relevance of *MAN2A2* and *CHD8* expression in CCA patient tissues, we analyzed the data from the Gene Expression Profiling Interactive Analysis (GEPIA) database (<http://gepia.cancer-pku.cn/index.html>) that showed that expression of both *MAN2A2* and *CHD8* were significantly higher in tumor tissues (T) compared with the adjacent normal tissues (NT) (Figure 4F). We next examined the correlation between *MAN2A2* and *CHD8*

expression. We found that these 2 genes were positively correlated ( $r = 0.51$ ;  $P = 1.4e-03$ ) (Figure 4G). *MAN2A2* and *CHD8* expression levels in various cancer tissues were also analyzed. Interestingly, relative gene expression of *MAN2A2* and *CHD8* was clearly upregulated in CCA tissues compared with adjacent NT (Figure S6). Compared with other cancers, high *MAN2A2* and *CHD8* expression levels are more likely to tend to be tumor tissue specific, especially in CCA.

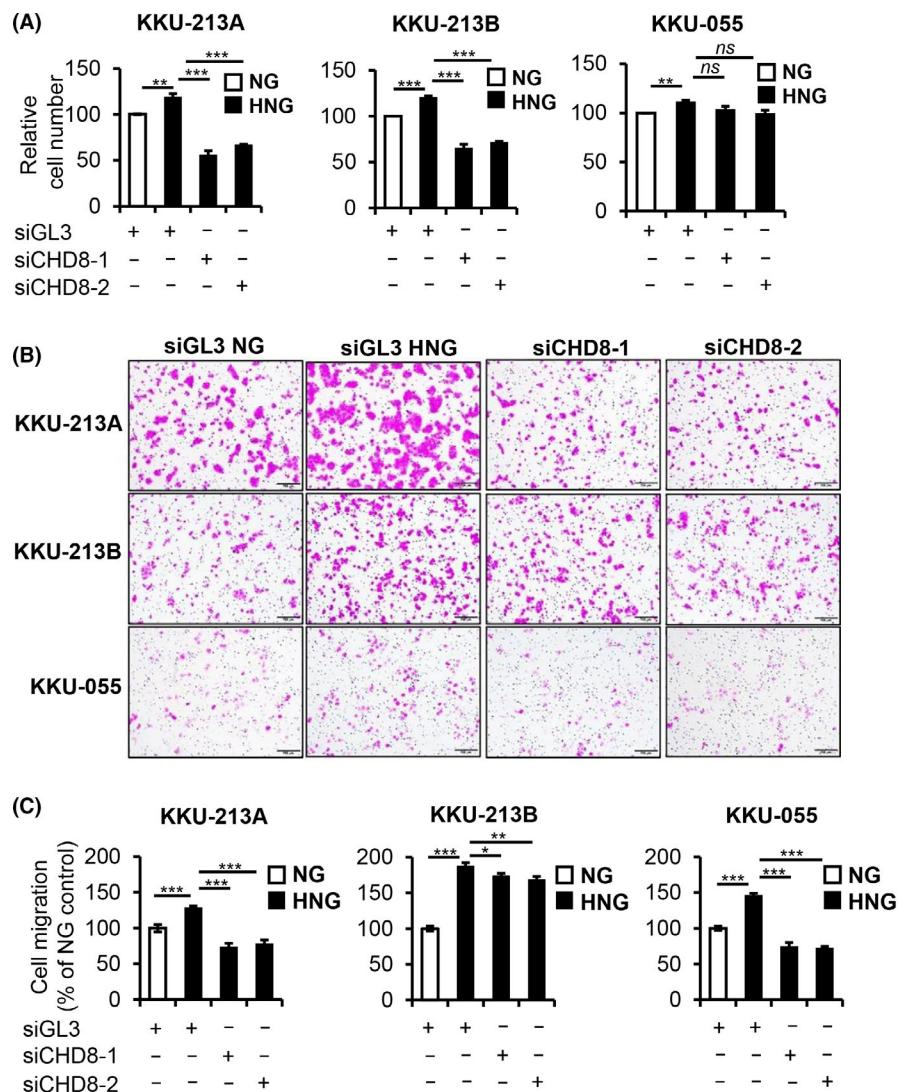
### 3.6 | CHD8 plays a crucial role for aggressive phenotypes of CCA cells

CHD family proteins have crucial roles in chromatin assembly and gene expression.<sup>31</sup> CHD8 has also been identified as a cancer driver gene involved in the epigenetics of pancreatic cancer. Upregulation of CHD8 and downregulation of *MAN2A2* were also observed in pancreatic cancer, as shown in Figure S6,<sup>32</sup> however the effect of CHD8 in CCA progression has not been reported. We wonder whether inhibition of *CHD8* would reduce the proliferation and migration ability of CCA cells. As shown in Figure 5A, *CHD8*

knockdown dramatically inhibited the proliferation rate in CCA cell lines (Figure 5A). Also, *CHD8* knockdown significantly inhibited cell migration (Figure 5B,C). After knockdown, *CHD8* protein levels were confirmed by western blot analysis (Figure S7). These observations suggested that consecutive upregulation of *CHD8* and *MAN2A2* played significant roles in the progression of CCA cells associated with high glucose-ROS signaling.

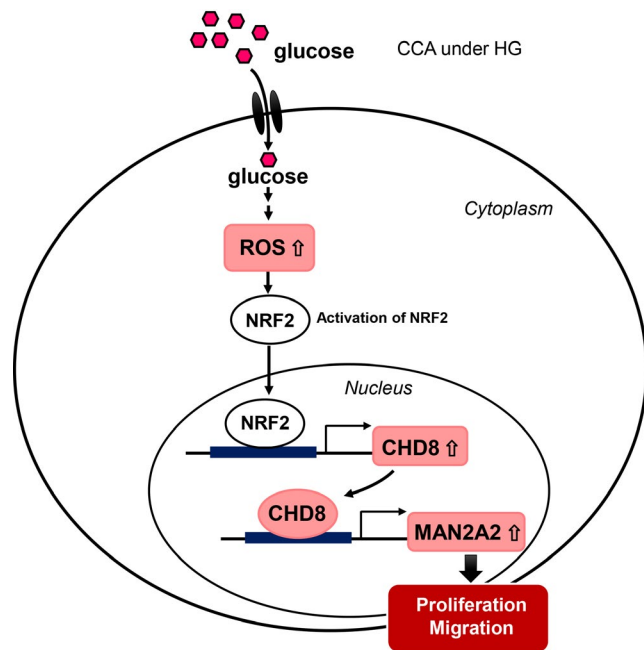
## 4 | DISCUSSION

In the present study, we investigated the mechanism by which high glucose-induced ROS could promote CCA progression. We identified that *MAN2A2* targeted high glucose-ROS signaling to promote cell proliferation and migration mediated by CHD8. Our data enabled us to draw several conclusions (Figure 6). First, high glucose-ROS signaling enhanced CCA progression through modulating *MAN2A2*. Second, there was regulation of *MAN2A2* by high glucose-ROS signaling. Third, high glucose-ROS signaling increased the NRF2-CHD8-*MAN2A2* pathway to promote CCA proliferation and migration.



**FIGURE 5** CHD8 is crucial for aggressive phenotypes of cholangiocarcinoma (CCA) cells. A, The relative cell number of siCHD8 knockdown after 24 h post-transfection in high glucose normalized by NG control cells. B, The representative image of migrated cells under microscope ( $\times 100$  magnification). C, The rate of migration quantitation analysis. Scale bar: 200  $\mu\text{m}$ . \* $P < .05$ , \*\* $P < .01$ , \*\*\* $P < .001$ , ns, not statistically significant ( $P > .05$ )





**FIGURE 6** Schematic model of how high glucose-ROS potentiates aggressive phenotypes of cholangiocarcinoma (CCA)

Finally, high glucose-ROS signaling was required for progression of CCA cells, and was consistent with previous reports.<sup>33</sup> In addition, *CHD8* and *MAN2A2* were highly expressed in CCA tissues and were positively correlated (Figure 4F,G). These findings suggested a promising therapeutic target for CCA under high glucose conditions.

Targeting ROS signaling in cancer is considered a double-edged sword, ie tumor-promoting and tumor-suppressing. Growing evidence has indicated the positive aspects of higher ROS levels in promoting high glucose-induced proliferation and migration of cancer cells, whereas proliferative effects of high ROS levels can be reduced by ROS inhibitor.<sup>25,34</sup> Consistent with the evidence from other cancers, our data also demonstrated that high glucose-induced ROS generation enhanced CCA cell aggressiveness and that NAC, a ROS inhibitor, significantly reduced ROS levels, proliferation, and migration in CCA cells. As to the mechanism, Luo et al demonstrated that high glucose-induced ROS markedly reduced the phosphorylation of c-Jun-N-terminal kinase (JNK), which enhances cell cycle and anti-apoptotic signals in pancreatic cancer.<sup>24</sup> In addition, another downstream mechanism of high glucose-ROS-dependent cancer progression is activation of the NRF2 pathway. NRF2 has been reported to be activated under oxidative stress conditions and leads to direct transcription of NRF2 target genes, contributing to cancer progression.<sup>35</sup> In this study, we showed that high glucose activated NRF2 stabilization and knockdown of NRF2 inhibited *CHD8* and *MAN2A2* in CCA cells, as found for *NQO1*, a common target gene of NRF2 signaling (Figure S4). In addition, NRF2 binding to the promoters for *CHD8*, *CTCF*, and *SMARCA4* was clearly observed using in silico analysis (Figure S3B). These data suggested that NRF2, at least in part, was involved in the regulation of *CHD8*, *CTCF*, and *SMARCA4* under

high glucose conditions in CCA cells. Future studies will focus on the investigation of downstream mechanisms of how *CHD8* and other molecules are induced under the high glucose-ROS signaling pathway.

A previous study showed that reduction of an alpha-mannosidase, *MAN1A1*, resulted in the accumulation of high mannose *N*-glycans in CCA cells and found a negative correlation with metastatic potential.<sup>36</sup> Consistently, our data showed that *MAN1A1* levels were significantly lower in CCA tissues (Figure S8A), suggesting that *MAN2A2* upregulation and *MAN1A1* downregulation were associated with progression of CCA cells. Fukuda et al reported that alpha-mannosidase IIx (MX) (encoded by *MAN2A2*) is an enzyme closely correlated with alpha-mannosidase II (MII) (encoded by *MAN2A1*), a Golgi *N*-glycan processing enzyme. *MAN2A2* and *MAN2A1* can compensate for each other's deficiencies.<sup>37</sup> *MAN2A2* was reported to mediate conversion of Man6GlcNAc2 to Man4GlcNAc2.<sup>37,38</sup> This evidence and our data suggested that decreased *MAN1A1* and increased *MAN2A2* levels resulted in aberrant high mannose-type *N*-glycan accumulation, ie accumulation of Man9GlcNAc2, Man6GlcNAc2 and then Man4GlcNAc2 (Figure S8B). This modification could cause inhibition of complex type *N*-glycans. Loss of *N*-linked glycosylation led to lack of protein function, stabilization, and had links to cancer progression including in CCA. A schematic diagram of this proposed pathway combined with related enzymes that are closely associated with *MAN2A2* is shown (Figure S8B). We will focus in the future on a more detailed understanding of how *MAN2A2* works in the progression of CCA.

Another interesting finding was the *CHD8* involvement in CCA progression. *CHD8* is a group of ATP-dependent chromatin remodeling enzymes from the chromodomain helicase DNA-binding protein (CHD) family, which plays a key role in the regulation of gene transcription and expression.<sup>39</sup> Although *CHD8* has been proposed to be an important epigenetic regulator by promoting histone H1 recruitment of  $\beta$ -catenin target gene and p53, resulting in suppression of their transcription,<sup>40,41</sup> even this suggestion is still controversial. *CHD8* also acts as a transcriptional activator of cyclin E2 (*CCNE2*) and thymidylate synthetase (*TYMS*) genes, required for cell proliferation.<sup>42,43</sup> However, only a few reports have investigated the role of *CHD8* in cancer and its function in CCA was totally unknown. Our data highlighted the importance of *CHD8* in CCA proliferation and migration, and the significance of *CHD8* as an upstream regulator for *MAN2A2*. We previously reported that *CHD8* interacts with CTCF insulator protein.<sup>28</sup> The *CHD8*-CTCF complex is associated with an enhancer-blocking mechanism that contributes to inactive chromatin states.<sup>44</sup> In addition, it has been reported that *CHD8* interacts with BRG1, a protein encoded by the *SMARCA4* gene<sup>45</sup> and CTCF also interacts with BRG1.<sup>46</sup> These reports and our data led us to hypothesize that *CHD8*, CTCF, and BRG1 might form a protein complex on the *MAN2A2* promoter and upregulate *MAN2A2* expression. Consistently, knockdown of these genes suppressed *MAN2A2* in CCA cells (Figures 4D and S3A,B). Moreover, *MAN2A2*, *CHD8*, CTCF, and *SMARCA4* (*BRG1*) were highly expressed in CCA tissues

compared with NT (Figure S6). However, a physical interaction among proteins was not observed (Figure S3C). It might be possible that CHD8, CTCF, and BRG1 positively regulate *MAN2A2* transcription independently to promote CCA progression. This point should be investigated in a future study.

In conclusion, our data demonstrated that high glucose increased *CHD8* and *MAN2A2* expression by accumulation of ROS, and that ROS production mediated proliferation and migration of CCA cells. Inhibition of these mechanisms might be a powerful therapeutic regime for CCA patients with DM. Future studies are needed to examine the potential mechanisms by which high glucose-upregulated *CHD8* and *MAN2A2* promote aggressive CCA cell growth.

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#### DISCLOSURE

The authors have no conflict of interest to declare.

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#### SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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