Transcriptional Activation of Human CDCA8 Gene Regulated by Transcription Factor NF-Y in Embryonic Stem Cells and Cancer Cells*

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The cell division cycle associated 8 (CDCA8) gene plays an important role in mitosis. Overexpression of CDCA8 was reported in some human cancers and is required for cancer growth and progression. We found CDCA8 expression was also high in human ES cells (hESCs) but dropped significantly upon hESC differentiation. However, the regulation of CDCA8 expression has not yet been studied. Here, we characterized the CDCA8 promoter and identified its cis-elements and transcription factors. Three transcription start sites were identified. Reporter gene assays revealed that the CDCA8 promoter was activated in hESCs and cancer cell lines. The promoter drove the reporter expression specifically to pluripotent cells during early mouse embryo development and to tumor tissues in tumor-bearing mice. These results indicate that CDCA8 is transcriptionally activated in hESCs and cancer cells. Mechanistically, two key activation elements, bound by transcription factor NF-Y and CREB1, respectively, were identified in the CDCA8 basic promoter by mutation analyses and electrophoretic motility shift assays. NF-Y binding is positively correlated with promoter activities in different cell types. Interestingly, the NF-YA subunit, binding to the promoter, is primarily a short isoform in hESCs and a long isoform in cancer cells, indicating a different activation mechanism of the CDCA8 transcription between hESCs and cancer cells. Finally, enhanced CDCA8 promoter activities by NF-Y overexpression and reduced CDCA8 transcription by NF-Y knockdown further verified that NF-Y is a positive regulator of CDCA8 transcription. Our study unearths the molecular mechanisms underlying the activation of CDCA8 expression in hESCs and cancer cells, which provides a better understanding of its biological functions.

The human cell division cycle associated 8 (CDCA8) gene is a component of the vertebrate chromosomal passenger complex (CPC). The CPC consists of at least four proteins as follows: Aurora B, INCENP, survivin, and CDCA8 (1), each of which has essential regulatory roles and dynamic cellular localization during mitosis (2). CDCA8 targets the CPC components to the centromeres (3), corrects kinetochore attachment errors, and stabilizes bipolar spindles in human cells (1). Overexpression of the CPC components Aurora B (4, 5), INCENP (6), and survivin (7–10) is associated with tumorigenesis in human cancers. CDCA8 is a putative oncogene that is up-regulated in many types of cancer tissues (11–14) but has very low or absent expression in normal tissues (12). Its overexpression is required for growth, survival, and the malignant nature of lung cancer cells (12). Overexpression and nuclear accumulation of CDCA8 are linked to the poor prognosis of lung cancer (12) and gastric cancer (11). Thus, CDCA8 was considered to be a promising target for the development of novel therapeutics and diagnostics (12).

We previously showed that CDCA8 is highly expressed in undifferentiated human ES cells (hESCs) and early mouse

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The abbreviations used are: CPC, chromosomal passenger complex; hESC, human ES cell; dhESC, differentiated human ES cell; TSS, transcription start site; TF, transcription factor; NF-Y, nuclear factor Y; iPSC, induced pluripotent stem cell; EB, embryoid body; RA, retinoic acid; hEF, human embryonic fibroblast; qPCR, quantitative PCR; RLM-RACE, RNA ligase-mediated rapid amplification of cDNA end; ChIP, chromatin immunoprecipitation; ICM, inner cell mass; EGFP, enhanced GFP; DOTAP-Chol, N-[1-(2,3-Dioleoyloxy)]-N,N,N-trimethylammonium propane methylsulfate; cholesterol.
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embryos but is expressed at low levels in differentiated hESCs (dHECs) (15, 16). Microinjection of anti-Borealin (encoded by CDCA8) antibody into mouse zygotes arrested development at the 2–4-cell stage (16). These results indicate that CDCA8 may play a crucial role in hESCs and early embryonic development. However, the mechanism governing CDCA8 up-regulation has not been studied yet. The aim of this study was to investigate the transcriptional regulation of CDCA8. We identified the transcription start sites (TSSs) of CDCA8, characterized its promoter, and determined regulatory cis-elements and transcription factors (TFs). We showed that CDCA8 promoter was activated in hESCs and cancer cells and that nuclear factor Y (NF-Y) was a functional activator by binding to a CCAAT-box in the CDCA8 promoter. We further showed that the isoforms of the NF-YA subunit responsible for CDCA8 activation differed between hESCs and cancer cells. Our results demonstrate that the up-regulation of CDCA8 in hESCs and cancer cells is mediated primarily at the transcriptional level and is positively regulated by NF-Y.

Materials and Methods

Cell Lines and Animals—Ethics approval and oversight was obtained from the Reproductive and Stem Cell Engineering Ethics Committee of Central South University and the Reproductive and Genetic Hospital of China International Trust and Investment Corp.-Xiangya. The hESC and induced pluripotent stem cell (iPSC) line used in this study were established and cultured in 6-well plate in triplicate. After 10 days in culture, the cancer cell lines (MCF-7, A549, K562, and H11032) were counted. The cancer cell lines (MCF-7, A549, K562, and H11032) were passaged on Matrigel (BD Biosciences). Briefly, 1000 cells/well were cultured on a feeder layer of mitotically inactivated mouse embryonic fibroblasts. The growth medium consisted of DMEM/F-12 supplemented with 15% knock-out serum replacement, 2 mM nonessential amino acids, 2 mM l-glutamine, 0.1 mM β-mercaptoethanol, and 4 ng/ml basic FGF (Invitrogen). Embryoid bodies (EB) were formed by suspension culturing, and chemical differentiation induction was performed with 0.1 μM retinoic acid (RA) (Sigma), both in the absence of bFGF. For the colony formation assay, hESCs were passaged on Matrigel (BD Biosciences). Briefly, 1000 cells/well were cultured in 6-well plate in triplicate. After 10 days in culture, the cancer cells (MCF-7, A549, K562, and HeLa) and normal cell lines (human umbilical vein endothelial cell (HUVECs); human skin fibroblasts (HSFs); amniotic epithelial cells (AECs), and human embryonic fibroblasts (hEFS)) were maintained in DMEM containing 10% fetal bovine serum (Invitrogen), C57BL/6, DBA/2, and nude mice were purchased from The Shanghai Laboratory Animal Center (Shanghai, China). All animal studies were approved by the Animal Care and Use Committee of Central South University and were conducted in accordance with national and international guidelines.

RT-PCR and Quantitative PCR (qPCR)—Total RNA from each cell line was isolated using TRI reagent (Sigma) and reverse-transcribed using the RevertAid First Strand cDNA synthesis kit (Fermentas Life Sciences, Burlington, Canada). Real-time qPCR was performed as described previously (19). The PCR primers used are as follows: human CDCA8 primers, sense 5'-TTGACTAATTCAGCTGCTGG-3' and antisense 5'-CTCTTTTCTCTTCTCCTACA-3'; NF-YA primers, sense 5'-GAGTCTCGGACGGCTCATG-3' and antisense 5'-CTTCACAGAGAAACG-3'; and antisense 5'-TGGTTGT-GACCTCGTGGT-3'; NF-YB primers, sense 5'-AGGTTGC-GCACTCTGAATGACT-3' and antisense 5'-CCTCTTCCAC-GCTGATTGT-3'; POU5F1 primers, sense 5'-AGCCGAACC-GTATCGAGAAC-3' and antisense 5'-TTACAGAACCAC-ACCTGGAC-3'; NANOG primers, sense 5'-AGCGAACGAC-GTACGAGAAC-3' and antisense 5'-TTACAGAACCACAC-CTGGAC-3'; KLF4 primers, sense 5'-AGGCAGACCTTATG-GAGAAC-3' and antisense 5'-TTACAGAACCACACTCG-GAC-3'; KRT17 primers, sense 5'-GGAGATGTGCCACTTACCG-3' and antisense 5'-CGCCAGTAGTTITTCATATTGTCG-3'; SOX17 primers, sense 5'-CAGTCGACGACCAGCGACC-ACC-3' and antisense 5'-CCAGCAGTCTCCGACCAGC-3'; CDX2 primers, sense 5'-CAGCGGACAGAAAGGAGA-3' and antisense 5'-CAGGGACAGAGGCAGACA-3'; and GAPDH primers, sense 5'-AACAGCTCTCATGATC-ACC-3' and antisense 5'-GGATGTGGTCTGAGAGGCC-3'.

Western Blotting—Total protein was prepared using RIPA buffer (Pierce), separated on SDS-polyacrylamide gels, and then transferred to PVDF membrane (Millipore Corporation, Billerica, MA). Target proteins were detected with primary antibodies against Borealin (sc-47955, Santa Cruz Biotechnology, Dallas, TX), OCT 3/4 (sc-5279, Santa Cruz Biotechnology), CDX2 (sc-17753x; Santa Cruz Biotechnology), OCT 3/4 (sc-5279, Santa Cruz Biotechnology), and GAPDH (sc-365062; Santa Cruz Biotechnology) and were detected by secondary antibodies (Santa Cruz Biotechnology) and ECL Western blotting kit (GE Healthcare).

5'-RNA Ligase-mediated Rapid Amplification of cDNA Ends (5'-RLM-RACE)—The TSSs of the CDCA8 gene were identified using the FirstChoice RLM-RACE kit (Ambion, Grand Island, NY) according to the manufacturer’s instructions. Total RNA was isolated from hESCs. We designed two nested antisense primers specific to exon 5 and exon 3 of the CDCA8 gene, 5'-GCCGAGTAGTTITTCATATTGTCG-3' and antisense 5'-CAGGGACAGAGGCAGACA-3'; and GAPDH primers, sense 5'-AACAGCTCTCATGATC-ACC-3' and antisense 5'-GGATGTGGTCTGAGAGGCC-3'.

Plasmid Constructions—The DNA sequence of the 5'-untranscribed region of the human CDCA8 was used to design nested primers shown in Table 1. The longest fragment was amplified first using outer primers (OS and OA). The 5'-truncated promoter fragments of 2041, 1593, 1071, 952, 619, 423, 269, and 143 bp in length were amplified by inner primers antisense IA and one of the sense primers IS1, IS2, IS3, IS4, IS5, IS6, IS7, and IS8, respectively. Primers IA9 and IS9 were used to amplify the 165-bp fragment. All of the inner sense and antisense primers contained the restriction endonuclease sites KpnI and Nhel at their 5'-ends, respectively. The template was genomic DNA isolated from hESCs. Plasmid products were digested by enzymes KpnI and Nhel and cloned into KpnI/Nhel sites of the pGL3-Basic reporter vector (Promega, Madison,
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TABLE 1

Primer used in amplification of CDCA8 promoter fragments

| Primer | Sequence (5'-3') | Position* | Promoter bp |
|--------|-----------------|-----------|-------------|
| O5     | GGCCTACTGATGCCTCCGAAAAT | ~ 2117 to ~ 2098 bp | ~ 2117 to 2098 |
| O6     | CCTGAAGATTTGCTCTTCTG  | 51 to 32 bp   | 51 to 32 |
| IS1    | ggGTTACCTGATGGGCGAGCTTTC | ~ 2093 to ~ 2074 bp | ~ 2093 to 2074 |
| IS2    | ggGTTACCTGATGGGCGAGCTTTC | ~ 1645 to ~ 1625 bp | ~ 1645 to 1625 |
| IS3    | gGATCCCTGATGGGCGAGCTTTC | ~ 1123 to ~ 1104 bp | ~ 1123 to 1104 |
| IS4    | ggGTTACCTGATGGGCGAGCTTTC | ~ 1004 to ~ 985 bp | ~ 1004 to 985 |
| IS5    | ggGTTACCTGATGGGCGAGCTTTC | ~ 671 to ~ 652 bp | ~ 671 to 652 |
| IS6    | ggGTTACCTGATGGGCGAGCTTTC | ~ 475 to ~ 456 bp | ~ 475 to 456 |
| IS7    | ggGTTACCTGATGGGCGAGCTTTC | ~ 321 to ~ 302 bp | ~ 321 to 302 |
| IS8    | ggGTTACCTGATGGGCGAGCTTTC | ~ 195 to ~ 176 bp | ~ 195 to 176 |
| IS9    | ggGTTACCTGATGGGCGAGCTTTC | ~ 53 to ~ 72 bp | ~ 53 to 72 |
| IS10   | gGATACCTGATGGGCGAGCTTTC | ~ 72 to ~ 53 bp | ~ 72 to 53 |

*The numbers refer to positions relative to the translation start site.

FIGURE 1. Expression of CDCA8 drops dramatically in differentiated hESCs. A, Western blot analysis of CDCA8 and Oct4 protein levels, and B RT-PCR analysis of differentiation-associated markers, including KRT17 (ectoderm), RUNX1 (mesoderm), SOX17 (endoderm), CDX2 (trophectoderm) in EBs and RA-treated hESCs for the indicated days.

er’s instructions (Invitrogen). Briefly, cultured cells at 90% confluence in 24-well plates were co-transfected with 0.04 μg of internal control pRL-CMV vector (Promega, Madison, WI) and 0.2 μg of CDCA8 reporter constructs, pGL3-control or pGL3-Basic vector (Promega). For the NF-Y overexpression assay, cells at 90% confluence in 24-well plates were co-transfected with 0.2 μg of CDCA8 reporter construct, 0.04 μg of pRL-CMV, and 0.6 μg of pAAV-NF-Y or empty-vector pAAV-MCS.

Luciferase Assays—Cell lysates were collected 48 h after transfection. The luciferase and Renilla activity of cell lysates was measured using the Dual-Luciferase reporter assay system (Promega) with a SIRUS luminometer (Berthold Technologies, Bad Wildbad, Germany). Renilla luciferase gene expression was used to normalize for transfection efficiency. Tissues collected from tumor-bearing mice (48 h after DNA-liposome complex injection) were immediately frozen on dry ice. The tissues were then homogenized using passive lysis buffer (Promega). Once thawed, the tissue suspension was centrifuged at 12,000 rpm at 4 °C for 3 min, and the supernatant was collected. The luciferase activity (relative light units) per mg of protein was used to compare gene expression in different tissues. The protein concentration was determined using a BCA protein assay kit (Pierce).

Transgenic Mouse Embryos Containing a CDCA8-EGFP Construct—The plasmid pEGFP-1071 was generated by replacement of the CMV promoter in pEGFP-C3 with the 1071-bp promoter fragment. The DNA fragment containing the CDCA8 promoter and the EGFP ORF was isolated by restriction enzyme digestion with AseI and MluI from the pEGFP-1071 vector and then was purified and microinjected into C57BL/6 (F) embryo eggs. The expression of EGFP driven by the CDCA8 promoter in embryos was observed during development in vitro. Fluorescent images were captured by microscopy (Olympus IX81 and CLSM) Olympus FV1000, Tokyo, Japan).

Establishment of Tumors and DNA Delivery—N-[1-(2,3-Di-}

Transient Transfections—Transient transfections were performed using Lipofectamine 2000 according to the manufacturer's instructions (Invitrogen). Briefly, cultured cells at 90% confluence in 24-well plates were co-transfected with 0.04 μg of internal control pRL-CMV vector (Promega, Madison, WI) and 0.2 μg of CDCA8 reporter constructs, pGL3-control or pGL3-Basic vector (Promega). For the NF-Y overexpression assay, cells at 90% confluence in 24-well plates were co-transfected with 0.2 μg of CDCA8 reporter construct, 0.04 μg of pRL-CMV, and 0.6 μg of pAAV-NF-Y or empty-vector pAAV-MCS.

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Establishment of Tumors and DNA Delivery—N-[1-(2,3-Di-}
null
three TSSs located 200, 194, and 175 bp upstream of the CDCA8 translation start site (Fig. 2, A and B). Additionally, the transcript initiated at −194 bp retained intron 1, indicating that CDCA8 can be alternatively spliced (Fig. 2B).

CDCA8 Promoter Is Active in Embryonic Stem Cells and Cancer Cells—To characterize the promoter activity and determine the promoter region critical for human CDCA8 transcription, 5′-truncated promoter fragments were amplified and cloned into a pGL3-Basic vector driving firefly luciferase gene expression (Fig. 3A). hESCs, four cancer cell lines, and four normal cell lines were transiently transfected with the CDCA8 reporter vectors and monitored for expression. As expected, the CDCA8 promoter was highly active in hESCs (Fig. 3B) and multiple cancer cell lines (Fig. 3C) with some variability between promoter fragment lengths. Promoter activity dropped dramatically in RA-induced dhESCs (Fig. 3B) and was almost undetectable in noncancer cell lines (Fig. 3D). Interestingly, the promoter activity significantly increased in hEFs induced to become iPSCs (Fig. 3E). RT-PCR analysis confirmed that the endogenous level of CDCA8 mRNA was consistent with the promoter activity experiments (Fig. 3F). Therefore, the CDCA8 promoter was activated in pluripotent stem cells and cancer cells in vitro.

In addition, the 1071-bp fragment (−1123 to −53) was more active than the 1593-bp fragment (−1645 to −53) in cancer cells (Fig. 3C), whereas the 952-bp fragment (−1004 to −53)
CDCA8 Promoter Activity Is Specific to Pluripotent Cells in Early Mouse Embryos—To observe promoter activity in developing embryos cultured in vitro, we created an EGFP reporter driven by the CDCA8 promoter (Fig. 4A) and microinjected it into mouse zygotes. CDCA8 promoter activity as determined by EGFP expression was observed from the two-cell to morula stages but was strongest in four-cell embryos (Fig. 4A). In blastocysts, EGFP expression was restricted to the pluripotent inner cell mass (ICM). These results indicate that during early mouse embryo development CDCA8 promoter activity was pluripotent cell-specific.

CDCA8 Promoter Is Active in Cancer Cells in Vivo—To examine the cancer activation of the CDCA8 promoter in vivo, we injected the CDCA8-Luc reporter plasmid (pGL3-1071) or the pGL3-basic plasmid (nonpromoter) and DOTAP-Chol cationic liposomes into breast tumor-bearing mice (Fig. 5A). Luciferase activity was assessed in tissue lysates 48 h after injection. As expected, the CDCA8 promoter was most active in tumor tissues (Fig. 5A). These results indicate that the CDCA8 promoter was activated in cancer cells in vivo.

Cis-elements in the CDCA8 Promoter Regulate Activity—To better understand the cis-elements regulating promoter activity, we analyzed the 269-bp promoter fragment containing the basic promoter region (−321 to −195). A number of potential TF-binding sites, some of which overlap one another, were identified using MatInspector and TESS (Fig. 6A). To determine the contribution of these predicted sites on transcriptional activity, individual mutations (Mut A to Mut G) were introduced in the basic promoter (Fig. 6A). Mut C increased promoter activity in HeLa cells, whereas Mut D to G reduced promoter activity (Fig. 6B). These suggested that site C acts as a negative regulatory element, whereas sites D to G act as positive elements.

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FIGURE 4. CDCA8 promoter activity is specific to pluripotent cells in early mouse embryos. A, representative images of early mouse embryos cultured in vitro with EGFP expression driven by the CDCA8 promoter. Embryos from two-cell to morula stages were photographed by transmission fluorescence microscope (upper panels, bright field; lower panels, EGFP), and blastocysts were photographed by confocal fluorescence microscope (bright field and EGFP were merged). Scale bar, 30 μm. B, 1071-EGFP construct used for microinjection was digested with restriction enzymes AseI and MluI. The numbers indicate promoter location relative to the translation start site.

FIGURE 5. CDCA8 promoter is active in cancer cells in vivo. A, representative image of breast tumor-bearing mouse 7 days after MCF-7 cell inoculation. B, luciferase assays performed on tissue lysates from blank (nonpromoter, injected with pGL3-basic, n = 5) and test mice (CDCA8 promoter, injected with pGL3-1071, n = 5). Each value is presented as relative light units (RLU) per mg of protein and shown as the mean ± S.D. *p < 0.05, as compared with other tissues from test mice.
regulatory elements. Notably, Mut E and Mut G dramatically decreased promoter activities, suggesting that sites E and G are key elements for CDCA8 transcription.

NF-Y and CREB1 Bind with Specificity to the CDCA8 Promoter—Sites E (CCAAT, antisense strand) and G (TGACG, antisense strand) are predicted as binding sites of NF-Y and CREB1 (Fig. 6A), respectively. To confirm this prediction, we performed EMSAs using probes, containing site E (biotin-labeled, Fig. 7A) or site G (radio-labeled, Fig. 7B) and flanking sequences. The mobility of each probe was shifted when incubated with HeLa cell nuclear extracts (Fig. 7, lane 2). However, there was no mobility shift when probes containing mutant site E (Fig. 7A, lane 3, CCAAT to AAAAT) or mutant site G (Fig. 7B, lane 5, TGACG to GACCA) were used. The addition of excess unlabeled probes eliminated the shift (Fig. 7, lane 4). This indicated that the nuclear extracts contained factors with specificity for site E or site G. To determine whether these factors might include NF-Y or CREB1, we performed an additional EMSA in which HeLa cell extracts were preincubated with anti-NF-YA or anti-CREB1 antibodies. Preincubation with TF-specific antibodies caused a supershift (Fig. 7, lane 5, and B, lane 6) that was not induced with nonspecific IgG antibody (Fig. 7, lane 6, and B, lane 7). Taken together, the data indicate that NF-Y and CREB1 bind with specificity to site E and site G from the CDCA8 promoter. The two bands observed during the shift and supershift assays using site E were due to the long (NF-YAL) and short (NF-YAS) isoforms of the NF-YA subunit (24).

NF-Y Binding Is Positively Correlated with CDCA8 Promoter Activity—NF-Y is a heterotrimeric factor, which is composed of NF-YA, NF-YB, and NF-YC subunits. NF-YA has been proved to play an important role in maintaining the stemness of stem cells by activating multiple stem cell genes (25–27). Thus, we next focused on the role of NF-Y on CDCA8 transcription. We also used an EMSA to compare NF-Y binding to site E in different conditions.
ent cell lines. The amount of NF-Y binding was much higher in hESCs and several cancer cell lines than in dhESCs (induced by RA for 4 days) and hEFs (Fig. 8A), which was positively correlated with the CDCA8 promoter activity in each cell line (Fig. 3).

In addition, the NF-YA subunit, binding to site E, was predominantly short isoform (NF-YAS) in hESCs and long isoform (NF-YAL) in cancer cells, dhESCs, and hEFs (Fig. 8A).

To verify the recruitment and direct binding of NF-Y to the endogenous CDCA8 promoter, we performed ChIP assay using anti-NF-YA or isotype control antibodies and specific primers for the CDCA8 promoter region involving site E. As expected, the results confirmed the binding of NF-Y to the CDCA8 promoter in vivo, which was also markedly increased in hESCs and cancer cell lines compared with dhESCs and hEFs (Fig. 8B).

Next, we assessed the expression of different NF-Y subunits to determine whether differential NF-Y binding might be regulated by expression level. RT-PCR analysis revealed no significant change in the expression of NF-YB and NF-YC among the cell lines tested (Fig. 8D). During hESC differentiation, there was an obvious decrease in the level of NF-YAS protein and a concurrent increase in the level of NF-YAL protein (Fig. 8C), which corresponded with decreased NF-YAS promoter binding and increased NF-YAL binding (Fig. 8A). However, cancer cell lines (A549, K562, and HeLa) expressed high levels of unbound NF-YAS, and hEFs expressed high levels of unbound NF-YAL (Fig. 8C), indicating that NF-YAS in cancer cells and NF-YAL in hEFs have low site E binding abilities.

NF-Y Is an Activator of CDCA8 Transcription—Because MutE dramatically decreased CDCA8 promoter activity (Fig. 6B), NF-Y may function as a key transcriptional activator. To test this prediction, we co-transfected CDCA8-Luc plasmids with or without an equimolar mixture of NF-YAS/B/C-expressing plasmids into hESCs (Fig. 9A) and NF-YAL/B/C-expressing plasmids into HeLa cells (Fig. 9B) and measured luciferase expression. Overexpression of NF-Y significantly activated the CDCA8 promoter in hESCs and HeLa cells (Fig. 9, A and B). These findings indicate that NF-Y is a transcriptional activator of CDCA8.

To further verify the role of NF-Y in activating the CDCA8 gene, we employed a doxycycline-inducible shRNA, which targets the conserved region of NF-YAL and NF-YAS mRNA, to knock down the expression of total NF-Y (23). qPCR analysis
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confirmed that NF-YA knockdown significantly reduced the endogenous mRNA level of CDCA8 in both hESCs (Fig. 9C) and HeLa cells (Fig. 9D).

Taken together, NF-Y is a functional activator for CDCA8 transcription. Furthermore, its differential binding contributes to the different CDCA8 promoter activities in different cell lines.

Knockdown of CDCA8 Induces hESC Differentiation toward Multiple Lineages—Overexpression of CDCA8 was proved to be essential for growth/survival and the malignant nature of lung cancer cells (12). Here, we analyzed the role of the activation of the CDCA8 gene in hESCs using a targeted shRNA. Knockdown of CDCA8 reduced the expression of several pluripotent markers (Oct4, Nanog, and KLF4) (Fig. 10A). Multiple germ layer markers were activated following CDCA8 knockdown, including KRT17 (ectoderm), SOX17 (endoderm), and CDX2 (trophoblast) (Fig. 10B). In addition, CDCA8 knockdown cells plated at low density formed fewer colonies (Fig. 10C) with differentiated cell morphology relative to control cells (Fig. 10D). Ki-67 is a nuclear cell proliferation-associated antigen. Flow cytometric analysis using CDCA8 knockdown cells revealed a decrease proportion of Ki-67+ cells (Fig. 10E). Taken together, these results suggest that the activation of CDCA8 was involved in maintaining hESCs in an undifferentiated state, and its down-regulation induces differentiation toward multiple lineages.

Discussion

In this study, we investigated the transcriptional regulation of the human CDCA8 gene in hESCs and cancer cells. We first identified three TSSs located within its first annotated exon. One transcript initiated 194 bp upstream of the translation start site and contained the first intron as a result of alternative splicing. This splice variant has been detected in 14 different cell lines. However, these transcripts have been proved to encode the same protein due to a strong Kozak consensus present in the second exon (28).

Next, we characterized the transcriptional activity of the upstream promoter region. Functional analysis revealed that the CDCA8 promoter had high activity in several cancer cell lines but not in normal mammalian cell lines. This cancer activation was further confirmed in tumor tissues of tumor-bearing mice. Moreover, the CDCA8 promoter was also active in pluripotent hESCs and iPSCs. Upon hESC differentiation, activity of the promoter decreased dramatically and was undetectable prior to hES reprogramming. Embryonic stem cells were isolated from the ICM of blastocysts (29). The physical and functional separation of the pluripotent ICM from trophectoderm cells is the first cell lineage specification of mammalian development. As expected, the CDCA8 promoter is active from the two-cell to the morula stages but is restricted to the pluripotent ICM in blastocyst. The above pattern of promoter activity is consistent with that of endogenous gene expression, indicating that up-regulation of CDCA8 in cancer cells and hESCs mainly occurs at the transcriptional level.

A common feature of human cancers, ES cells, and early embryonic cells is that they retain high proliferation activity. As a mitotic regulatory gene, the activation of CDCA8 transcription should contribute to the rapid cell growth. Indeed, CDCA8 was proved to be essential for the growth of lung cancer cells, which was significantly suppressed by small interfering RNA against CDCA8 (12). Here, proliferation of hESCs was also reduced by CDCA8 knockdown. Furthermore, two previous studies suggested that CDCA8 plays a crucial role in cancer progression, although the mechanism is largely unknown. Patients with gastric or lung cancer showing higher nuclear accumulation or expression of CDCA8 represented a shorter tumor-specific survival period (11, 12). Compared with chemosensitive tumors, CDCA8 has higher expression in chemoresis-

FIGURE 9. NF-Y activates CDCA8 transcription in hESCs and cancer cells. A and B, transient transfection with NF-Y induces CDCA8 promoter activation in hESCs (A) and HeLa (B). Upper panel, relative luciferase expression under different experimental conditions. Lower panel, overexpression of NF-Y as detected by Western blotting. C and D, relative mRNA levels of total NF-YA and CDCA8 were determined by real time qPCR in doxycycline (DOX)-inducible shGFP/shNF-YA hESCs (C) and HeLa cells (D) cultured in the presence or absence of doxycycline. All values represent the means ± S.D. from three independent experiments. *, p < 0.05, and **, p < 0.01, as compared with shNF-YA without doxycycline.

confirmed that NF-YA knockdown significantly reduced the endogenous mRNA level of CDCA8 in both hESCs (Fig. 9C) and HeLa cells (Fig. 9D).

Taken together, NF-Y is a functional activator for CDCA8 transcription. Furthermore, its differential binding contributes to the different CDCA8 promoter activities in different cell lines.

Knockdown of CDCA8 Induces hESC Differentiation toward Multiple Lineages—Overexpression of CDCA8 was proved to be essential for growth/survival and the malignant nature of lung cancer cells (12). Here, we analyzed the role of the activa-
Knockdown of CDCA8 induces hESC differentiation toward multiple lineages. hESCs infected with shRNA control (shCtrl) or shCDCA8 were analyzed. A, real time qPCR analyses of pluripotent markers. B, differentiation-associated markers were determined by RT-PCR. Representative results are shown. Signal intensities were normalized with values for GAPDH and are presented as the percent observed in shCtrl. C, colony formation capability of hESCs. Infected cells were plated at low density (1000 cells per well of a six-well plate) in triplicate. Colonies were counted after 10 days of culture. D, morphology of hESCs colonies. E, percentages of Ki-67+ cells were analyzed by flow cytometry. All values represent the means ± S.D. from three independent experiments. *, p < 0.05, and **, p < 0.01.
tant epithelial ovarian tumors, suggesting its role in the chemoresistance of cancer (13). In this study, the high levels of CDCA8 transcription in hESCs and early embryonic cells dropped dramatically after differentiation. Knockdown of CDCA8 in hESCs reduced pluripotent marker expression and colony formation ability, as well as induced hESC differentiation toward multiple lineages. These results might be a reflection of a role for CDCA8 activation in maintaining hESCs in an undifferentiated status. Further studies need to be directed at clarifying the function and mechanism of CDCA8 in undifferentiated phenotypes of hESCs.

Our detailed analyses of the regulatory mechanisms demonstrated that heterotrimeric transcription factor NF-Y was a key activator of CDCA8 transcription in hESCs and cancer cells. First, mutation of the CCAAT-box, located 249 bp upstream of the translation start site, greatly reduced CDCA8 promoter activity at the trough level. Second, NF-Y bound to this CCAAT-box in vivo and in vitro. Its binding amount was positively correlated to promoter activity. Third, overexpression of NF-Y significantly activated the promoter. Fourth, shRNA against NF-YA reduced the endogenous mRNA levels of CDCA8.

The CCAAT-box, regarded as a binding site for NF-Y, was recently found to be a common cis-regulatory motif in the promoters of genes up-regulated in pluripotent stem cells (30), and it was also highly enriched in regulatory regions of genes over-expressed in tumors (31). Subunit NF-YA has two different isoforms, NF-YAL (long) and NF-YAS (short), resulting from alternative splicing (24). Both NF-YA isoforms were similarly effective in activating transcription (24), but they were not functionally identical. Sp1 activated the cystathionine-β-synthase promoter synergistically only with NF-YAL (32). NF-YAS is down-regulated, whereas NF-YAL is up-regulated during differentiation of hESCs (30), mouse ES cells (25, 30), and hematopoietic stem cells (26, 27). NF-YAS has a crucial role in maintaining stemness by directly activating key stem cell genes in mouse ES cells (25). NF-YAS also plays a central integrating role in the self-renewal of mouse hematopoietic stem cells by activating multiple pathways (27). Here, NF-YAS was highly expressed and bound the CDCA8 promoter in hESCs; however, after differentiation, NF-YAS expression decreased dramatically, whereas NF-YAL became slightly expressed and bound the promoter. These findings are consistent with the reported expression pattern and regulatory mechanism of NF-YA isoforms in stem cells and suggest that CDCA8 probably belongs to a set of stem cell genes regulated by NF-YAS.

Interestingly, the CDCA8 promoter is activated predominantly by NF-YAL in cancer cells suggesting that CDCA8 transcription is differentially regulated between hESCs and cancer cells. Given that CDCA8 was reported to be a promising target for anticancer drug development (12), selective suppression of the NF-YAL-CDCA8 pathway might be a useful strategy. The low promoter binding abilities of NF-YAS in cancer cells or NF-YAL in hEFs may be explained by post-translational modifications or other trans-activating factors. Other predicted cis-elements and TFs for the CDCA8 promoter need to be further explored.

In conclusion, we, for the first time, characterized the promoter of the human CDCA8 gene and demonstrated its transcriptional activation in hESCs and cancer cells. Furthermore, we identified NF-Y as a key activator for the transcription of CDCA8. Our study reveals some molecular mechanisms conferring the CDCA8 up-regulation in hESCs and cancer cells, which provides a better understanding of its biological functions and may be useful for the development of cancer therapies targeting CDCA8.

Author Contributions — C. D. and C. X. M. conceived and designed the study and acquired and analyzed data. C. D. wrote the manuscript. X. M. X. and Y. F. G. performed the microinjections. L. J. L. acquired and analyzed the ChIP assay data. D. Z. provided human ES cells. L. S. C., G. L., and G. X. L. conceived and designed the study and analyzed the data. G. L. and G. X. L. gave final approval of the manuscript. All authors reviewed the results and approved the final version of the manuscript.

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