Elevated Glucose Promotes DNA Replication and Cancer Cell Growth

through pRB-E2F1

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Research Article

ABSTRACT

Although epidemiological studies have highlighted a link between hyperglycemia and increased risk of cancer, knowledge about the molecular mechanism behind the link remains limited. Moreover, while High Glucose (HG) is known to promote cell growth, the overall transcription regulation involved in this process is less clear. In this study, through genome-wide analyses, we identify E2F1 as the core transcription factor for the HG-induced cell growth.

Inhibition of E2F1 abrogates the HG-induced DNA synthesis and cell growth, supporting the role of E2F1 in this process. Furthermore, we demonstrate that elevated glucose levels enhance pRB phosphorylation, which plays a role in E2F1 activation. Interestingly, among HG-induced E2F1 target genes, RRM2 (Ribonucleotide Reductase regulatory subunit M2) participates in the nucleotide synthesis by catalyzing the generation of the essential dNTP for DNA replication. We show that HG increases cellular dNTP levels in E2F1-RRM2 dependent manner, which correlates to enhanced DNA synthesis and cancer cell growth. Collectively, our findings decipher a pRB-E2F1-RRM2 dependent link between hyperglycemia and cancer cell proliferation and provide a molecular mechanism by which hyperglycemia directs tumor cells to DNA replication.

Keywords: E2F1 transcription factor; pRB phosphorylation; Ribonucleotide Reductase regulatory subunit M2 (RRM2); High glucose; Cancer

INTRODUCTION

Cancer is characterized by uncontrolled cell proliferation with sustaining proliferative signaling as a classic hallmark ^[1–3]. In many circumstances, transcription factors are pivotal effectors for altered proliferative signaling pathways and function to promote uncontrolled cell growth through regulation of transcription. Ultimately, altered signaling cascades lead to the process of DNA synthesis and cell cycle progression for excessive cell proliferation.

Diabetes mellitus is a group of metabolic disorders triggered by dysregulation of glucose metabolism resulting in hyperglycemia. It is categorized into two main types: Type 1 (T1DM) and Type 2 (T2DM), both have been linked to an increased risk of various cancers ^[4,5]. Given that glucose is a key nutrient source for cell survival, especially for the rapidly proliferating tumor cells ^[6], it is not surprising that elevated glucose level has been suggested as a leading risk factor for cancer in the past decade ^[7]. In fact, hyperglycemia has been widely accepted as a major biological link between diabetes and cancer due to the Warburg effect: A higher dependency of tumor cells on glycolysis for continuously producing ATP energy molecule ^[4,8–10]. Besides the direct effect on the cancer cells, elevated glucose levels can sustain an uncontrolled and everlasting chronic inflammatory state, creating a tumor favorable microenvironment to facilitate tumor development and metastasis ^[11]. While hyperglycemia's role in cancer has been studied, a comprehensive understanding of the molecular pathway by which hyperglycemia induces cancer growth remains lacking.

To systematically investigate this and to identify the role of key transcription factors in this process, we carried out high throughput RNA-seq analysis. Our data revealed that the core transcriptional regulator E2F1 plays a critical role in directing cancer cells to DNA synthesis and cell proliferation under elevated glucose conditions. Moreover, we showed that elevated glucose enhances pRB hyper-phosphorylation, leading to E2F1 activation. Interestingly, among HG induced E2F1 target genes, RRM2 has been shown to participate in the nucleotide synthesis by generating essential dNTP for DNA replication. Our data suggested that HG leads to RRM2 dependent increase of cellular dNTP levels, which correlates to DNA synthesis and cancer cell growth. Together, our finding uncovers a molecular mechanism for enhancing cell growth by elevated glucose levels and sheds light on the significance of the pRB/E2F axis as a potential therapeutic target in the tumor-bearing diabetic patients.

MATERIALS AND METHODS

Cell culture and reagents

HCT116 and U2OS cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) containing 5 mM glucose. H460 cells were cultured in RPMI 1640 containing 11 mM glucose.

HEK293T cells were cultured in DMEM containing high glucose (25 mM). All medium were supplemented with 10% FBS. For HCT116 and U2OS cells, HG treatments were carried out by adding 25mM glucose (#BM-675, Boston BioProducts) into the complete medium for 6 h or as indicated. For H460 cells, HG treatments were carried out by culturing cells in RPMI 1640 medium containing either 5 mM (Mock) or 25 mM glucose (HG). To inhibit E2F activity, cells were treated with 40 μ M pan-E2F inhibitor HLM006474 (Sigma-Aldrich) for 9 h. To inhibit RRM2 activity, HCT116 cells were treated with 250 or 500 nM Triapine (Selleck Chemicals) as described. To inhibit pRB phosphorylation, HCT116 cells were treated with 1 μ M PF-3600 (PF-06873600, Cayman Chemical) as indicated ^[12].

The following antibodies were used for IB: anti-Vinculin (V9131, Sigma-Aldrich), anti-RRM2 (sc-398294, Santa Cruz), anti-E2F1 (sc-251, Santa Cruz), anti-CHAF1A (sc-133105, Santa Cruz), anti-Rb1 (#9309, Cell Signaling Technology), anti-phospho Rb1 (S807/811) (#8516, Cell Signaling Technology) and anti-β-actin (A3854, Millipore Sigma).

RNA-seq and data analysis

Total RNA was isolated and purified from HCT116 cells in biological triplicates using Direct-zol RNA Miniprep Plus Kit (Zymo Research) according to the supplier's instruction.

RNA-seq libraries were then prepared following the manufacturer's protocol of NEBNext Ultra II Directional RNA Library Prep Kit for Illumina (New England BioLabs). The sequencing was performed on Illumina NextSeq using single end 75 bp read

length. Sequencing reads were mapped to the human reference genome (GENCODE v34) using Salmon ^[13], followed by differential expression analysis using DESeq2 ^[14]. Gene Set Enrichment Analysis (GSEA) (v4.2) was used to analyze the enrichment of REACTOME pathways and transcription factors among differentially expressed genes ^[15]. Volcano plot was generated using ggplot2 (v3.4.0).

GeneOntology biological process analysis was performed in ShinyGO (v0.75) [16].

shRNA-mediated knock down

E2F1-targeting and scramble shRNA (Table 1) were cloned into pLK0.1 vector with puromycin selection marker. Lentivirus were produced by transfection of HEK 293T cells with the transfer plasmid and lentiviral packaging and VSVG plasmids. The virus was harvested 48 h post-transfection. H460 cells were transduced with the lentivirus in the presence of 10 ug/ml polybrene (Millipore). After 48 h, transduced cells were selected with 1 μ g/ml puromycin for 7 days.

Туре	Name	Sequence	
qPCR primer	GAPDH_F	ACAACTTTGGTATCGTGGAAGG	
	GAPDH_R	GCCATCACGCCACAGTTTC	
	RRM2_F	GTGGAGCGATTTAGCCAAGAA	
	RRM2_R	CACAAGGCATCGTTTCAATGG	
	CHAF1A_F	TTAGACCGAAACTTGTCAACGG	
	CHAF1A_R	GTCTGGCTGCTCATTCGAGT	
	CHAF1B_F	AGAGGCAAGAAGCTACCGGAT	
	CHAF1B_R	CTGGCGTGAGAAGCAAAGA	
	PCNA_F	CCTGCTGGGATATTAGCTCCA	
	PCNA_R	CAGCGGTAGGTGTCGAAGC	
	CCNE2_F	GGAACCACAGATGAGGTCCAT	
	CCNE2_R	CCATCAGTGACGTAAGCAAACT	
	CLSPN_F	TGGAGAGTGGGGTCCATTCAT	
	CLSPN_R	CCGGGGTTTACGTTTGAAGAAA	
	RBM14_F	CTACCAGCAGGCTTTTGGCA	
	RBM14_R	GTCATGGGCTGAGTCCGATAG	
	18S rRNA_F	CTCAACACGGGAAACCTCAC	
	18S rRNA_R	CGCTCCACCAACTAAGAACG	
shRNA	Scramble	GCGTACATCACTCGTTAATAT	
	shE2F1	CGCTATGAGACCTCACTGAAT	
ChIP-qPCR	RRM2_E2F1_F	ACGGGGGTGTCCCCGGGGGT	
	RRM2_E2F1_R	CTTCCCATTGGCTGCGCCTT	

Table 1. List of oligonucleotide sequences.

Cell cycle and cell growth analysis

Cell cycle analysis was performed using the Click-iT Plus Edu Kit (#C10632, Thermo Scientific) according to the manufacturer's protocol. In brief, 2 million cells post-treatment were harvested and fixed. EdU was then labeled with Alexa Fluor 488 picolyl azide for 30 min at room temperature. After labeling, total DNA content was stained with 20 ng/ml Pl (#P3566, Invitrogen) for 1 h at room temperature. Samples were then analyzed by flow cytometry on the NovoCyte platform (ACEA).

For cell growth assays, cells were seeded at 2 x 10⁴ cells per well in a 24-well plate in medium containing either 5 mM or 25

mM glucose. At each time point, cells were trypsinized and counted on the automated cell counter (Thermo Fisher Scientific).

DNA fiber assay

Cells were pulse-labeled with 25 μ M CldU (Sigma-Aldrich) for 20 min, followed by a second pulse of 250 μ M IdU (Fisher Scientific) for another 20 min. Cells were harvested, lysed and DNA spread on slides as previously described ^[17]. DNA fibers were further denatured in 2.5M HCl and blocked with 1% BSA in PBS with 0.1% Tween-20 (PBST) to reduce background. The labeled CldU and IdU fibers were immunoblotted with the following primary antibodies (1:500 dilution) for 1 h at room temperature: Rat monoclonal anti-BrdU antibody [BU1/75 (ICR1)] (#ab6326, Abcam) and mouse monoclonal anti-BrdU antibody (clone B44) (#BDB347580, Fisher Scientific). Secondary antibodies of Alexa Fluor 555 goat anti-rat IgG (#A21434, Thermo Scientific) and Alexa Fluor 488 F (ab')2 goat anti-mouse IgG (#A-11017, Thermo Scientific) were used to at 1:500 dilution for 2 h at room temperature. Images of well spread DNA fibers were taken using Leica microscope with X 40 oil immersion objective. 100-150 well spread DNA replication fork was estimated using the conversion of 2.59 kb/µm as described ^[18].

RNA isolation and RT-qPCR

RNA was extracted with the TRIzol reagent (#15596018, Invitrogen) and then reverse-transcribed with the reverse transcription supermix (#1708841, Bio-Rad) according to the manufactures' protocols. qPCR was performed using the SYBR supermix (#1708882, Bio-Rad) in the Bio-Rad CFX cycler with CFX Maestro software. Primers for qPCR are listed in Table 1. Results of mRNA relative levels were calculated by 2^{-ΔΔCt} in normalization to the GAPDH and relative to the control samples. All PCR reactions were performed in technical triplicates.

ChIP-qPCR

Cells were cross-linked in 1% formaldehyde (#F1635, Sigma-Aldrich) for 10 min at room temperature and followed by attenuation of 125 mM glycine. Nuclei were isolated in NP-40 cell lysis buffer (50 mM Tris pH 8, 150 mM NaCl, 1% NP40, 15 mM EDTA) for 20 min on ice and spun down 5000 rpm for 5 min at 4°C. Nuclei were then lysed in nuclear lysis buffer (50 mM Tris pH 8, 150 mM NaCl, 10 mM EDTA, 1% SDS) for 10 min on ice. Lysed nuclei were then subjected to sonication to generate ~300bp chromatin fragments by confirmation on agarose gel. Immunoprecipitation was continued by incubating the sheared chromatin with E2F1 antibody (#3742, Cell Signaling) and IgG control (#2729, Cell Signaling) overnight at 4°C. Protein G beads (#10003D, Thermo Scientific) were added the following day and incubated on a rotator for 4 h at 4°C. Immunoprecipitated beads were then washed twice in Iow salt wash buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris pH 8, 150 mM NaCl), high salt wash buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM NaCl), LiCl wash buffer (0.25 M LiCl, 1% NP40, 1% deoxycholate, 1 mM EDTA, 10 mM Tris pH 8) and once in TE buffer (1 mM EDTA, 10 mM Tris pH 8) and finally eluted in elution buffer (1% SDS, 0.1 M NaHCO3). The eluted immunoprecipitations and previously saved input samples were reverse cross-linked in a 65°C water bath overnight. Reverse cross-linked DNA was isolated by PCR purification kit (Qiangen).

ChIP-qPCR was performed using the SYBR supermix (Bio-Rad) in the Bio-Rad CFX cycler with CFX Maestro software. Primers for ChIP-qPCR are listed in Table 1. 1% of starting chromatin was used as input and technical triplicates were performed. The ChIP-qPCR data was analyzed with the percent input method including normalization for both IgG levels and input chromatin going into the ChIP.

Three-Dimensional (3D) cultures

Cells were seeded at 2000 cells per well in a 96-well U-bottom plate (#353077, Corning).

The 3D cell culture media was a mixture of the media for 2D cell culture supplemented with 10% matrix (#A1413201, Thermo Scientific) containing 5 mM or 25 mM glucose. On day 6, sphere colonies were firstly observed under microscope and images were taken using Leica microscope with X 5 bright field objective. The length and width for each spheroid were measured afterwards in Image J (v1.53k). The viability for spheroids was determined by CellTiter-Glo® 3D cell viability assay

kit (#G9681, Promega). Briefly, 100 μl CellTiter-Glo[®] 3D reagent was added into the wells to be determined, followed by shaking for 5 min. After incubation at room temperature for 25 min, the plate was read on the luminometer plate reader (Promega). The luminescence signals were measured and collected for evaluating the 3D cell growth viability.

Intracellular dNTPs measurement

Intracellular dNTP levels were determined as previously described ^[19]. Briefly, cell pellets were resuspended in 60% methanol (Fisher Scientific) and then incubated at 95°C for 3 min. The supernatant was collected after centrifugation and transferred into the Amicon Ultra-0.5 ml centrifugal filter (#UFC500396, Millipore) for centrifugation again. After centrifugation, the flow through was saved and dried using Speed-Vac. The dried pellet was dissolved in 300 µl of sterile water and stored at -80°C. Determination of dNTP levels were performed by following the PCR based assay as previously described ^[19].

Statistical analysis

All the data are represented as mean ± SD. All the statistical tests were done using Graphpad Prism 9. P values were also generated in Graphpad Prism 9, with *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001, ns represents non-significance.

RESULTS

Elevated glucose prompts DNA replication and cell growth

To understand the underlying molecular mechanism for HG-induced cell proliferation, we performed RNA-seq analysis on HG treated colon cancer HCT116 cells. Differential expression analysis revealed 1150 upregulated and 975 downregulated mRNA transcripts, with a cutoff of -log10(p value) \geq 1.3 and log2(FC) at \pm 0.58 (Figure 1A). Furthermore, Gene Set Enrichment Analysis (GSEA) ^[15] revealed that cell cycle checkpoints and DNA synthesis were the top two REACTOME pathways that are strongly enriched following HG treatment (Figures 1B and C). Consistently, Gene Ontology (GO) analysis of all up-regulated genes in HG-treated cells also revealed significant enrichment in the DNA replication promoting genes (Supplementary Figure 1A).

Those identified genes are illustrated on volcano plot (Figure 1A) and listed in Table 2. Interestingly, GO analysis of all downregulated genes revealed significant enrichment in cellular response to glucose starvation (Supplementary Figure 1B). Those analyses suggest that, upon HG treatment, cancer cells are directed to DNA replication for cell proliferation through transcription activation.

Gene symbol	Gene name	log2FC	padi
done cymbol		IOBEI O	paaj
BLM	BLM RecQ like helicase	1.09	1.42E-09
CCNA2	cyclin A2	1.06	6.84E-14
CCNE1	cyclin E1	1.06	6.78E-08
CCNE2	cyclin E2	1.74	1.19E-08
CDK1	cyclin dependent kinase 1	0.98	6.53E-08
CDK2	cyclin dependent kinase 2	0.96	3.79E-18
CHAF1A	chromatin assembly factor 1 subunit A	1.43	7.16E-24
CHAF1B	chromatin assembly factor 1 subunit B	1.13	3.25E-09
CLSPN	claspin	1.43	2.93E-15
DSCC1	DNA replication and sister chromatid cohesion 1	1.03	1.58E-18
DTL	denticleless E3 ubiquitin protein ligase homolog	1.11	2.93E-07
E2F7	E2F transcription factor 7	0.91	3.68E-06

E2F8	E2F transcription factor 8	1.21	1.01E-09
ESCO2	establishment of sister chromatid cohesion N-acetyltransferase 2	1.18	6.94E-08
EX01	exonuclease 1	1.63	4.70E-12
FEN1	flap structure-specific endonuclease 1	1.23	2.20E-09
GINS1	GINS complex subunit 1	1.19	9.64E-12
GINS2	GINS complex subunit 2	1.21	9.74E-12
GMNN	geminin, DNA replication inhibitor	1.25	7.47E-11
MCM10	minichromosome maintenance 10 replication initiation factor	1.57	1.71E-11
MCM2	minichromosome maintenance complex component 2	0.92	0.000146
MCM4	minichromosome maintenance complex component 4	1.18	7.50E-10
MSH6	mutS homolog 6	1	1.12E-07
ORC1	origin recognition complex subunit 1	1.3	1.06E-12
ORC6	origin recognition complex subunit 6	1.11	8.59E-14
PCNA	proliferating cell nuclear antigen	1.09	2.68E-11
PRIM2	DNA primase subunit 2	0.95	1.19E-08
RAD51	RAD51 recombinase	1.26	2.23E-13
RFC4	replication factor C subunit 4	0.95	2.83E-15
RRM2	ribonucleotide reductase regulatory subunit M2	1.13	2.28E-22
TICRR	TOPBP1 interacting checkpoint and replication regulator	0.9	2.49E-10
TONSL	tonsoku like, DNA repair protein	0.99	8.74E-07
TREX1	three prime repair exonuclease 1	1.39	3.46E-07

To verify this result, we investigate the effect of HG on DNA replication by measuring the percentage of cells undergoing DNA synthesis using 5-ethynyl-2'-deoxyuridine (EdU) incorporation assay. The results show, compared to 5mM glucose control, an increased percentage of HCT116 cells in the S phase at both 6 h and 24 h following HG treatment (Figure 1D), indicating an enhanced G1/S progression. Moreover, cell proliferation assays revealed that cells treated with HG displayed a growth advantage compared to their counterparts in control conditions (Figure 1E). To provide direct evidence for enhanced DNA replication in HG-treated cells, we determined replication fork speed of a progressing fork in the cell using the DNA fiber assay. The results clearly show an accelerated replication fork speed in an undergoing replication fork upon HG treatment (Figure 1F). Measuring of 100-150 spread DNA fibers for each condition further confirmed the results (Figure 1F). To exclude the possibility that HG-induced phenotype is specifically observed in HCT116 cells, we tested the HG response in another cell line H460 derived from lung cancer. The results suggest that HG confers similar enhancing effects on DNA synthesis and cell proliferation in H460 cells as observed in HCT116 cells (Supplementary Figures 1C-E).



Figure 1. Elevated glucose prompts DNA replication and cell growth.

A



A) Volcano plot depicting differential gene expression in HCT116 cells following HG exposure (Green: up, Red: down). Dashed lines denote the cutoffs for log2(FC) (\pm 0.58) and -log10(p value) (1.3). Triangles (Δ) represent data points exceeding the cap. The positions of DNA synthesis genes are labeled in blue. Mock: 5 mM glucose, HG: 25 mM glucose. B) GSEA analysis of Reactome pathway of increased (left) and reduced (right) expression of genes after HG treatment. The data are displayed as a scatterplot, with the normalized p value (right y-axis), false discovery q value (left y-axis), and the Normalized Enrichment Score (NES) (x-axis) for each assessed gene set displayed. The gene sets highlighted in red indicate cell cycle checkpoints and DNA synthesis pathway. C) The top 2 significant enriched gene set of REACTOME pathways from GSEA analysis. D) Cell cycle profiles of EdU incorporation and DNA content in HCT116 treated with HG at the indicated time. The left panel is the representative analysis of flow cytometry. G₁, S+ and G₂ of the cell cycle were gated as indicated. The right panel indicates the percentage of EdU incorporated cells in the S phase. E) Cell growth curve of HCT116 cells under 5mM and 25 mM conditions. F.) DNA replication fork progression of control and HG-treated HCT116 cells was determined by DNA fiber assay at the indicated time. The right panel summarizes measuring of 100-150 spread DNA fibers in HCT116 cells for each condition. Results are displayed in mean \pm SD for n=3 replicates.

Together, these results support the role of HG in re-directing cells to DNA synthesis and cell proliferation.

E2F1 plays a crucial role in high glucose-induced DNA replication and cell growth

Next, we performed GSEA analysis to identify key transcription factors responsible for this HG-induced cell adaptation. Our analyses revealed E2F1 as the top transcription factor (Figure 2A and Table 3). To confirm the role of E2F1 in HG-induced DNA synthesis and cell proliferation, we employed lentivirus-mediated RNAi approach to knockdown E2F1 (Fig. 2B). Our results show that the HG-induced cells entering into S phase were significantly reduced upon E2F1 inhibition (Figure 2B). Similarly, HG-induced increases in DNA replication fork speed and cell growth were also reduced in E2F1 knockdown cells (Figures 2C and D).

Name	NES	NOM p-val	FDR q-val		
E2F_Q6	-2.67537	0	0		
E2F_Q4	-2.67462	0	0		
SGCGSSAAA_E2F1DP2_01	-2.63266	0	0		
E2F1DP1RB_01	-2.61037	0	0		
E2F4DP1_01	-2.57304	0	0		
E2F1_Q6	-2.54446	0	0		
E2F_02	-2.53889	0	0		
E2F1DP1_01	-2.51966	0	0		
E2F1_Q6_01	-2.51628	0	0		
E2F1DP2_01	-2.51625	0	0		
E2F1_Q3	-2.51108	0	0		
E2F4DP2_01	-2.50157	0	0		
E2F_Q3_01	-2.48993	0	0		
E2F_03	-2.48384	0	0		
E2F_Q4_01	-2.43934	0	0		
E2F_Q3	-2.39982	0	0		
E2F_Q6_01	-2.37582	0	0		
E2F1_Q4_01	-2.36182	0	0		
PPARGC1A_TARGET_GENES	-2.06926	0	2.42E-04		
HSF2_TARGET_GENES	-2.05118	0	2.86E-04		
Note: NES: Normalized Enrichment Score; NOM p-val: Nominal p-value; FDR q-val: False Discovery Rate q-value.					

 Table 3. List of top 20 transcription factors identified in Gene Set Enrichment Analysis (GSEA).

We note that, compared to controls, E2F1 knocked down alone led to more EdU incorporated cells in the S phase (Figure 2B) as well as increased DNA replication fork speed (Figure 2C). Since the activator E2F family members (E2F1-3) have been shown to have extensive functional redundancy and overlap, we speculate that other E2F family members may offset the E2F1 knocked down effect, resulting in increased cells in the S phase and DNA synthesis. To test this possibility, we employed a pan-E2F inhibitor HLM006474 that blocks chromatin accessibility of E2F family members in cells ^[20]. Strikingly, HLM006474 completely abolished HCT116 cells entering into S phase in the presence and absence of HG (Figure 2E). Similarly, a remarkable DNA replication fork arrest was also detected in HLM006474-treated cells (Figure 2F). To exclude the potential cell type specific effect, we knock-downed E2F1 in HCT116 cells (Supplementary Figure 2A) and inhibited E2F1 with the inhibitor in H460 (Supplementary Figure 2B) and obtained similar HG effect on DNA replication and cell proliferation. Together, these results indicate that E2F1 plays a crucial role in promoting DNA replication and cell proliferation following HG treatment.

Figure 2. E2F1 plays a crucial role in high glucose-induced DNA replication and cell growth.





A) The most significant enriched transcription factor revealed by GSEA. B) Cell cycle profiles of EdU incorporation and DNA content in HG-treated H460 cells in the presence of scramble control or shE2F1. The right panel indicates the relative percentage of EdU incorporated cells in the S phase following HG exposure, with or without E2F1 knock down. The E2F1 levels were verified by Western analysis. C) DNA replication progression of HG-treated H460 cells with or without E2F1 knock down. D) Cell growth assay of control or E2F1 knockdown H460 cells following HG. E) HCT116 cells were treated with DMSO or the E2F inhibitor HLM006474. EdU incorporation and DNA staining were analyzed by flow cytometry following HG exposure. The right panel indicates the percentage of EdU-positive cells in the S phase. F) DNA replication fork progression of HCT116 cells treated with HLM006474 was determined by DNA fiber assay following HG exposure. The bottom panel summarizes measuring of 100-150 spread DNA fibers in H460 cells for each condition. Results are displayed in mean ± SD for n=3 replicates.

Elevated glucose induces E2F1-dependent transactivation through pRB phosphorylation

To further establish the role of E2F1 as key transcription activator in HG-induced DNA synthesis and cell growth, we assessed its effect on up-regulation of 7 identified DNA replication genes identified from GSEA analysis (Table 2 and Figure 1A) by RT-PCR. As shown in Figure 3A, compared to controls, HG significantly increased the mRNA levels of RRM2, CHAF1A, CHAF1B, PCNA, CCNE2, CLSPN and RBM14. E2F1 knockdown, however, significantly reduced the HG-induced up-regulation. Importantly, E2F1 knockdown also reduced HG-induced RRM2 and CHAF1A protein levels (Figure 3B), suggesting functional relevance of the regulation. Furthermore, we showed that treating cells with the E2F1 inhibitor HLM006474 also blocks HG-induced mRNA and protein levels of DNA replication-associated genes (Figures 3C and D).

Consistently, overexpression of E2F1 up-regulated the mRNA and protein levels of the DNA replication genes and reduced HG-induced up-regulation in cells (Supplementary Figures 3A and B). These results suggest that E2F functions as a transcription factor up-regulating DNA replication genes following HG treatment.

Several groups have reported that E2F1 binds to the RRM2 promoter and activates its transcription [21-23]. We thus carried out ChIP analysis to test whether elevated glucose levels affect the ability of E2F1 to bind to DNA. The assay confirmed the binding of E2F1 to the RRM2 promoter (Figure 3E) as well as to the PCNA promoter (Figure 3F). Importantly, exposure of cells to HG significantly enhanced the binding of E2F1 to both promoters. Of note, while E2F has been suggested to target genes involved in glucose metabolism ^[24,25], we did not observe any effect of HG on expression of those genes in our RNA-seq analysis. Together, these results suggest that elevated glucose levels up-regulate DNA replication genes through E2F1-dependent transactivation.



Figure 3. E2F1 activates transcription of DNA replication genes in response to elevated glucose.

A) RT-qPCR of top DNA replication genes in control (scramble) or E2F1 knock down H460 cells following HG exposure. B) Western analysis of CHAF1A, RRM2 and E2F1 protein levels from the same treatment as indicated in 3A. C) RT-qPCR of top DNA replication genes in control or HLM006474 treated HCT116. D) Western blot analysis for CHAF1A, RRM2 and E2F1 from the same treatment as indicated in 3C. E and F) Top: Schematic representation of the RRM2 (3E) or PCNA (3F) promoters. ChIP-qPCR analysis of E2F1 binding to the RRM2 and PCNA promoters in the HCT116 cells following HG exposure. Results are displayed in mean ± SD for n=3 replicates.

Interestingly, a previous study has suggested that treating cells with HG leads to pRB hyper-phosphorylation in murine pancreatic cells ^[26]. pRB is the target of phosphorylation by CDK2/4/6 in the G1 phase of the cell cycle. Generally, once hyper-phosphorylated, pRB alleviates repression of E2F, leading to activation of E2F1-dependent transcription ^[27-29]. To test the role of pRB phosphorylation in HG-mediated E2F transactivation, we treated cells with HG and assay pRB phosphorylation using phosphorS807/S811-specific antibody. As shown in Figure 4A, increased pRB phosphorylation was indeed observed following HG treatment in HCT116 cells, suggesting pRB hyperphosphorylation potentially contributed to E2F1 activation. Interestingly, perhaps due to higher levels of pRB phosphorylation in the cell, treating U2OS cells with HG didn't further enhance pRB phosphorylation (Figure 4A). Significantly, compared to HG-treated HCT116 cells, treating U2OS cells with HG also failed to up-regulate DNA replication genes (Figures 4B and Supplementary Figure 4A) and to enhance DNA synthesis (Figure 4C) and cell proliferation (Figure S4C). To confirm these results, we treated HCT116 cells with the CDK2/4/6 inhibitor PF-3600 and showed inhibition of pRB phosphorylation (Figure 4E) also blocks HG-induced up-regulation genes (Figures 4D and Supplementary Figure 4B).

These results suggest that elevated glucose up-regulates DNA replication associated genes through regulating the pRB-E2F pathway.



Figure 4. HG-induced Rb1 phosphorylation is required for activation of DNA replication genes.

A) Western blot analysis of total pRB, Ser807/Ser811-phosphorylated pRB and RRM2 in HCT116 and U2OS cells following HG exposure. B) RT-qPCR of top DNA replication genes in HCT116 and U2OS cells following HG treatment. C) Cell cycle profiles of EdU incorporation and DNA content in U2OS cells following HG treatment at the indicated time. The right panel indicates the percentage of EdU-labeled cells in the S phase. D) HCT116 cells were treated with the CDK inhibitor PF-3600 and RT-qPCR of top DNA replication genes was performed following HG exposure. E) Western blot analysis of total pRB and Ser807/Ser811-phosphorylated pRB from the same treatment as indicated in 4D. Results are displayed in mean ± SD for n=3 replicates

Regulation of intracellular dNTP levels by HG is dependent on the E2F1-RRM2 axis

The role of RRM2 as a proto-oncogene has been recently recognized. RRM2 is an essential component in the holoenzyme Ribonucleotide Reductase (RNR) that is important for reducing the 2' carbon of NDP to produce dNDP, a rate-limiting step in the DNA de novo pathway. Our finding that HG up-regulates both RRM2 mRNA and protein levels prompts us to test its role in regulating intracellular dNTP levels. Using a previously described PCR-based method ^[19] the average intracellular dATP, dGTP, dCTP and dTTP levels were measured and calculated at 2.86, 1.04, 3.07, and 10.82 pmol/106 cells, respectively. Importantly, treating cells with HG led to a rapid and robust increase of all four dNTP levels in the cells (Figure 5A). To establish the role of RNR in the HG-induced upregulation of dNTP, we treated cells with the RNR inhibitor Triapine at two concentrations, 250 or 500 nM, and observed reduced intracellular dATP and dGTP levels under both conditions. The Triapine treatment also reduced dCTP and dTTP, but to a lesser extent, which is consistent with a previous report ^[30]. Importantly, inhibition of RNR clearly prevented HG-induced DNA replication fork progression (Figure 5B).

Finally, we verified the role of E2F1 in HG-induced dNTP up-regulation. As shown in Figure 5C, treating cells with the E2F inhibitor 6476 clearly blocks dATP and dGTP up-regulation following HG treatment. Together, these results suggest that elevated glucose enhances dNTP levels through activation of the E2F1-RRM2 axis.



Figure 5. Regulation of intracellular dNTP levels by HG is dependent on the E2F1-RRM2 axis.

A) Intracellular dNTP levels in HCT116 cells treated with or without Triapine and HG as indicated. B) DNA replication fork progression of Triapine-treated HCT116 cells following HG exposure. The right panel summarizes measuring of 100-150 spread DNA fibers for each condition. C) HCT116 cells were treated with the E2F inhibitor HLM006474. Intracellular dATP and dGTP levels were determined following HG. Results are displayed in mean ± SD of three separate experiments.

Inhibition of E2F1-RRM2 axis alleviates high glucose-induced cancer 3D spheroid growth

We next investigated the contribution of the E2F1-RRM2 axis to cancer cell growth following HG treatment. To better mimic the in vivo environment, we established a short-term Three Dimensional (3D) tumor spheroid culture ^[31]. As shown in Figure 6A, H460 cells formed spheroid-like round or spherical structures in the 3D cultures. Upon HG treatment, increased size of the spheroids was observed, indicating a stronger cell growth compared with non-HG treated spheroids (Figure 6A). To confirm the cell growth potential, overall cell viability of total spheroids in the 3D cultures was further determined following HG treatment (Figure 6B). These results provide additional support for the finding that elevated glucose re-directs cells to cell growth.

To investigate the role of E2F1 in HG-induced spheroid growth, we employed lentivirus-mediated RNAi approach to knockdown E2F1. The results show that compared with scramble controls, HG-induced spheroid growth was reduced upon E2F1 inhibition (Figure 6A). Importantly, HG-induced cell viability of total spheroids in the 3D cultures was also reduced in E2F1 knockdown cells (Figure 6B).

Next, we investigated the role of RRM2 in HG-induced spheroid growth by treating cells with the RNR inhibitor Triapine. As shown in Figure 6C, HG-induced spheroid growth was indeed blocked upon the RNR inhibition. As expected, HG-induced cell

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viability of total spheroids in the 3D cultures was also blocked by the inhibition (Figure 6D). Taken together, these results demonstrate elevated glucose promotes cancer cell growth through E2F1-RRM2 activation.



Figure 6. Inhibition of the E2F1-RRM2 axis alleviates high glucose-induced cancer cell growth.

A) Representative bright field images of H460 derived spheroids in the presence of scramble control or shE2F1 for 6 days following HG exposure. Scale bar: 100 μ m. B) ATP luminescence signals represented for the total spheroid's viability on the plate. C) Representative bright field images of HCT116-derived spheroids in the presence or absence of Triapine for 6 days following HG exposure. D) ATP luminescence signals represented for the total spheroid's viability on the plate. Results are displayed in mean \pm SD of three separate experiments.

DISCUSSION

Diabetes mellitus and cancer have a tremendous effect on human health worldwide. Although a number of epidemiological studies have highlighted the link between two diseases ^[5,8,32], the molecular mechanism by which hyperglycemia promotes cancer cell growth remains well defined. In addition, while High Glucose (HG) is known to promote cell growth, the overall transcription regulation involved in this process is less clear. In this study, we identified E2F1 as the core transcriptional regulator involved in re-directing cells to DNA replication and cell proliferation under elevated glucose conditions. Among HG-induced E2F1 target genes, we show that activation of RRM2 leads to up-regulation of intracellular dNTP levels, which plays a role in DNA synthesis and cancer cell growth. Together, our findings provide a molecular mechanism by which hyperglycemia promotes cancer cell proliferation.

E2F transcription factors are downstream effectors of the tumor suppressor pRB and have a pivotal role in controlling cellcycle progression ^[27-29]. E2Fs also participate in cellular processes beyond the cell cycle, including apoptosis, differentiation and development. However, the role of E2Fs in directing cancer cells to proliferation following HG has not been well investigated. By examining the transcriptome data of the HG-treated cells, DNA replication emerges as a significant signature. Furthermore, GSEA analysis revealed E2F1 as the core transcription factor, suggesting hyperglycemia potentially directs cancer cells into DNA replication through E2F1-mediated transactivation. Interestingly, consistent with this notion, regulation of DNA replication genes has been reported in fin tissues in the diabetic zebrafish model ^[33]. Furthermore, inhibition of GLUT1, a key rate-limiting factor for glucose uptake, blocked growth of pRB-positive Triple Negative Breast Cancer (TNBC) ^[34]. Notably, pathway enrichment analysis of gene expression data in TNBC also suggests that the functionality of the E2F pathway contributed to the process. Together, those results imply a HG-regulated pRB-E2F1 axis in cancer cells. Clearly, it will be intriguing to further assess its contribution in pRB-positive cancer patients.

Emerging evidence has indicated that RRM2, the small subunit of RNR, is an important proto-oncogene and cancer therapeutic target ^[35]. The importance of RNR for DNA replication relates to its central role in regulating dNTP levels. RRM2 expression is elevated in several cancer types and the level of the expression is highly correlated with tumor grades.

CONCLUSION

Furthermore, overexpression of RRM2 is often associated with chemo-resistant cancers. Interestingly, RRM2 has been shown a direct transcriptional target of several transcription factors including E2F1. Herein, our result that RRM2 can be transcriptionally activated by E2F1 following HG provides a new link between diabetes and cancer and potentially suggests a new avenue for targeted cancer therapy for diabetes patients. In fact, the RNR inhibitor Triapine employed in our study is currently undergoing clinical trials. It has been shown to increase sensitivity of chemotherapy and radiation therapy in cervical cancer cells. Our data that Triapine treatment inhibits HG-induced DNA replication fork further validated its application in targeted cancer therapy for diabetes patients. Together, our data suggest a model of how cancer cells exert adaptation to hyperglycemia and illustrate the molecular mechanism of E2F to be the core transcription factor in activating the transcription of the downstream DNA replication genes, therefore promoting the DNA replication and cellular proliferation.

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