A G-quadruplex structure at the 5′ end of the H19 coding region regulates H19 transcription

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The H19 gene, one of the best known imprinted genes, encodes a long non-coding RNA that regulates cell proliferation and differentiation. H19 RNA is widely expressed in embryonic tissues, but its expression is restricted in only a few tissues after birth. However, regulation of H19 gene expression remains poorly understood outside the context of genomic imprinting. Here we identified evolutionarily conserved guanine (G)-rich repeated motifs at the 5′ end of the H19 coding region that are consistent with theoretically deduced G-quadruplex sequences. Circular dichroism spectroscopy and electrophoretic mobility shift assays with G-quadruplex-specific ligands revealed that the G-rich motif, located immediately downstream of the transcription start site (TSS), forms a G-quadruplex structure in vitro. By using a series of mutant forms of H19 harboring deletion or G-to-A substitutions, we found that the H19-G-quadruplex regulates H19 gene expression. We further showed that transcription factors Sp1 and E2F1 were associated with the H19-G-quadruplex to either suppress or promote the H19 transcription, respectively. Moreover, H19 expression during differentiation of mouse embryonic stem cells appears to be regulated by a genomic H19-G-quadruplex. These results demonstrate that the G-quadruplex structure immediately downstream of the TSS functions as a novel regulatory element for H19 gene expression.

The H19 gene encodes a ~2.5 kb transcript that is capped, spliced, and polyadenylated. H19 RNA lacks an evolutionarily conserved open reading frame but has a conserved secondary RNA structure indicating it has a functional role as a non-coding RNA1,2. It has been established that the first exon of H19 encodes microRNAs: miR-675-3p and miR-675-5p3. H19 RNA is widely expressed in embryonic and extra-embryonic tissues. Shortly after birth, however, H19 RNA expression is drastically repressed in the majority of tissues and is sustained only in specific organs, including mammary gland, uterus, cardiac and skeletal muscles4–8. Mice lacking H19 are viable and fertile with an overgrowth phenotype at birth9,10. Recent studies have revealed the physiological functions of H19 and miR-675, such as adult hematopoietic stem cell quiescence11, skeletal muscle cell differentiation12,13, and limitation of placental growth14.

Upon tumorigenesis, the expression of H19 becomes dysregulated. H19 is highly expressed in various cancers, such as breast6,15, lung16, bladder17,18 and colon cancers19,20. In addition, H19 enhances tumor cell proliferation, colony formation and tumor metastasis21–25, indicating a proto-oncogenic function of H19 and/or miR-675. Therefore, the regulation of H19 gene expression appears to be critical for cell fate decisions and tissue homeostasis. The H19 gene is under the control of genomic imprinting, whereby H19 is expressed only from the maternal allele. It has been established that monoallelic expression of H19 genes is regulated by a differentially methylated region (DMR) located between −2 kb and −4 kb upstream of the H19 gene locus26–28. Outside of the context of genomic imprinting, however, little is known about how H19 gene expression is regulated, and there is limited information on the genomic structure of H19 gene.

The G-quadruplex is a non-canonical B-DNA structure, consisting of stacked square planar arrays of a guanine (G)-tetrad complexed with a monovalent cation such as Na+ and K+ (reviewed in refs 29 and 30). G-quadruplex structures are found in telomeres, promoters and regions proximal to transcription start sites (TSS) of genes31–34. Accumulating evidence has revealed a pivotal role of the DNA G-quadruplex in telomere

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maintenance, replication, chromosome fragility, and transcriptional regulation of cancer-related genes, including c-Myc, K-Ras, and YY1. In this report, we identify preserved G-rich motifs located immediately downstream of the H19 TSS that forms a G-quadruplex structure. This G-quadruplex structure regulates H19 transcription through binding to transcription factors Sp1 and E2F1. This study demonstrates for the first time the regulation of H19 gene expression via G-quadruplex structure within its coding region.

Methods

Circular dichroism spectroscopy. DNA oligonucleotides (Japan Bio Services Co.) were dissolved in buffer (50 mM Tris-HCl 20 mM KCl) at concentration of 10 μM. Before analysis, the oligonucleotides were heated at 95 °C for 5 min, then slowly cooled down to room temperature, and incubate overnight. Circular dichroism (CD) spectra were recorded on a J-720 spectropolarimeter (JASCO, Tokyo, Japan) using a quartz cell of 1 mm optical path length and an instrument scanning speed of 500 nm/min with a response time of 1 s, and over a wavelength range of 220–320 nm. Finally CD spectra are representative of five averaged scans taken at 25 °C, then a stepwise increase of 10 °C from 25 °C to 95 °C.

Circular dichroism melting assay. A solution of the oligonucleotides was prepared in 50 mM Tris-HCl with 20 mM KCl at concentration of 10 μM. The solution was annealed at 95 °C for 5 min, then slowly cooled down to room temperature, and incubate overnight. Melting curves were obtained by monitoring the CD intensity at 260 nm on a J-720 spectropolarimeter (JASCO, Tokyo, Japan) by using a quartz cell of 1 mm optical path length; the temperature was changed as follows, 25 °C to 95 °C at 1.0 °C/min.

Electrophoretic mobility shift assays (EMSA). DNA samples were heated at 95 °C for 5 min in 10 mM Tris-HCl, pH 8.0, with or without 100 mM KCl, and slowly cooled to room temperature. Samples were electrophoresed on a 12% native polyacrylamide gel in 0.5 × TBE buffer at 100 V (constant voltage). The gels were stained using 2D-Silver stain-β (Cosmo Bio). For EMSA using a fluorescent ligand (L1BOD-7OTD)44, DNA samples were incubated with 100 μM L1BOD-7OTD in 10 mM Tris-HCl, pH 8.0. The fluorescent signals were detected using a Typhoon 9410 imager (Amersham). C-myc oligonucleotide (GAG GGG CGC TTA TGG GGA GGG TGG GGA GGA GAC) and mutant C-myc oligonucleotide (GAG GGG CCG TTA TGC TTA CGC TCT TGA ATC TCA TGA AGG TGG GGA GGA GAC) were used as a positive and negative control, respectively45.

Cell culture and transfections. HEK293T cells, HeLa cells, U2OS cells and EpH4 cells (a gift from Dr. Ernst Reichmann, University of Zurich) were cultured in DMEM (Nissui) containing 10% fetal bovine serum (Nichirei Bio Science), 4% L-glutamine (Nacalai), 0.15% sodium bicarbonate and antibiotics. Mouse embryonic stem cells (mESCs) (OLV2-1 cell46, Riken BRC, AES0153) were cultured in G-MEM containing 15% fetal bovine serum, 0.1 mM non-essential amino acids, 1 mM sodium pyruvate, 0.1 mM 2-mercaptoethanol (all GIBCO) and leukemia inhibitory factor (LIF, Wako) on a 0.1% (w/v) gelatin-coated dish. For neural differentiation of mESCs,Cells were transfected using calcium phosphate, Lipofectamine LTX (Invitrogen) in Low Ca²⁺ DMEM, or PEI max (Polyscience), respectively. mESCs were transfected by using Lipofectamine2000 (Invitrogen).

Plasmid construction. Genomic regions of the H19 locus (−49 to +2287 and +56 to +2287 relative to the mouse H19 TSS) were amplified by PCR and cloned into pEF1α/pENTR (Addgene 17427)48. Mutagenesis within the H19 G-quadruplex sequence was performed by PCR using the mutant oligonucleotides (see Fig. 1B). Flag-conjugated full-length mouse Sp1, NCL and E2F1 were cloned into pcDNA3.

siRNA experiments. The siRNA sequences were as follows: Sp1-1, 5′-GGC UGG UGG UGG UGG AAU ATT-3′; Sp1-2, 5′-UGG AGU GAT GUA GCC UAA UAU Utt-3′; Luciferase, 5′-CUU ACG CUG AGU UCG Att-3′. HeLa cells were transfected with siRNA by using Oligofectamine (Invitrogen).

Dual luciferase assays. The promoter region of H19 (−840 to +14 and −840 to +84) was amplified by PCR and cloned into pGL4.10 (Promega). HEK293T and EpH4 cells were transfected with the plasmids together with pRL [Renilla luciferase] plasmid (Promega). Forty-eight hours after transfection, luciferase assays were performed using the Dual-Luciferase® Reporter Assay System (Promega) and an ARVO X-3 plate reader (PerkinElmer). Firefly luciferase activity was normalized against control Renilla luciferase activity following the manufacturer’s instructions.

PCR, and quantitative RT-PCR (qRT-PCR). Total DNA was isolated using phenol:chloroform and ethanol and then subjected to PCR (Taq DNA polymerase, TOYOBO). The transfection efficiency was analyzed by PCR using a following primer pair setting on vector backbone (pEF1α/pENTR): 5′-CGG TTG CAT TGG ATT CCT GT-3′ and 5′-TTC CGA CTC GTC CAA CAT CA-3′. Total cellular RNA was extracted using an RNase mini kit (Qiagen). cDNAs were prepared using 0.4–1 μg of RNA and M-MLV reverse transcriptase (Invitrogen).
Chromatin Immunoprecipitation (ChIP). HeLa cells were cross-linked with 1% formaldehyde at room temperature for 10 min. Glycine was added at a final concentration of 0.125 M, followed by incubation for 10 min at room temperature. The cells were washed with PBS, then lysed with SDS lysis buffer (50 mM Tris, 167 mM NaCl, 1.1% Triton X-100, 0.11% sodium deoxycholate, 1 mM PMSF, 10 μg/mL aprotinin, and 1 mM DTT). Samples were sonicated, centrifuged at 15,000 rpm at 4 °C for 10 min and recover supernatant. Rabbit anti-Sp1, rabbit anti-NCL, rabbit anti-E2F1 or rabbit IgG (invitrogen) and Dynabeads M-280 Sheep anti-Rabbit IgG (life technologies) were preincubated with 1% BSA and 200 μg/ml of the oligonucleotides for 1 hour at 4 °C, followed by the addition of 20 μl streptavidin sepharose beads (GE Healthcare) pre-blocked with 1% BSA and 10 μg/ml Aprotinin, and 1 mM DTT) and diluted with ChIP dilution buffer (50 mM Tris, 167 mM NaCl, 1.1% Triton X-100, 0.11% sodium deoxycholate, 1 mM PMSF, 10 μg/mL aprotinin, and 1 mM DTT). Pull-down assays and western blotting. The single and double stranded biotin-labeled H19 G-quadruplex-WT-oligonucleotides (5′-biotin-ACC GGG GGT GGG GAG GAT CC-3′) or the single and double stranded biotin-labeled H19 G-quadruplex-mutant-oligonucleotides (5′-biotin-ACC GAG TGG GCC CTA GCC AGC ATC GCC ATC CC-3′) (20 μM) were heated at 95 °C for 5 min in 10 mM Tris–HCl, pH 8.0 with 100 mM KCl, and cooled to 4 °C. HeLa cells and mESCs were washed with PBS, then resuspended in binding buffer (50 mM Hepes, pH 7.3, 2 mM EGTA, 2 mM MgCl2, 1 mM EDTA, 15 mM NaF, 10 mM β-glycerophosphate, 10% glycerol, 100 mM KCl, 1 mM DTT, 10 μg/ml Aprotinin, 1 mM PMSF, and 1% NP-40). After incubation for 15 min on ice, the cell suspensions were sonicated (10 sec at 1.2 output, 4 cycles), and the insoluble fractions were removed by centrifugation. The obtained cell extracts were incubated with 10 μl of the oligonucleotides for 1 hour at 4 °C, followed by the addition of 20 μl streptavidin sepharose beads (GE Healthcare) pre-blocked with 1% BSA and 200 μg/ml salmon sperm DNA, and incubated for another 30 min at 4 °C. The beads were washed three times with binding buffer without NP-40 and once with PBS, and resuspended in 100 μl SDS sample buffer and boiled. Aliquots of 20 μl were subjected to western blotting using the following primary antibodies: rabbit anti-Sp1 (21962-1-AP, Protein tech), rabbit anti-NCL (H-250, Santa Cruz), rabbit anti-E2F1 (sc-193, Santa Cruz), and mouse anti-α-tubulin (T6557, Sigma). Primary antibodies were detected with HRP-conjugated secondary antibodies (GE Healthcare) using ECL-Plus reagents (PerkinElmer). Figure 1. (A) Evolutionarily conserved G-rich sequences immediately downstream of the H19 TSS in mammals. The guanine tandem repeats are underlined. (B) Oligonucleotides used in this study. Guanine-to-adenine substitutions are indicated in red.
Construction of H19 G-quadruplex disrupted mESC lines. Genomic mutagenesis of the H19 G-quadruplex sequence was performed using the Crispr/Cas9 system. Briefly, mESCs were co-transfected with a plasmid encoding Cas9 and a guide RNA (Addgene 44248) targeting the first exon of H19 (5’-GAG GAG AGT CGT GGG GTG CGG-3’) and pEF1α/pENTR containing Mut2-H19 (−840 to +2287) (see Fig. 1B) using Lipofectamine 2000 (Invitrogen). The transfected cells were selected by incubation with puromycin and clonally expanded. Bi-allelic mutations were confirmed by genomic sequencing. Two lines of mESCs harboring genomic Mut2 mutation were established and used for the analysis (Mut2-G4-a cell and Mut2-G4-b cell).

Statistical analysis. All data are representative of at least three independent experiments. P values were calculated by applying Dunnett’s multiple-comparison test or two-tailed t-test. Data are presented as the mean ± standard error of the mean.

Results
The G-rich motifs located immediately downstream of the H19TSS form a G-quadruplex structure. H19 harbors G-rich sequences immediately downstream of the TSS, which are conserved in mammalian species (Fig. 1A). The G-rich sequence in the region between +14 and +39 of mouse H19 displays a G-score of 84, calculated by QGRS Mapper (Max length: 30, Min G-group: 2, Loop size: 0 to 36), a software that provides information on the composition of putative G-quadruplex forming G-rich sequences50, suggesting that this region forms a G-quadruplex structure. To assess this possibility, we prepared single-stranded oligonucleotides corresponding to the regions between +1 to +78, +12 to +42, +42 to +74 and +1 to +27 of mouse H19, and analyzed them by circular dichroism (CD) spectroscopy. The CD spectrum of the oligonucleotide for the region between +1 to +78 was characteristic for parallel G-quadruplex structures51 in the presence of 20 mM KCl [Fig. 2A, WT-1 to 78] or within the region between +12 to +42 [Fig. 1B, WT-1 to 78], resulted in disruption of the CD spectrum patterns [Fig. 2A, WT-1 to 78 and Mut2-1 to 78]. The CD spectra at 25 °C (Fig. 2B) and at their respective Tm values (Fig. 2C) support the notion that WT-1 to 78 and Mut3-1 to 78, but not Mut1-1 to 78 or Mut2-1 to 78, form G-quadruplex structures. In addition, the oligonucleotide for the region between +12 to +42 [Figs 1B and 2D, WT-12 to 42] exhibited a characteristic spectrum for parallel G-quadruplex structures. In contrast, the oligonucleotide for the region between +42 to +74, as well as the mutant oligonucleotides with G-to-A substitutions in this region, barely formed G-quadruplex structures [Figs 1B and 2D, WT-42 to 74]. These results suggest that the region between +12 to +27 within the H19 gene forms a DNA G-quadruplex structure. The region between +1 to +27 is capable of forming a G-quadruplex structure in the short oligonucleotides, but the region between +1 to +12 is dispensable for G-quadruplex structure formation in the longer oligonucleotides.

We next performed an EMSA to investigate the conformational change of the H19 G-rich sequence resulting from the addition of physiologically relevant levels of KCl (100 mM). In the absence of 100 mM KCl, WT-, Mut1-, Mut2-, Mut3-, Mut4-, and Mut5- (+1 to +78) oligonucleotides exhibited a single band at the corresponding single-stranded (ss) DNA size (Fig. 3A, KCl−). In the presence of 100 mM KCl, however, WT-, Mut3-, Mut4- and Mut5- (+1 to +78) oligonucleotides, but not Mut1- and Mut2- (+1 to +78) oligonucleotides, exhibited additional slow-migrating bands corresponding to inter- and intra-molecular G-quadruplex structures (Fig. 3A, KCl+). In the presence of 100 mM KCl, only WT-1 to 78, Mut3-1 to 78, and Mut5-1 to 78 exhibited additional bands at the corresponding positions (Fig. 3A, KCl+). The additional bands were confirmed by silver staining (Fig. 3B). The fluorescence signals in the slow-migrating bands (Fig. 3B, fluorescence) were detected with L1BOD-7OTD and Fluorolight-7OTD, which specifically interacts with and stabilizes G-quadruplex structures44. In the presence of L1BOD-7OTD, WT-, Mut3-, Mut4-, and Mut5- (+1 to +78) oligonucleotides, but not Mut1- and Mut2- (+1 to +78) oligonucleotides, again exhibited the additional slow-migrating bands even in the absence of KCl (Fig. 3B, silver staining). We also detected fluorescence signals in the slow-migrating bands (Fig. 3B, fluorescence). Consistent with the results of CD assay, the short form of oligonucleotides WT- (+12 to +42) exhibited slow-migrating bands with fluorescence signals (Supplementary Figure 1), confirming the ability of this sequences to form G-quadruplex structures. WT- (+1 to +27) also partially exhibited slow-migrating bands with fluorescence signals (Supplementary Figure 1), although this region is dispensable for G-quadruplex structure formation in longer stretch of DNA sequences. Notably, Mut6- (+12 to +42), the short form of mutant oligonucleotides with G-to-A substitutions in the central G-quadruplex sequence, exhibited the partially disrupted CD spectra but still forms G-quadruplex [Figs 1B and 2D, Mut6- (+12 to +42)]. However, the EMSA analysis with L1BOD-7OTD showed that Mut6- (+12 to +42) formed an intermolecular, rather intramolecular, G-quadruplex structure (Supplementary Figure 1). These results, taken together, demonstrate that the G-rich sequence within the region between +12 to +42 of H19 forms a G-quadruplex structure in vitro.

The H19 G-quadruplex regulates H19 gene transcription. To determine whether the G-quadruplex structure at the H19 TSS regulates H19 gene transcription, we constructed a series of plasmids encoding mouse WT-H19, Mut2-G4-H19, Mut3-G4-H19, or ΔG4-H19, where the +1 to +56 region of H19 was deleted, under the control of the EF1α promoter (Fig. 4A). 293T cells were transfected with each plasmid and the expression levels of H19 RNA were analyzed by qRT-PCR. PCR analysis confirmed the same degree of transfection efficiency of the plasmids (Fig. 4C). The results showed that the expression levels of H19 RNA were significantly higher in the cells transfected with ΔG4-H19 and Mut2-G4-H19 compared with the WT-H19-transfected cells (Fig. 4B) at the various concentrations of the plasmids (Supplementary Figure 2). Mut3-G4-H19 exhibited the similar level of H19 RNA as WT-H19 (Fig. 4B), suggesting that the G-rich sequences within the region between +43 to +
78 is dispensable for regulating H19 gene transcription. Next, we constructed luciferase assay vectors, in which the H19 promoter element together with G-quadruplex sequence (−840 to +84) (H19 pro-G4-Luc) or the H19 promoter element alone (−840 to +14) (H19 pro-Luc) was fused to the luciferase-coding sequence (Fig. 4D). We transfected these vectors into 293T or EpH4 cells, and found that luciferase activity was much higher in the cells transfected with H19 pro-Luc compared with that of H19 pro-G4-Luc or the control plasmid-transfected cells.
These results indicate that the \( H19 \) G-quadruplex sequence in the regions between +1 to +42 has a function to suppress \( H19 \) gene transcription.

Identification of proteins associated with the \( H19 \) G-quadruplex. To gain insight into the molecular mechanisms, we next determined the \( H19 \) G-quadruplex-associate proteins. To this end, we performed a pull-down assay using the biotinylated WT- and Mut2-oligonucleotides. As the region between +43 to +78 was dispensable for suppressing \( H19 \) gene transcription (see Fig. 4B), we used the biotinylated WT- and Mut2-oligonucleotides for the region between +1 to +43 of \( H19 \) for a pull-down assay (Fig. 5A, bio-WT and bio-Mut2, respectively). We confirmed that both single- and double-stranded bio-WT, but not bio-Mut2, exhibit mobility-shift bands in EMSA in the presence of 100 mM KCl (Fig. 5B) or L1BOD-7OTD (Fig. 5C). We incubated the single- or double-stranded biotinylated oligonucleotides with the cell lysates in the presence of 100 mM KCl. By using whole cell lysates from HeLa cells and mouse embryonic stem cells (mESCs), we examined the...
association of the oligonucleotides with proteins that have been reported to interact with G-quadruplex, including Sp152–54, Nucleolin (NCL)52,53,55, and Poly(ADP-ribose) polymerase-1 (PARP1)56–58. We found that Sp1 and NCL bound to bio-WT, but not bio-Mut2 (Fig. 5D). On the other hand, PARP1 bound to both bio-WT and bio-Mut2 double-stranded oligonucleotides, but not single-stranded oligonucleotides (Fig. 5D), indicating that PARP1 is associated with double-stranded oligonucleotides in a DNA sequence-independent manner. ChIP-qPCR analysis showed binding of endogenous Sp1, but not NCL, to the genomic region of $H19$ G-quadruplex (Fig. 5E and F). These observations indicate that Sp1 is associated with the $H19$ G-quadruplex both in vitro and in vivo. Notably, Sp1 bound to bio-WT more efficiently in the lysates prepared from the G1/S phase-synchronized cells than that from M phase-synchronized cells (Supplementary Figure 3B), suggesting the cell cycle-dependent association of Sp1 with the $H19$ G-quadruplex. We found that the ectopic expression of Sp1 in HeLa cells resulted in the suppression of transcription. (A) Schematic diagram of the $H19$ expression vectors used in this study. The full length $H19$ gene (WT-$H19$), G-quadruplex sequence-truncated $H19$ gene (ΔG4-$H19$), or $H19$ gene with Mut2 or Mut3 G-quadruplex sequence (Mut2-G4-$H19$, Mut3-G4-$H19$) were driven by the EF1α promoter. (B) qPCR analysis of $H19$ RNA levels in 293T cells transfected with the indicated plasmids. Values are normalized against G3PDH. (C) PCR analysis for transfection efficiency of the plasmids in (B) using a primer pair specific to the vector backbone. (D) Schematic diagram of the luciferase reporter plasmids. $H19$ G-quadruplex sequence was inserted just upstream of the luciferase gene (Luc). Luc or G-quadruplex-Luc (G4-Luc) was driven by the $H19$ promoter (the region between $-840$ to $+14$). (E,F) 293T cells (E) or EpH4 cells (F) were transfected with the luciferase reporter plasmids together with the control RL-SV40 plasmid, and the luciferase activities measured. Values are normalized against the activity of co-transfected Renilla luciferase. (B,E,F) mean ± s.d. from three experiments; *P < 0.05, ***P < 0.005 analyzed by Dunnett’s multiple-comparison test.
Figure 5. Identification of H19 G-quadruplex associated factors. (A) Biotin-labeled oligonucleotide sequences used in the pull-down assays. (B) Single-stranded (ss) or double-stranded (ds) biotin-labeled oligonucleotides in (A) were prepared by heating at 95 °C for 5 min, followed by cooling to 4 °C. The oligonucleotides were incubated with or without 100 mM KCl, electrophoresed, and subjected to silver staining. (C) Each oligonucleotide prepared as in (B) was incubated with or without L1BOD-7OTD, and electrophoresed. Silver stained images and fluorescent signals are shown. (D) Western blot analysis of Sp1, NCL, PARP1 and E2F1 for the pull-down assay of each oligonucleotide. The input is 10% of the total fraction used for each sample. Uncropped images are in Supplementary Figure 6A. (E,F) ChIP-qPCR analysis against Sp1 (E), NCL (F) or control IgG in HeLa cells using primer pairs for amplifying the region between +113 and +282. The obtained qPCR values are normalized by input DNA. Fold enrichment relative to IgG is shown. (G) qPCR analysis for endogenous H19 RNA levels in HeLa cells transiently expressing Sp1, NCL and E2F1. Values are normalized against G3PDH. (H) qPCR analysis for endogenous H19 RNA levels in HeLa cells depleted with Sp1 by siRNA. Western blot analysis of Sp1 and control tubulin are shown in the bottom. Uncropped images are in Supplementary Figure 6B. (I) ChIP-qPCR analysis against E2F1 or control IgG in HeLa cells ectopically expressing E2F1. The obtained qPCR values are normalized by input DNA. Fold enrichment relative to IgG is shown. (E–I) mean ± s.d. from three (E–H) or two (I) independent experiments; **P < 0.01, ***P < 0.005 analyzed by two-tailed t-test (E,F) or Dunnett’s multiple-comparison test (G,H).
decrease of endogenous H19 RNA level (Fig. 5G). Conversely, knockdown of Sp1 by siRNA resulted in upregulation of the H19 RNA level (Fig. 5H), indicating that Sp1 suppresses H19 gene transcription. We further found that E2F1, which is reported to regulate H19 gene transcription23, also bound to single- and double-stranded bio-WT, but not bio-Mut2 in a pull-down assay (Fig. 5D). Importantly, the ectopically expressing E2F1 in HeLa cells binds to the genomic region of H19 G-quadruplex (Fig. 5I) and increased the endogenous H19 RNA level (Fig. 5G). The ectopic expression of NCL had no effect on the H19 RNA level (Fig. 5G). These results taken together indicate that, through binding to the H19 G-quadruplex, Sp1 and E2F1 regulate H19 transcription in an opposite way; Sp1 suppresses whereas E2F1 promotes H19 gene transcription.

Genomic H19 G-quadruplex regulates H19 transcription during mESC differentiation. It has been reported that the expression level of H19 RNA increases during differentiation of mESCs8,59–61. Consistently, we observed that levels of H19 RNA increased during neural differentiation of mESCs by SFEBq at day 0, day 3, day 5 and day 7. (B) mESCs were differentiated in SFEBq medium in the presence or absence of L1H1-7OTD (5 μM). The qPCR analysis of H19, Oct4 and Sox1 RNA levels at differentiation Day 0 or Day 5 are shown. Values are normalized against β-actin. mean ± s.d. from three experiments; *P < 0.05, ***P < 0.005 analyzed by Dunnett’s multiple-comparison test.

Figure 6. L1H1-7OTD suppresses H19 transcription during mESC differentiation. (A) qRT-PCR analysis of RNA levels of H19, Oct4 and Sox1 during neural differentiation of mESCs by SFEBq at day 0, day 3, day 5 and day 7. (B) mESCs were differentiated in SFEBq medium in the presence or absence of L1H1-7OTD (5 μM). mESCs were differentiated in SFEBq medium in the presence or absence of L1H1-7OTD (5 μM).
Figure 7. The genomic H19 G-quadruplex sequence regulates H19 expression. (A, B) Control mESCs (WT-G4 cell) or Mut2-G4-mESCs (Mut-G4-a cell) were cultured in mESC maintenance medium (undifferentiated) or differentiated in EB medium for 6 days (EB Day6) in the presence or absence of L1H1-7OTD (5 μM). The qPCR analyses of H19 RNA levels are shown. (C) qRT-PCR analysis of H19 RNA levels in WT- and Mut-G4-a, or -b cells in EB culture on day 6. (D–F) qRT-PCR analysis for the RNA levels of H19 (D), E2F1 (E) and Sp1 (F) in WT- and Mut2-G4 cells in mECS maintenance medium (undifferentiated) or in SFEBq culture on day 5. (G, H) qRT-PCR analysis for H19 RNA levels in WT- (G) and Mut2-G4 cells (H) transfected with or without the E2F1-coding plasmids. (A–H) Values are normalized against β-actin. mean ± s.d. from three experiments; *P < 0.05, **P < 0.01, ***P < 0.005 analyzed by Dunnett’s multiple-comparison test (A–F) and two-tailed t-test (G, H).

(Fig. 7C) and in SFEBq culture on Day5 (Fig. 7D), suggesting an H19 transcription-promoting function of the H19 G-quadruplex sequence. Consistent with this notion, the level of E2F1, which promotes H19 gene transcription (see Fig. 5G and H), was decreased during neural differentiation of both WT- and Mut2-G4 cells. Furthermore, ectopic expression of E2F1 significantly increased the endogenous H19 RNA level in WT-G4 cells, but not in Mut2-G4 cells in the
mESC maintenance medium. These results demonstrate that the genomic H19 G-quadruplex structure immediately downstream of TSS regulates H19 transcription during mESC differentiation in a dual opposite way.

Discussion

The H19 gene is located 200 kb downstream of the Insulin-like growth factor 2 (Igf2) gene on chromosome 7 in mice and 11p15.5 in humans. The H19-Igf2 locus is under the control of genomic imprinting, whereby H19 is expressed from the maternal allele and Igf2 is expressed from the paternal allele. The mechanism of the monoallelic expression of H19 by the epigenetic modification within DMR is well established and the methylation pattern within DMR is generally maintained indefinitely. However, the mechanism that explains the differential expression of H19 among cell types or tissues, which would be relevant to cell differentiation condition, remains unclear.

In this report, we show that H19 gene transcription is regulated by a G-quadruplex which is located at the region immediately downstream of H19 TSS. H19 expression is increased during mESC differentiation, which is attenuated by the G-quadruplex stabilizing compound Li1H1-7OTD in WT-G4 mESCs but not in Mut2-G4 mESCs, indicating the functional H19 G-quadruplex-mediated H19 transcription regulation. It has been reported that the monoallelic expression of H19 is maintained during ESC differentiation. Therefore, the H19 G-quadruplex-mediated H19 transcription regulation during mESC differentiation seems independent of genomic imprinting. In Mut2-G4 cells, H19 expression level is lower than that in WT-G4 cells, suggesting the promoting role of G-quadruplex in H19 transcription. Consistently, our results show that E2F1 binds to the H19 G-quadruplex, and promotes H19 transcription. Notably, however, the H19 expression level was partially upregulated in the Mut2-G4 cells during differentiation (see Fig. 7B and D). Therefore, in addition to the G-quadruplex-mediated mechanism, H19 gene transcription would also require H19 promoter activation during mESC differentiation. On the other hand, our results show that Sp1 also binds to the H19 G-quadruplex, and suppresses H19 transcription. It is worth to note that the expression levels of E2F1 and Sp1 are increased and decreased, respectively, during mESC differentiation (see Fig. 7E and F). Therefore, the balance of E2F1/Sp1 expression levels would determine the function of the H19 G-quadruplex on H19 gene transcription regulation.

How E2F1/Sp1 regulates H19 gene transcription through the H19 G-quadruplex remains open question. Sp1 is known to recruit a large number of proteins including transcription initiation complex and transcription repressor complex. Our data show that Sp1 acts as H19 transcription repressor in conjunction with H19 G-quadruplex. Although we could not determine whether Sp1 recognizes the G-quadruplex structure or Sp1-target sequence within the H19 G-rich motif, it would be possible that Sp1 recruits transcription repressor complexes to the H19 G-quadruplex to suppress H19 transcription. It has previously shown that E2F1 binds to H19 promoter region. We show that E2F1 is associated with the H19 G-quadruplex downstream of H19 TSS. It would be interesting to determine which region or both plays a pivotal role in promoting H19 transcription.

This study describes a regulatory mechanism for H19 gene transcription via the G-quadruplex that has not been described before. Putative G-quadruplex sequences are distributed throughout the genomic regions of non-coding RNA; therefore, these G-quadruplexe structures would function as regulatory elements of transcription. H19 RNA is highly expressed in various cancers and plays a proto-oncogenic function in several tumors. Therefore, our findings implicate that G-quadruplex-mediated transcription regulation of H19 gene would be an effective target for anti-cancer agent.

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Author Contributions

M.F. and Y.M. performed the experiments. M.F., Y.M., K.N. and F.T. analyzed the data. M.F., K.N. and F.T. wrote the manuscript.

Additional Information

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