

GATA6-AS1 Regulates GATA6 Expression to Modulate Human Endoderm Differentiation

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SUMMARY

Transcriptome analysis has uncovered a series of long noncoding RNAs (lncRNAs) transcribed during cell differentiation, but how lncRNA is integrated with known transcriptional regulatory network is poorly understood. Here, we utilize human definitive endoderm differentiation as a model system and decipher the functional interaction between lncRNA and key transcriptional factor. We have identified *GATA6-AS1*, an lncRNA divergently transcribed from the *GATA6* locus, is highly expressed during endoderm differentiation. Knockdown of *GATA6-AS1* in human pluripotent stem cells has no influence on morphology and pluripotency; however, *GATA6-AS1* depletion causes the deficiency of definitive endoderm differentiation. *GATA6-AS1* positively regulates the expression of endoderm key factor *GATA6*. Further investigation shows *GATA6-AS1* interacts with SMAD2/3 and activates the transcription of *GATA6*. In addition, overexpression of *GATA6* is able to rescue the defect of endoderm differentiation due to the absence of *GATA6-AS1*, suggesting that *GATA6* is the functional target of *GATA6-AS1* during endoderm differentiation. Ultimately, our study reveals that *GATA6-AS1* is necessary for human endoderm specification and reveals the underlying mechanism between *GATA6-AS1* and *GATA6*.

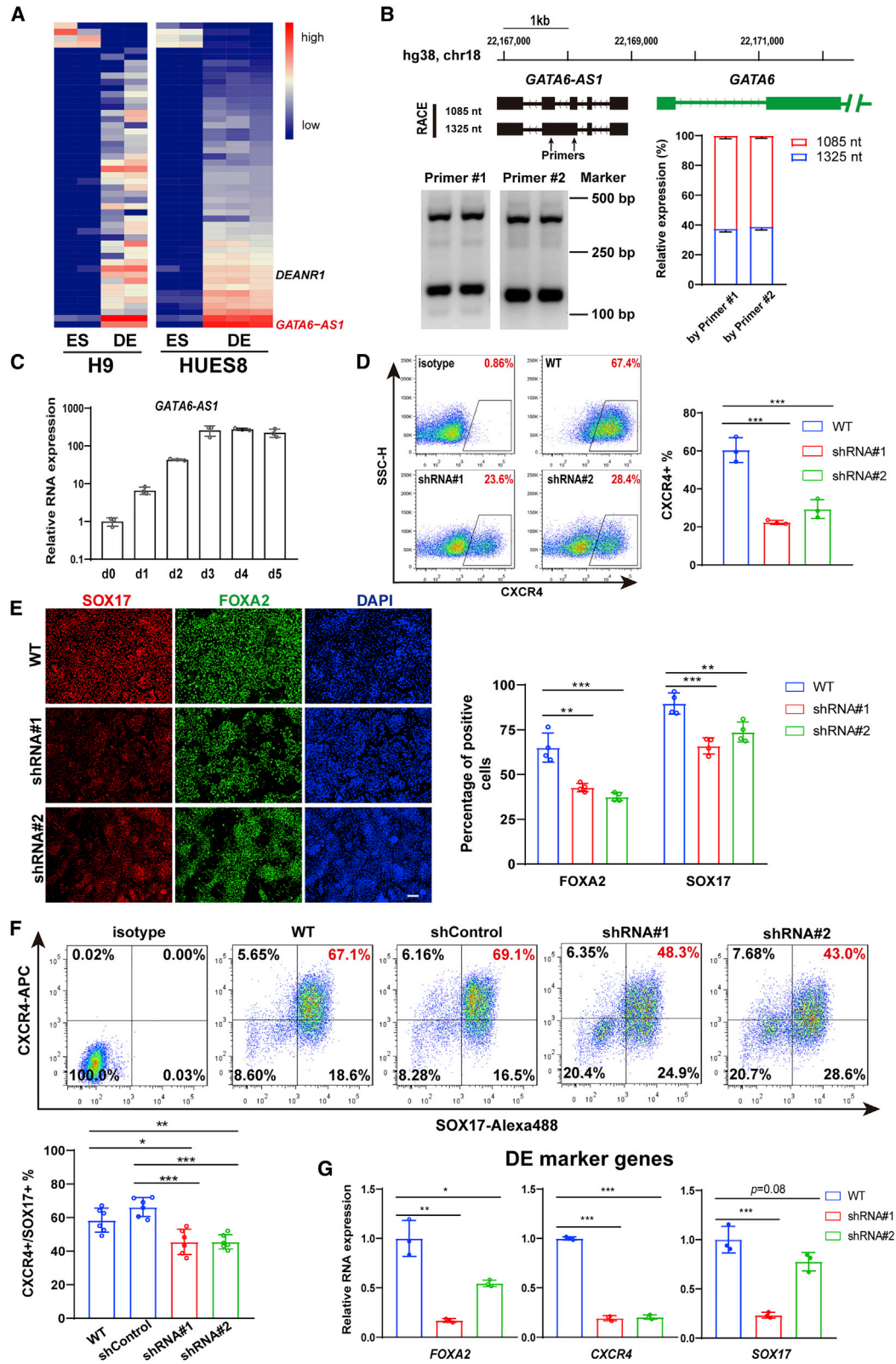
INTRODUCTION

Long noncoding RNAs (lncRNAs) are longer than 200 nucleotides and with low protein-coding potential, and they are expressed in a more cell-type-specific manner during development and in certain cancers (Delas and Hannon, 2017; Rinn and Chang, 2012). More than 77,000 lncRNA transcripts have been identified according to the RefLnc database (Jiang et al., 2019), but the majority have not been functionally dissected yet. During the last decade, lncRNAs have been uncovered as an important functional player in tissue development and cell differentiation (Fatica and Bozzoni, 2014). Among them, many are *cis*-acting lncRNAs that regulate local gene expression in a manner dependent on the location from where they originate (Gil and Ulitsky, 2019). Recently, *yy1ncT*, a lncRNA localizing to the BRACHYURY (T) locus, regulates the expression of the key mesoderm specifier T and thus contributes to mesoderm commitment (Frank et al., 2019); *CCR5-AS*, the antisense lncRNA of the *CCR5* locus, protects *CCR5* mRNA from degradation and facilitates the HIV infection of CD4-positive T cells (Kulkarni et al., 2019). Mechanistically, *cis*-acting lncRNAs could promote or repress local transcription by recruiting transcription regulatory protein or bridging proximal enhancers into the promoter of the target gene or competitively binding transcription factors or epigenetic modifiers (Gil and Ulitsky, 2019).

Germ layer specification is one of the most critical events during early development. Sperm-egg fusion generates the

zygote and through multiple rounds of mitotic division the embryo goes through two-cell, four-cell, morula stage, and subsequently enters blastocyst stage. The inner cell mass in blastocyst undergoes differentiation and generates three germ layers: ectoderm, mesoderm, and definitive endoderm (Kiecker et al., 2016; Okabe, 2013; Wamaitha and Niakan, 2018). The definitive endoderm contributes to the respiratory and gastrointestinal tracts and their derived tissues and organs, and endoderm dysplasia is associated with numerous human diseases that torment millions of people every year (Zorn and Wells, 2009). Due to the limitation to obtain clinical embryonic materials, very few studies on human endoderm development are performed. Embryonic stem cells (ESCs) are established from human blastocysts and provide a suitable model to study early development, disease modeling, and drug screening, as well as cell-based therapy (Thomson et al., 1998; Yiangou et al., 2018).

Transcription factors regulation network and epigenetic modifications have been extensively reported to contribute to definitive endoderm differentiation (Li et al., 2019; Tsankov et al., 2015). However, whether and how lncRNAs could function in endoderm differentiation was largely unknown for a long time. In 2013 Young group reported that more than 60% of lncRNA species expressed in human and murine ESCs were divergently transcribed from active protein-coding genes (Sigova et al., 2013). A following study together revealed that these divergently transcribed lncRNA/mRNA gene pairs exhibited coordinated



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transcriptional changes during differentiation into mesoderm and endoderm lineage through genome-wide analysis (Luo et al., 2016; Sigova et al., 2013). For example, lncRNA *Evx1as* bound to regulatory sites on chromatin and interacted with Mediator to facilitate *EVX1* transcription and eventually contributed to mouse mesoderm differentiation (Luo et al., 2016). In 2015, lncRNA *DEANR1* was first identified to contribute to human endoderm differentiation by facilitating *FOXA2* transcriptional activation (Jiang et al., 2015). Subsequently, Daneshvar et al. (2016) uncovered that *DIGIT*, an lncRNA transcribed from the Goosecoid (*GSC*) locus, regulated *GSC* expression to manipulate endoderm differentiation.

GATA6-AS1 is an lncRNA divergently transcribed from the *GATA6* locus. *GATA6* is an essential transcription factor during definitive endoderm and pancreas development and β cell function (Chia et al., 2019; Fisher et al., 2017; Schrode et al., 2014; Shi et al., 2017; Tiyafoonchai et al., 2017). However, the function of *GATA6-AS1* is unknown in endoderm and derived organs. Here, using a definitive endoderm differentiation system, in which both *GATA6-AS1* and *GATA6* were highly expressed, we dissected the biological role of *GATA6-AS1* and further investigated the functional link between *GATA6-AS1* and *GATA6*.

RESULTS

GATA6-AS1 Is an lncRNA Highly Expressed in Definitive Endoderm

First, we performed transcriptomic analysis during definitive endoderm differentiation using human ESC line HUES8. By combining the data with previous published data based on H9 cells (GSE44875) (Jiang et al., 2015), we screened a number of endoderm-specific lncRNAs (Table S1). Among these, *GATA6-AS1*, a lncRNA divergently transcribed from *GATA6* locus, exhibited the highest expression in definitive endoderm (Figure 1A). Since the current annotation of lncRNAs is not as accurate as coding genes,

we performed 5' and 3' RACE experiments and identified two isoforms of *GATA6-AS1* with one region difference: one was 1,085 nucleotides and the other was 1,325 nucleotides (Supplemental Information). We further determined the expression levels of both isoforms and found that the longer form is approximately 40%, while the shorter form is about 60% (Figure 1B). Because both isoforms were largely similar in sequence and no isoform preference was obviously observed, we decided to not make a distinction between the isoform types in following studies. In addition, both isoforms were analyzed with low protein-coding potential by Coding Potential Calculator 2 (CPC2) (Kang et al., 2017) and Coding Potential Assessment Tool (CPAT) (Wang et al., 2013), respectively (Figure S1A).

By qRT-PCR, we validated the expression pattern of *GATA6-AS1* during endoderm differentiation, and the results showed that *GATA6-AS1* was gradually increased and achieved the highest expression level at day 3 (Figure 1C), when the endoderm differentiation was considered almost completed. Consistent with the endoderm-specific expression pattern, we surveyed available databases including most human tissues (Fagerberg et al., 2014) and found *GATA6-AS1* was highly expressed in endoderm-derived tissues and organs, such as stomach, small intestine, and duodenum (Figure S1B). These results indicated that *GATA6-AS1* is an lncRNA highly expressed in definitive endoderm.

GATA6-AS1 Is Required for Definitive Endoderm Differentiation

To further determine the biological function of *GATA6-AS1* in definitive endoderm differentiation, we utilized a quick definitive endoderm differentiation system. Time course experiment showed that most endoderm-specific genes achieved the expression peaks and both pluripotency and mesoderm-associated genes quickly decreased at day 3 (Figure S1C). Immunofluorescence assay of 3-day differentiated cells showed there were about 74% of *FOXA2*-positive

Figure 1. *GATA6-AS1* Is Highly Expressed in and Required for Human Endoderm Differentiation

(A) Heatmap showing the differentially expressed lncRNAs between ESCs and definitive endoderm (DE) cells ($\text{abs}(\log_2(\text{fold-change})) > 5$, and $p < 0.05$). Red represents higher expression and blue represents lower expression. The list of differentially expressed lncRNAs is shown in Table S1.

(B) Diagram of the *GATA6-AS1* and *GATA6* gene locus. Two isoforms of *GATA6-AS1* were identified by 5' and 3' RACE and validated by RT-PCR. The relative expression levels of two isoforms were calculated according to PCR analysis ($n = 3$).

(C) Time course (day 0 to 5) expression pattern of *GATA6-AS1* during endoderm differentiation ($n = 3$). All data were normalized to day 0.

(D) Flow cytometric analysis showed the percentage of *CXCR4*-positive cells in differentiated HUES8 and *GATA6-AS1* knockdown cells ($n = 3$).

(E) Immunofluorescence of endoderm markers *SOX17* and *FOXA2* in differentiated HUES8 and *GATA6-AS1* knockdown cells ($n = 4$ for quantification). Scale bar, 100 μm .

(F) Co-flow cytometric analysis of *SOX17* and *CXCR4* in differentiated HUES8, shControl, and *GATA6-AS1* knockdown cells ($n = 6$).

(G) mRNA levels of endoderm marker genes, including *FOXA2*, *SOX17*, and *CXCR4* were detected by qRT-PCR ($n = 3$). All data were normalized to control.



and 82% of SOX17-positive cells (Figure S1D). These results together with our previous studies (Jiang et al., 2013a, 2013b, 2015) showed that the differentiation protocol was a simple, fast, and effective system to further investigate lncRNA function in definitive endoderm. In addition, CXCR4 was a widely used surface marker to evaluating the differentiation efficiency of definitive endoderm (Chu et al., 2016; D'Amour et al., 2005; Drukker et al., 2012; Jiang et al., 2015; Jiang et al., 2013a; Jiang et al., 2013b). We also confirmed this notion in our differentiation system by immunostaining with both CXCR4 and SOX17 (Figure S1E), and intracellular flow cytometric analysis using fluorescence-labeled antibody against SOX17 and CXCR4 (Figure S1F). Next, we designed five shRNAs targeting *GATA6-AS1* and two control shRNAs targeting unrelated sequences in our initial two rounds of screening (Figure S2A). The results showed three shRNAs effectively interfered with endoderm differentiation, while the other two shRNAs with ineffective knockdown efficiency and the control shRNAs did not (Figure S2A). We also compared, side-by-side, the control shRNA and wild-type cells, and the results showed no difference in terms of the differentiation capability toward definitive endoderm (Figure S2B), suggesting the lentiviral vector has no effect on the phenotype. Next, we evaluated the knockdown efficiency of shRNAs to target the common regions of both isoforms of *GATA6-AS1* in the endoderm and 293T cells and obtained two efficient shRNAs (Figure S2C). Then, accordingly, we established two human ESC lines with stable *GATA6-AS1* knockdown (Figure S2D). *GATA6-AS1* knockdown ESCs had no influence on colony morphology or expression of pluripotent genes (Figures S2D–S2F), which is consistent with the notion that *GATA6-AS1* is not expressed in undifferentiated pluripotent stem cells.

Next, we subjected the *GATA6-AS1* knockdown ESCs to endoderm differentiation assay using our differentiation scheme described above. As CXCR4-positive cells could represent human definitive endoderm cells in such a system (Figures S1E and S1F) and be easily detected, we first performed flow cytometric analysis of CXCR4 and the results showed a significant decrease in the percentage of CXCR4-positive cells in *GATA6-AS1* knockdown cells compared with wild-type cells (Figure 1D). Consistently, immunofluorescence assay showed significantly decreased endoderm markers, including SOX17 and FOXA2, in knockdown cells (Figure 1E). In addition, the intracellular flow cytometry analysis of CXCR4 and SOX17 showed that CXCR4-positive/SOX17-positive cells were significantly reduced (Figure 1F), which was further supported by qRT-PCR analysis of endoderm marker genes (Figure 1G). Meanwhile, both pluripotent genes (*SOX2* and *OCT4*) and mesoderm marker genes (*T* and *MIXL1*) showed increased expression in a certain extent in differentiated *GATA6-*

AS1 knockdown cells (Figure S2G). To further confirm the *GATA6-AS1* function in definitive endoderm, we performed the definitive endoderm differentiation in another differentiation system supplemented with 1 μ M WNT inhibitor XAV-939 at day 3, which was a more efficient definitive endoderm differentiation protocol (Jiang et al., 2013b). The results of flow cytometry showed that *GATA6-AS1* knockdown cells exhibited a significant decrease of CXCR4-positive/SOX17-positive cells, similar to the results described above (Figure S2H). These results suggested that *GATA6-AS1* was a functional lncRNA in endoderm differentiation, and that depletion of *GATA6-AS1* blocked human endoderm differentiation from ESCs.

***GATA6-AS1* Influences the Transcriptome Profile of Definitive Endoderm**

To further confirm the important role of *GATA6-AS1* and investigate the downstream targets in endoderm differentiation, we performed RNA sequencing (RNA-seq) experiment using differentiated endoderm cells from *GATA6-AS1* knockdown cells and wild-type cells. Transcriptome analysis indicated that approximately 8% of coding genes exhibited differential expression levels, including pluripotent genes and endoderm marker genes (Figure 2A). Moreover, the expression of ESC-specific genes was still maintained in differentiated *GATA6-AS1* knockdown cells, but the expression levels of endoderm-specific genes were lower compared with differentiated wild-type cells (Figure 2B; Table S2). Consistently, genes with higher expression in wild-type cells were significantly enriched in regulation of gastrulation by gene set enrichment analysis (GSEA) (Figure 2C; Table S3). In addition, gene ontology (GO) terms of downregulated genes in *GATA6-AS1* knockdown endoderm cells included pattern specification process, embryonic organ development, cell fate commitment, and stem cell differentiation (Figure 2D; Table S3), supporting that *GATA6-AS1* played an important role in early development. Further analysis of differently expressed transcription factors showed pluripotent genes and endoderm-specific genes, especially *GATA6*, were interacting with other proteins or transcription factors (Figure 2E). Taken together, these results provide transcriptome evidence that *GATA6-AS1* is a functional lncRNA in human early endoderm differentiation.

To exclude the effect of *GATA6-AS1* in endoderm differentiation was not due to the genetic background of individual cell lines, we re-constructed another stable cell line with one shRNA achieving effective *GATA6-AS1* knockdown in H9 ESCs (Figure S3A), while the *GATA6-AS1* knockdown cells maintained typical colony morphology (Figure S3B). After being subjected to endoderm differentiation, we observed a reduced percentage of CXCR4-positive cells in *GATA6-AS1* knockdown cells (Figure S3C), along with

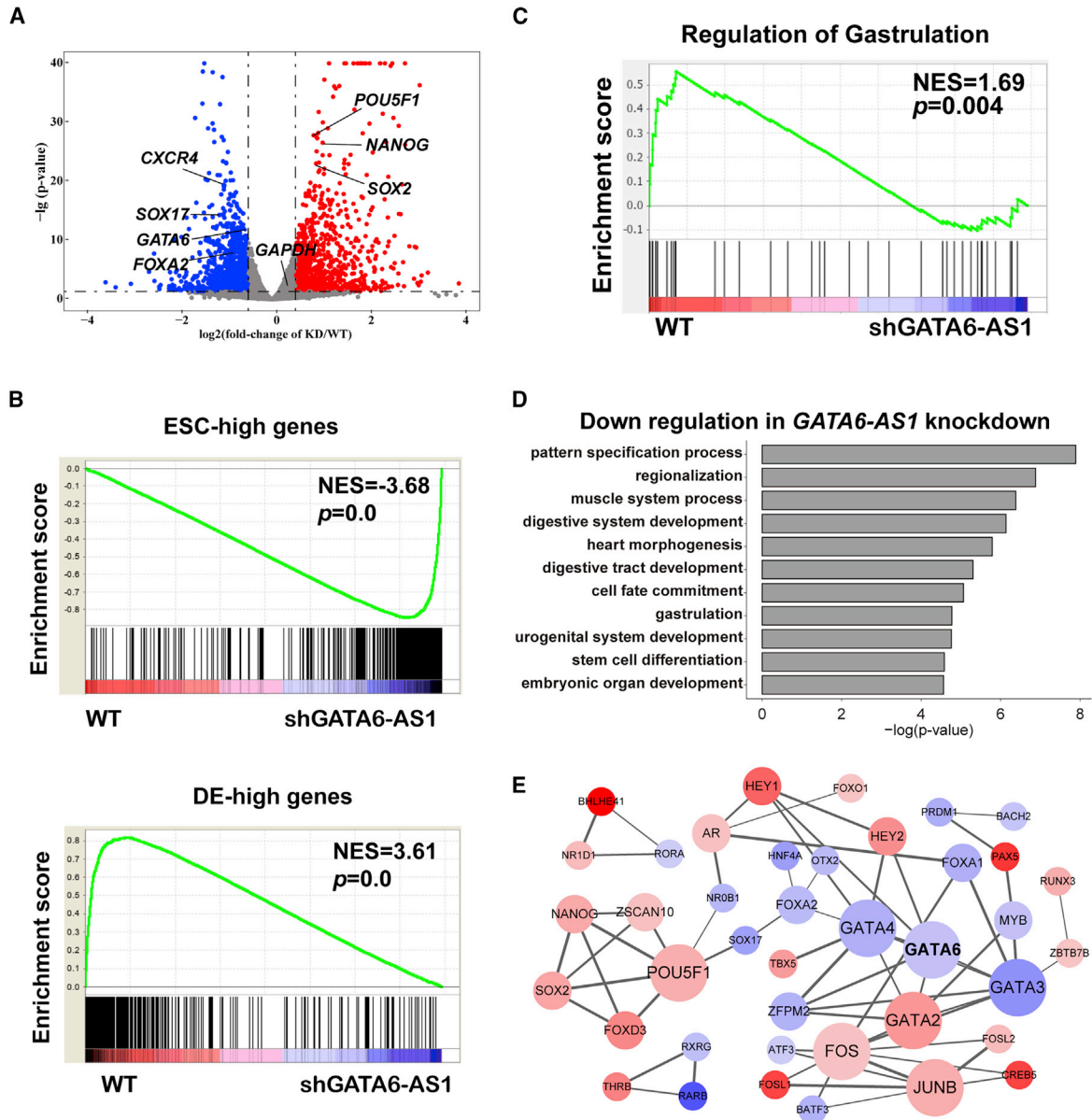


Figure 2. *GATA6-AS1* Influences the Transcriptome Profile of Human Endoderm

(A) Scatterplot showing differentially expressed genes ($p < 0.05$ and $abs(\log_2(\text{fold-change})) > 0.5$) between differentiated control cells and *GATA6-AS1* knockdown cells. Up- and downregulated genes in *GATA6-AS1* knockdown cells are shown in red and blue, respectively.

(B) Gene set enrichment analysis (GSEA) profile of ESC-high and DE-high genes. The gene list is shown in Table S2.

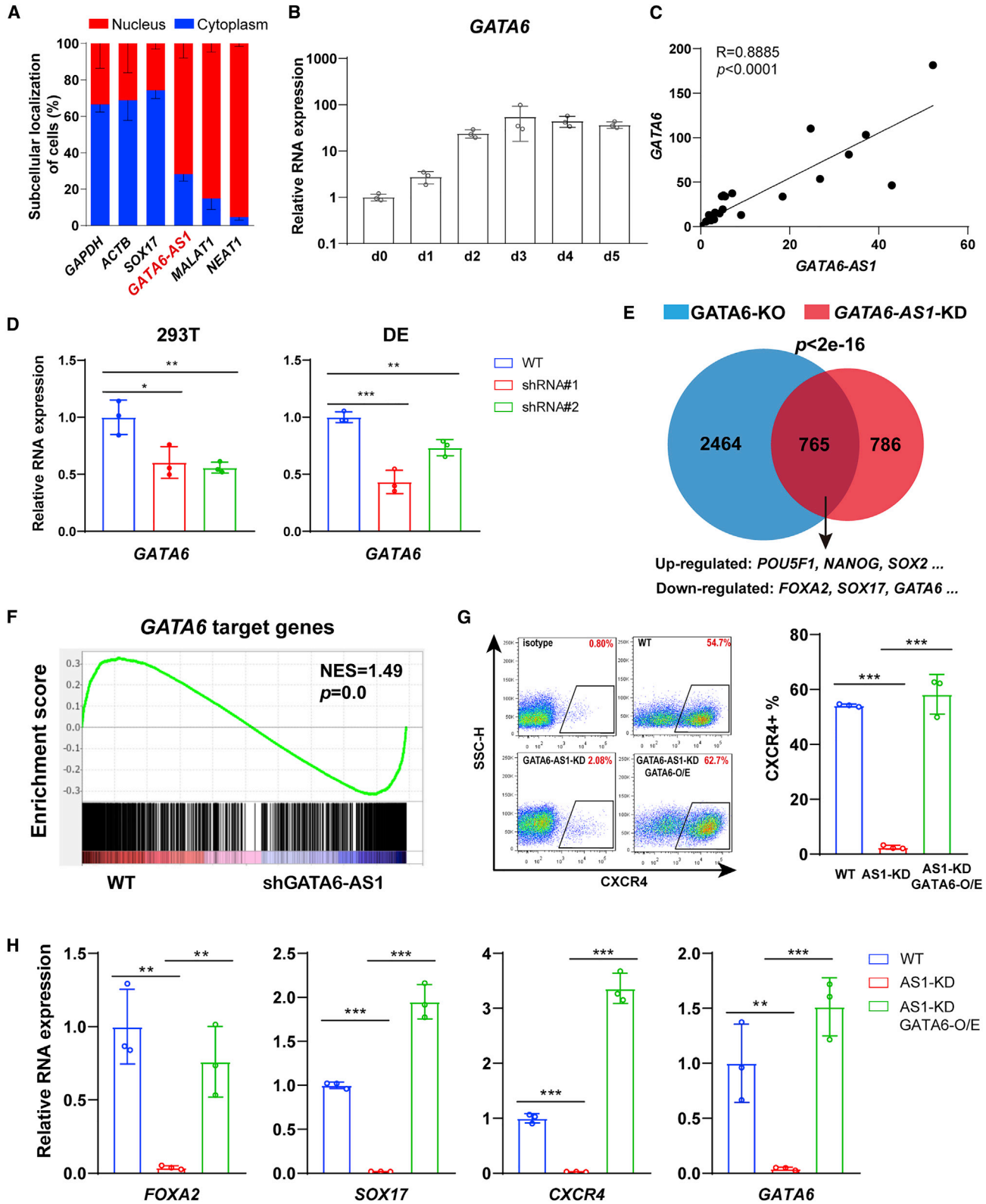
(C) GSEA profile of “regulation of gastrulation” set. The gene list is shown in Table S3.

(D) Gene ontology (GO) enrichment analysis of downregulated genes upon *GATA6-AS1* knockdown. The full GO terms are listed in Table S3.

(E) Differently expressed transcription factors interaction network. Red and blue represent upregulated and downregulated transcription factors after *GATA6-AS1* knockdown, respectively. The circle size represents the number of interaction proteins and the line width represents combined score of proteins.

significantly decreased mRNA levels of *SOX17*, *GATA6*, *FOXA2*, and *CXCR4* (Figure S3D). The protein levels of *GATA6* and *SOX17* were obviously reduced after *GATA6-AS1* depletion (Figure S3E). Moreover, when we performed

the following differentiation toward pancreatic lineage based on our previously established protocols (Jiang et al., 2015; Tan et al., 2019), we found severe defects in pancreatic lineage marked by *PDX1* and hepatic lineage marked



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by AFP in *GATA6-AS1* knockdown cells (Figure S3F), supporting the notion that *GATA6-AS1* is important for endoderm fate. These data together confirmed that *GATA6-AS1* was required for human endoderm differentiation, and that deficiency of *GATA6-AS1* caused the downregulation of definitive endoderm signature genes and blocked stem cell differentiation and cell fate commitment.

GATA6 Is the Target of GATA6-AS1

Since the subcellular distribution of lncRNAs is usually correlated with different action models (Chen, 2016), we asked whether *GATA6-AS1* was localized in the nucleus or cytosol. We separated cytoplasmic and nucleus fractions of endoderm cells and determined the expression levels of *GATA6-AS1* by qRT-PCR. The result showed that the *GATA6-AS1* transcript was mainly localized in the nucleus (Figure 3A). Given that *GATA6-AS1* was divergently transcribed from the *GATA6* locus, and *GATA6* is one of the key nodes of the protein-protein interaction network affected by *GATA6-AS1* (Figure 2E), we proposed that *GATA6* might be the target of *GATA6-AS1*. Consistent with this hypothesis, *GATA6* was upregulated during endoderm differentiation (Figure 3B) in a similar trend to *GATA6-AS1* (Figure 1C). We also found a significant correlation between *GATA6-AS1* and *GATA6* expression from either different RNA-seq samples (Jiang et al., 2015; Li et al., 2018; Xin et al., 2013) (Figure 3C; Table S4) or our qRT-PCR results (Figure S3G). More importantly, knockdown of *GATA6-AS1* led to a decreased expression of *GATA6* in both 293T cells and differentiated endoderm cells (Figures 3D and S3D), suggesting that *GATA6-AS1* might regulate *GATA6*.

To further investigate the relationship between *GATA6-AS1* and *GATA6*, we downloaded and re-analyzed the publicly available RNA-seq data of human endoderm cells with *GATA6* depletion (E-MTAB-5958 [Chia et al., 2019]), and identified 3,229 differentially expressed genes due to *GATA6*-KO (Table S1). We found that there was significant

overlap between *GATA6-AS1*-regulated genes (Table S1) and *GATA6*-regulated genes (Figure 3E). In addition, we identified *GATA6* direct targets by combining the RNA-seq data of *GATA6*-KO and *GATA6* chromatin immunoprecipitation sequencing (ChIP-seq) data (Chia et al., 2019; Fisher et al., 2017; Li et al., 2019; Tsankov et al., 2015) (Figure S3H), and performed GSEA, which indicated that *GATA6* target genes (Table S3) were significantly enriched in those genes affected by *GATA6-AS1* knockdown in differentiated endoderm (Figure 3F). These data together supported that *GATA6-AS1* likely regulated *GATA6* and shared similar downstream targets in endoderm differentiation.

To confirm *GATA6* was the functional target of *GATA6-AS1*, we put *GATA6* coding region back to *GATA6-AS1*-depletion cells and checked whether the phenotype of endoderm differentiation defect could be rescued. Overexpression of *GATA6* did not disrupt the pluripotency in ESC (Figures S3I–S3K). However, *GATA6* overexpression indeed rescued the gene expression of endoderm markers (Figure 3H) and recovered endoderm differentiation in *GATA6-AS1* knockdown cells (Figure 3G) as well. These data demonstrated that *GATA6* was the functional target of *GATA6-AS1* in human endoderm differentiation.

GATA6-AS1 Interacts with SMAD2/3 to Promote Definitive Endoderm Differentiation

Previous research reported that SMAD2/3 transcriptionally activated endoderm-specific transcription factors, including *GATA6*, to promote endoderm differentiation (Chia et al., 2019; Kim et al., 2011; Li et al., 2019). Therefore, we were wondering whether *GATA6-AS1* was involved in the SMAD2/3-mediated regulation of *GATA6*. To this end, we performed SMAD2/3 ChIP assay on *GATA6-AS1* knockdown and wild-type endoderm cells. The ChIP-qPCR results showed that SMAD2/3 indeed bound to the promoter region of *GATA6* (Figure 4A); moreover, *GATA6-AS1* deletion reduced SMAD2/3 occupation on the *GATA6* locus (Figure 4A). We further performed an SMAD2/3

Figure 3. GATA6 Is the Functional Target of GATA6-AS1 in Endoderm Differentiation

- (A) Subcellular localization of *GATA6-AS1* in human endoderm cells. *GAPDH*, *ACTB*, and *SOX17* transcripts served as cytoplasm-located control, and *MALAT1* and *NEAT1* served as nuclei-located controls (n = 6). Red represents nuclei fraction and blue represents cytoplasmic fraction.
- (B) Time course (day 0 to 5) expression pattern of *GATA6* RNA during endoderm differentiation (n = 3). All data were normalized to day 0.
- (C) The correlation analysis of *GATA6* and *GATA6-AS1* transcripts in different samples. The data presented in Figure 3C are shown in Table S4.
- (D) The expression level of *GATA6* RNA upon *GATA6-AS1* knockdown in 293T, differentiated endoderm cells (HUES8) (n = 3).
- (E) Venn diagram indicates the significant overlap of differentially expressed genes due to *GATA6*-KO or *GATA6-AS1* knockdown in endoderm cells. The gene list is shown in Table S3.
- (F) GSEA profile of *GATA6* target genes in differentiated control endoderm cells and *GATA6-AS1* knockdown endoderm cells. The list of *GATA6* target genes is shown in Table S3.
- (G) Flow cytometric analysis showed the percentage of CXCR4-positive cells in differentiated HUES8 (WT), *GATA6-AS1* knockdown (AS1-KD), and *GATA6*-overexpressing cells with *GATA6-AS1* knockdown (AS1-KD; *GATA6*-O/E) (n = 3).
- (H) mRNA levels of endoderm marker genes, including *FOXA2*, *SOX17*, and *CXCR4* detected by qRT-PCR for the samples shown in G (n = 3).

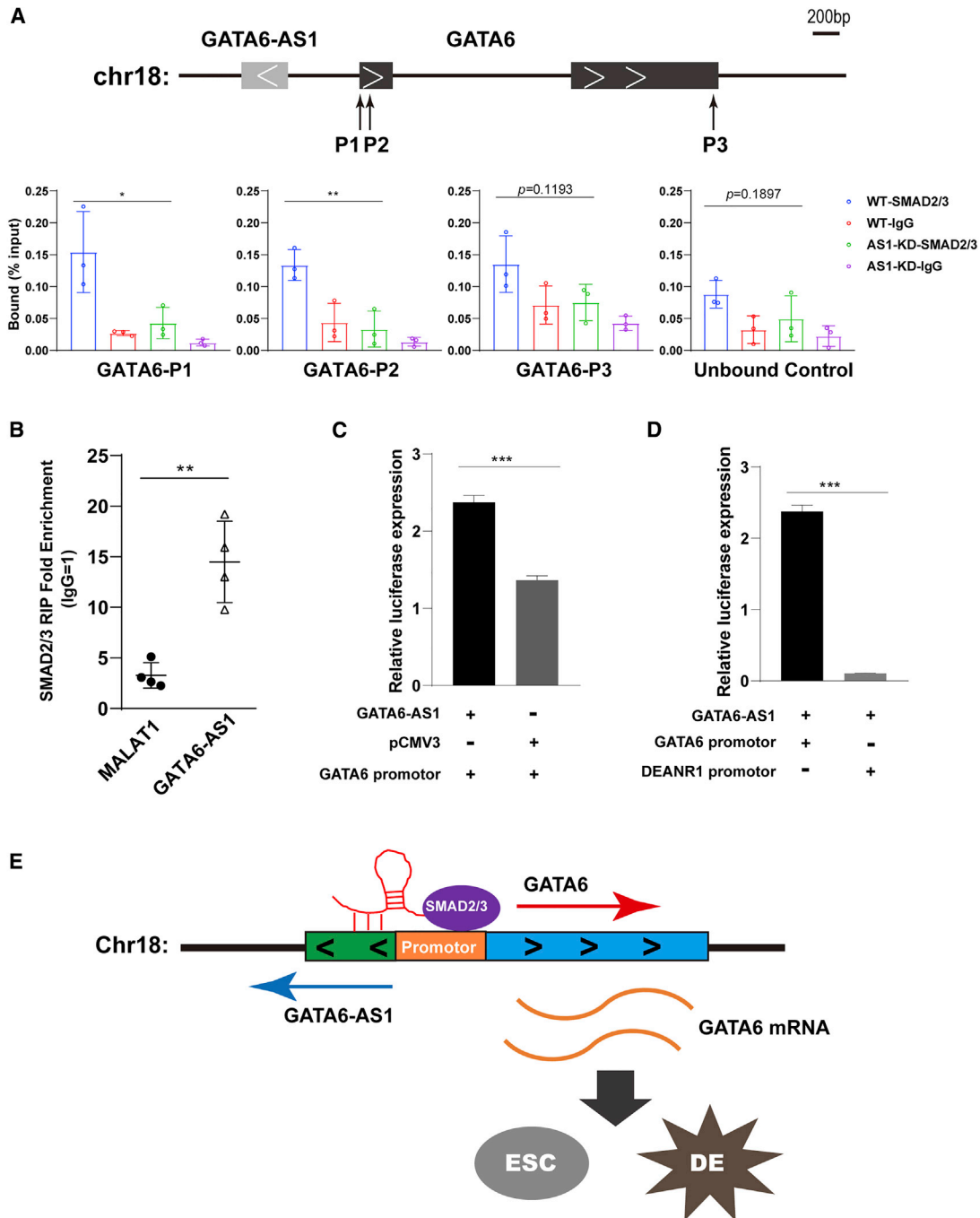


Figure 4. *GATA6-AS1* Interacts with SMAD2/3 and Regulates *GATA6* Transcription Activity

(A) ChIP-qPCR analysis of SMAD2/3 bound to *GATA6* locus upon *GATA6-AS1* depletion ($n = 3$). Arrows represent the genomic position of PCR primers.

(B) Immunoprecipitation demonstrated that SMAD2/3 interacted with *GATA6-AS1*. *MALAT1* served as lncRNA control ($n = 4$).

(C) *GATA6-AS1* transcript increased the transcription activity of *GATA6* promoter in 293T cells ($n = 3$).

(D) *GATA6-AS1* transcript showed specific activation for *GATA6* promoter rather than *DEANR1* promoters ($n = 3$).

(E) Model of *GATA6-AS1*-regulated *GATA6* via interacting with SMAD2/3 and promoting the transcription activity. *GATA6-AS1* might form an RNA-DNA triple helix with the *GATA6* promoter and interacted with SMAD2/3 to activate *GATA6* transcription, thus further promoting human ESCs to differentiate into definitive endoderm.



immunoprecipitation assay in endoderm cells and found that SMAD2/3 interacted with *GATA6-AS1*, rather than unrelated lncRNA, such as *MALAT1* (Figure 4B). Luciferase assay showed that the *GATA6-AS1* transcript could activate the transcription of the *GATA6* promoter (Figure 4C), and that this activation was specific to the *GATA6* promoter rather than the *DEANR1* promoter (Figure 4D). These results demonstrated that *GATA6-AS1* was required for SMAD2/3-mediated *GATA6* transcriptional activation to modulate human endoderm differentiation (Figure 4E).

DISCUSSION

lncRNAs, especially *cis*-acting lncRNAs, have attracted more attention in studies of development and differentiation; however, the function of the majority of lncRNAs is still unclear due to numerous transcripts and diverse cell types (Gil and Ulitsky, 2019). Here, we revealed that *GATA6-AS1* was functional in regulating *GATA6* expression by interacting with and mediating SMAD2/3 to bind to the *GATA6* promoter region. Biologically, depletion of *GATA6-AS1* severely blocked human endoderm differentiation, and *GATA6* overexpression rescued the endoderm differentiation deficiency. Our study identified a novel lncRNA in human endoderm differentiation and replenished a regulation model of key factor *GATA6*.

Recently *GATA6-AS1* was studied in two different biological systems. Neumann et al., (2018) showed that *GATA6-AS1* was upregulated in endothelial cells during hypoxia and that *GATA6-AS1* knockdown disrupted endothelial-mesenchymal transition and promoted formation of blood vessels. They further found that *GATA6-AS1* negatively regulated *LOXL2*, which led to the deamination of active marker H3K4me3, and *GATA6-AS1* knockdown led to reduced H3K4me3 and repressed the expression of angiogenesis-related genes in endothelial cells (Neumann et al., 2018). Zhu et al. (2018) studied *GATA6-AS1* (named *lncGata6*) in the mouse intestine system and reported that *lncGata6* was highly expressed in mice intestine stem cells and contributed to regeneration and colorectal tumorigenesis. In this context, *lncGata6* recruited the NURF complex onto the *Ehf* promoter and promoted *Lgr4/5* expression by *Ehf*. In our endoderm differentiation context, we also checked whether those two mechanisms existed. First, we found that expression of both *LOXL2* and *EHF* was very low during endoderm differentiation (Figure S3L), and that *GATA6-AS1* knockdown had no significant effect on *LOXL2* expression (Figure S3M). Second, our data clearly suggested that *GATA6-AS1* regulated *GATA6* expression demonstrated by both RNA and protein analysis (Figures 2G and 3D) and, most importantly, that overexpression of *GATA6* was able to rescue the phenotype of defected

endoderm differentiation due to *GATA6-AS1* depletion (Figures 3G and 3H). Supporting our conclusion, several studies showed the expression levels of *GATA6-AS1* was tightly correlated with *GATA6* in diverse cell types, including stem cells and cancer cells (Kuo et al., 2019; Liu et al., 2018; Luo et al., 2016). These data suggested that the relationship between *GATA6-AS1* and *GATA6* might be more universal. Recently, Kuo et al. (2019) reported that *GATA6-AS1* could form RNA-DNA triple helices in the *GATA6* promoter through a computational method called the Triplex Domain Finder. This raised a hypothesis that the *GATA6-AS1* transcript might form RNA-DNA triple helices on its own gene body region, recruiting and assisting SMAD2/3 bound at the promoter of *GATA6* to activate *GATA6* transcription, which further contributed to various biological contexts, including endoderm differentiation.

Definitive endoderm differentiation is mainly induced by the transforming growth factor β /SMAD signaling pathway. SMAD regulates target gene expression via binding to SMAD-binding elements in promoter regions. Recent studies showed that lncRNA was also necessary in the SMAD regulation network. lncRNA *GAS5* competitively bound SMAD3 protein via multiple RNA SBEs to inhibit SMAD3 binding to the SMC gene promoter to block smooth muscle cell differentiation (Tang et al., 2017). *lnc-TSI* interacted with MH2 domain of SMAD3, blocking interaction of SMAD3 with SMAD7 in renal fibrogenesis (Wang et al., 2018). In addition, lncRNA *ELIT-1* bound to SMAD3 and functioned as a cofactor to recruit SMAD3 to the promoters of *Snail* and other SMAD target genes to boost epithelial-mesenchymal transition progression (Sakai et al., 2019). In our study, we revealed that *GATA6-AS1* was able to function as a cofactor of SMAD2/3 to positively regulate *GATA6* for endoderm differentiation. This finding together with the *DEANR1* report (Jiang et al., 2015) suggested that lncRNAs may play a special role for SMAD functions in pluripotency exit and differentiation initiation in human pluripotent stem cells, which provides a meaningful insight in understanding SMAD signaling and the switch between pluripotency and differentiation.

Recently, *GATA6* has been well studied in human pancreatic development by different groups, and the dosage-sensitive requirement of *GATA6* in the differentiation of both definitive endoderm and pancreas has been identified (Chia et al., 2019; Shi et al., 2017; Tiyaboonchai et al., 2017). However, there were very few reports showing how *GATA6* was regulated in endoderm and pancreas differentiation as well as in disease pathogenesis. We noticed that many SNPs located in the *GATA6/GATA6-AS1* locus were highly associated with the clinical traits, including pancreatic agenesis, congenital heart disease, or acute myocardial infarction (Allen et al., 2011; Sun et al., 2019). The variants within coding region and changing the



protein sequence very likely act by affecting the function of the GATA6 protein (Allen et al., 2011), but how those SNPs located in noncoding regions contribute to disease are poorly studied. Very recently, Gadue's group reported that a noncoding SNP rs12953985 located at the downstream target of the GATA6 locus contributed to the regulation of GATA6 and thus to pancreas differentiation and pancreatic agenesis (Kishore et al., 2020). Here, we reported that *GATA6-AS1* positively regulated GATA6 transcription; moreover, *GATA6-AS1* knockdown cells failed to differentiate into endodermal pancreatic lineage (Figure S3F). The fact that several noncoding SNPs locate in the gene body of *GATA6-AS1* and our functional dissection of *GATA6-AS1* indicate a possibility that *GATA6-AS1* might be a target for these diseases, and further studies on the relationship of these noncoding variants and *GATA6-AS1* in different contexts would facilitate our understanding on the role of noncoding variants in disease.

In summary, we dissected the biological function of *GATA6-AS1* in human endoderm differentiation. Moreover, we found that *GATA6-AS1* regulated GATA6 expression by interacting with SMAD2/3 and mediating the transcriptional activation of *GATA6*. Our results not only uncovered a new lncRNA contributing to early lineage specification, but also provided evidence to support the regulation ship between GATA6 and the divergent transcript *GATA6-AS1*.

EXPERIMENTAL PROCEDURES

Cell Culture and Differentiation

Two different human ESC lines, HUES8 and H9, were used in this study. They were both cultured with mTeSR1 (STEMCELL Technologies, cat. no. AB217641) on Matrigel-coated plates. Human embryonic kidney 293T cells were cultured with DMEM containing 10% FBS and 1% penicillin-streptomycin. The protocol to differentiate human ESCs into definitive endoderm was based on a previous report (Jiang et al., 2013a) with minor modifications. Generally, DMEM-F12 (Gibco, cat. no. C11330500BT) or RPMI 1640 (Gibco, cat. no. C22400500BT) was used as basal medium, supplemented with 0.2% BSA (YEASEN, cat. no. B57370), 1% penicillin-streptomycin (Gibco, cat. no. 15140163). Activin A (100 ng/mL, PeproTech, cat. no. 120-14P) and 2.5 μ M CHIR99021 (Selleck, cat. no. S2924) were added at day 1, followed by 100 ng/mL Activin A alone for the next 2 or 4 days. For following pancreatic lineage differentiation, the differentiated definitive endoderm cells were cultured in MCDB131 (Gibco, cat. no. 10372019) supplemented with 1.5 g/L sodium bicarbonate (Gibco, cat. no. 25080094), 10 mM glucose (Invitrogen, cat. no. A2494001), 0.5% BSA, 0.25 mM ascorbic acid (Sigma, cat. no. A5960), 1 \times ITS-X (BasalMedia, cat. no. S452J7), 1 \times GlutaMAX (Gibco, cat. no. 35050061), 1% penicillin-streptomycin, 50 ng/mL KGF (PeproTech, cat. no. 100-19), and 2 μ M XAV-939 (Selleck, cat. no. S1180) for 2 days. Next, 0.5 μ M SANT1 (Selleck, cat. no.

S7092), 100 nM TTNPB (Selleck, cat. no. S4627), and 500 nM PDBU (Sigma, cat. no. P1269) were additionally added to the above medium and cultured for 5 days.

Construction of Knockdown

We utilized shRNA for knockdown. A lentiviral vector system, containing the murine U6-shRNA cassette and a puromycin selection marker, was used in this study. After infection, we used 2 μ g/mL puromycin for selection to establish stable knockdown human ESC lines. We constructed two efficient shRNA vectors against *GATA6-AS1*. The sequences of oligoes were listed as below:

shRNA no. 1: GAAACCGTCTCATCCAAT
shRNA no. 2: GAAAGGATTCTCCGACA
shControl: GAAGTATCCGCGTACGTT.

RNA-Binding Protein Immunoprecipitation and ChIP

RNA-binding protein immunoprecipitation (RIP) assay was performed using Magna RIP Kit (Millipore) according to the manufacturer's instruction. For each sample, cell lysates from 2×10^7 cells were prepared and 5 μ g control IgG (R&D, cat. no. AB-108-C) or antibody against SMAD2/3 (R&D, cat. no. AF3797) was used.

ChIP was performed using the protocol reported previously (Chia et al., 2019). For each sample, cell lysates from 1×10^7 definitive endoderm cells were prepared. For each IP, 10 μ g SMAD2/3 (R&D, cat. no. AF3797) or IgG was used. Primers used to detect chromatin enrichment are listed in Table S5.

RNA-Seq and Data Analysis

We performed RNA-seq for undifferentiated ESCs, differentiated definitive endoderm samples from *GATA6-AS1* knockdown and wild-type genotypes on Illumina HiSeq X Ten PE150. To identify differentially expressed genes among ESCs, definitive endoderm, and *GATA6-AS1* knockdown cells, we used Bioconductor package DESeq2 to determine gene expression differences (Love et al., 2014). GSEA was performed on GSEA software to identify specifically enriched signal pathways (Mootha et al., 2003; Subramanian et al., 2005).

To identify GATA6 target genes, we downloaded the following datasets: RNA-seq data of GATA6-KO (E-MTAB-5958 [Chia et al., 2019]), ChIP-seq data of GATA6 (GSE77360 [Fisher et al., 2017], GSE109524 [Li et al., 2019], and GSE61475 [Tsankov et al., 2015]). The genes with both GATA6 binding and changed expression upon GATA6 knockout were identified as GATA6 target genes, which were used in Figure 3F.

Statistical Methods

All experiments were performed as at least three independent experiments. Data were analyzed in GraphPad Prism 8. Data are shown as means \pm SD. Comparisons were conducted via Student's t test (two-tailed, equal variance) and p values are shown with * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Data and Code Availability

The Gene Expression Omnibus accession numbers for the RNA-seq raw data reported in this work are GSE137208 (undifferentiated HUES8) and GSE143499 (endoderm samples).



SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at <https://doi.org/10.1016/j.stemcr.2020.07.014>.

AUTHOR CONTRIBUTIONS

W.J. conceived the project, and designed the experiment together with J.Y. and P.L. J.Y. performed most of the bench experiments with help from P.L. and M.L. P.L. performed the initial screening. J.Y. analyzed the next-generation sequencing data with help from C.Y. and T.Z. J.Y. drafted the manuscript, and W.J. and J.Y. finalized the manuscript. All authors contributed to and approved the final manuscript.

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