MEG8 long noncoding RNA contributes to epigenetic progression of the epithelial-mesenchymal transition of lung and pancreatic cancer cells

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Long noncoding RNAs (lncRNAs) are important regulatory molecules in various biological and pathological processes, including cancer development. We have previously shown that the MEG3 lncRNA plays an essential role in transforming growth factor-β (TGF-β)-induced epithelial-mesenchymal transition (EMT) of human lung cancer cells. In this study, we investigated the function of another lncRNA, MEG8, which shares the DLK1–DIO3 locus with MEG3, in the regulation of EMT. MEG8 lncRNA expression was immediately induced during TGF-β–mediated EMT of A549 and LC2/ad lung cancer and Panc1 pancreatic cancer cell lines. MEG8 overexpression specifically suppressed the expression of microRNA-34a and miRNA-203 genes, resulting in up-regulation of SNAIL family transcriptional repressor 1 (SNAI1) and SNAI2 transcription factors, which repressed expression of cadherin 1 (CDH1)/E-cadherin. Mechanistic investigations revealed that MEG8 associates with enhancer of zeste 2 polycomb repressive complex 2 subunit (EZH2) protein and induces its recruitment to the regulatory regions of the two microRNA genes for histone H3 methylation and transcriptional repression. Interestingly, expression of both MEG8 and MEG3, but not each individually, could induce EMT-related cell morphological changes and increased cell motility in the absence of TGF-β by activating the gene expression program required for EMT. MEG8 knockdown indicated that endogenous MEG8 lncRNA is indispensable for TGF-β–induced EMT in A549 lung cancer and Panc1 pancreatic cancer cells. Our findings indicate that MEG8 lncRNA significantly contributes to epigenetic EMT induction and increase our understanding of the lncRNA-mediated regulatory mechanisms involved in malignant progression of cancer.

Epithelial-mesenchymal transition (EMT) is a highly dynamic and reversible mechanism conductive to cancer malignancies such as invasion and metastasis. A major inducer of EMT is transforming growth factor-β (TGF-β), along with cytokines and growth factors secreted by the tumor microenvironment. EMT results in loss of cell–cell adhesion, abnormal apical-basal polarity, and cytoskeletal reorganization, thereby increasing motility, invasiveness, and stem cell–like properties of cancer cells. The switch in gene expression during EMT is characterized by repression of epithelial genes and induction of mesenchymal genes. One of the well-known hallmarks of EMT is the functional loss of E-cadherin, a defining marker of epithelial cells. The EMT program is initiated by EMT-inducing transcription factors (EMT-TFs) such as SNAI1, SNAI2, ZEB1, ZEB2, and TWIST1 through the down-regulation of E-cadherin. Besides, the phenotypic plasticity of EMT is thought to be determined by epigenetic regulation, including DNA methylation and histone modifications. This work was supported in part by Grants-in-aid for Scientific Research C 15K08263 (to T. S.) and 16K19030 (to M. T.) from the Ministry of Education, Culture, Sports, Science and Technology of Japan. The authors declare that they have no conflicts of interest with the contents of this article.

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1 The abbreviations used are: EMT, epithelial-mesenchymal transition; TGF-β, transforming growth factor-β; PRC2, polycomb repressive complex 2; H3K27me3, histone H3 trimethylated Lys-27; miR-200, microRNA-200; IncRNA, long noncoding RNA; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; shRNA, small hairpin RNA; RIP, RNA immunoprecipitation; ChIRP, chromatin isolation by RNA purification; SNAIL, SNAI1 family transcriptional repressor; CDH1, cadherin 1; EZH2, enhancer of zeste 2 polycomb repressive complex 2 subunit; EMT-TF, EMT-inducing transcription factor; KMT, lysine methyltransferase; KDM, lysine demethylases (KDMs) called “writers” and “erasers,” respectively. Indeed, aberrant regulation of these enzymes has been implicated in the developmental defects and the pathogenesis of cancer.

Retroviral insertional mutagenesis in mice has enabled us to identify hundreds of candidate cancer genes, including many...
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Results

Expression of MEG8 long noncoding RNA was immediately and transiently up-regulated in TGF-β–induced EMT of lung and pancreatic cancer cells

In the previous study, we found that the expressions of MEG8 and MEG9 IncRNAs were clearly up-regulated as with MEG3 during TGF-β–induced EMT in A549 lung cancer cells but were not detected in another cell line, LC-2/ad, under our usual quantitative RT-PCR (QRT-PCR) condition (19). We tried to examine the expression changes of these IncRNAs again in LC2/ad cells by using a highly sensitive QPCR enzyme mixture. This efficient QRT-PCR enabled us to detect the expression of MEG8 (Fig. 1B) but not MEG9 (data not shown) in LC-2/ad cells. We further examined the expression of MEG8 in some of the human cell lines showing EMT phenotype in response to TGF-β. QRT-PCR analysis showed that MEG8 expression was clearly detected in a pancreatic cancer cell line, Panc1 (Fig. 1C), but was not detected in a colon cancer cell line, HT29, and in an immortalized breast epithelial cell line, MCF10A (data not shown). Next we performed time course expression analysis of MEG8 during the TGF-β–mediated EMT process of A549, LC-2/ad, and Panc1 cells (Fig. 1, A, B, and C). The expression of MEG8 was immediately and transiently induced by TGF-β as with MEG3 (19), implying its possible role in EMT induction. Therefore, we decided to elucidate the function of MEG8 IncRNA as a candidate regulator in the EMT process of lung and pancreatic cancer cells.

Overexpression of MEG8 induced E-cadherin down-regulation by influencing a subset of EMT-related genes

We first examined whether overexpression of MEG8 would influence the EMT process in lung and pancreatic cancer cells. We observed the cell morphologies and the status of E-cadherin and actin in A549, LC-2/ad, and Panc1 cells overexpressing MEG8, whose expression was confirmed by QRT-PCR (Fig. S1). As reported previously, TGF-β treatment induced scattered, elongated, or enlarged cell morphology characteristic of EMT in A549, LC-2/ad, and Panc1 cells (Fig. 1, D, E, and F, upper panel) (15, 16). The induced changes were further confirmed by immunofluorescence of E-cadherin, an epithelial cell marker, and phalloidin staining of actin reorganization. Control cells revealed loss of E-cadherin staining and appearance of actin stress fibers in the presence of TGF-β (Fig. 1, D, E, and F, middle and lower panels). Cells expressing MEG8 looked slightly scattered compared with the control cells and showed reduction of E-cadherin staining but did not induce actin remodeling (Fig. 1, D, E, and F). The effect of TGF-β seemed to be slightly enhanced by MEG8 for EMT cellular phenotypes (Fig. 1, D, E, and F). Based on the E-cadherin down-regulation, we presumed that MEG8 overexpression by itself could be sufficient to partially proceed with the EMT program in lung and pancreatic cancer cells.

EMT is associated with a decrease of epithelial cell markers such as CDH1/E-cadherin and a gain of mesenchymal markers such as fibronectin and vimentin (1). Next we analyzed the expression level of these genes in A549, LC-2/ad, and Panc1 cells overexpressing MEG8 with or without TGF-β. QRT-PCR

genes encoding histone KMTs and KDMs (12, 13). We have demonstrated that several of these candidate KMTs and KDMs are implicated not only in cancer initiation but also in malignant progression such as cell invasion and EMT (7, 14). Especially, the regulation of histone H3K27 methylation by polycomb repressive complex 2 (PRC2) was shown to be requisite for the EMT-inducing gene expression program in lung and colon cancer cells (15, 16). PRC2 is critical to initiate and maintain a repressed gene state during development and cancer; it is composed of four core subunits, SUZ12, EED, RBBP4/7, and the catalytic EZH2 (17, 18). During TGF-β–induced EMT, we reported that EED and JARID2, an accessory factor of PRC2, were essential for proper recruitment of PRC2 to the target loci, including CDH1/E-cadherin and microRNA-200 (miR-200) family genes, which are important for epithelial phenotypes, H3K27 methylation, and transcriptional repression (15, 16).

Furthermore, we have recently discovered that long noncoding RNA (IncRNA) MEG3 interacts with JARID2 and functions as an “initiator” to recruit PRC2 to the specific target genes during the EMT process (19).

IncRNAs, noncoding RNAs longer than 200 nucleotides, have been reported to play key roles in diverse biological processes (20). Expression of IncRNAs is strikingly cell type– and developmental stage–specific, and aberrant expression has been found associated with malignant tumors (21–23). Although the precise functions of the vast majority of IncRNAs are still under investigation, recent studies have revealed that a subset of IncRNAs can regulate specific gene expression by interacting with transcription factors and chromatin regulators (23). Among histone-modifying enzymes, PRC2 is the most well-known factor shown to be recruited and regulated by IncRNAs such as HOTAIR and RepA (24–26). We also found that MEG3 IncRNA could tether the PRC2 complex to the regulatory regions of CDH1 and miR-200 family genes for transcriptional repression (19), showing an important role of IncRNAs in the epigenetic regulation of EMT. However, MEG3 expression by itself could not promote EMT completely, probably due to its restricted influence on a subset of the EMT-responsible genes. MEG3 gene belongs to the DLK1–DIO3 locus located at chromosome 14 in human (27, 28). This locus contains other IncRNAs, MEG8 and MEG9. In our previous experiment, the expressions of MEG8 and MEG9 IncRNAs were also up-regulated as with MEG3 during TGF-β–induced EMT in A549 lung cancer cells (19). Therefore, we tried to examine whether these IncRNAs are implicated in the EMT process of cancer cells.

Here, we discovered that MEG8 IncRNA played an essential role in the TGF-β–induced EMT program of lung and pancreatic cancer cells. MEG8 could interact with EZH2 enzyme and stimulate its recruitment on a different subset of EMT-related genes for H3K27 methylation and gene repression compared with MEG3. Remarkably, coexpression of MEG8 and MEG3 was shown to promote the EMT process in the absence of TGF-β by activating SNAIL and ZEB family EMT-TFs, respectively.
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Figure 1. The expression of MEG8 long noncoding RNA during TGF-β–induced EMT of A549, LC-2/ad, and Panc1 cancer cells and the effects of MEG8 overexpression in cell morphologies of these cells. A–C, QRT-PCR analysis was performed to detect the expression of MEG8 lncRNA in A549 (A), LC-2/ad (B), and Panc1 (C) cells before and after treatment with 1 ng/ml TGF-β (6, 12, 24, 48, and 72 h). Statistical analyses are described in detail in Table S2. p values are based on one-way ANOVA with Bonferroni post-test (*, p < 0.05; **, p < 0.01; *** , p < 0.001 compared with control). Error bars represent S.D.

D–F, cell morphological changes of A549 (D), LC-2/ad (E), and Panc1 (F) cells after TGF-β treatment. The cells were infected with control retrovirus or retrovirus expressing MEG8 without or with 1 ng/ml TGF-β treatment for 72 h. Cells were stained with 0.4% crystal violet (upper panel). The infected cells were immuno-stained with anti-E-cadherin antibody and DAPI (middle panel) and stained with TRITC-phalloidin and DAPI, showing reconstruction of the actin cytoskeleton (lower panel). Scale bars, 10 μm.
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Figure 2. The expressions of a subset of EMT-related genes were affected by MEG8 in A549 cells. A, QRT-PCR analysis was performed to detect the expression of CDH1/E-cadherin, fibronectin, vimentin, SNAI1, SNAI2, ZEB1, ZEB2, miR-34a, miR-203, miR-200a, miR-200c, PMEPA1, SMAD7, ID1, and ID2 in A549 cells infected with control retrovirus or retrovirus expressing MEG8 with or without 1 ng/ml TGF-β treatment for 24 h. p values are based on two-way ANOVA with Bonferroni post-test (**, p < 0.01; ***, p < 0.001; ns, not significant) (see Table S2). Error bars represent S.D. B, immunoblotting revealed the expression of E-cadherin, fibronectin, vimentin, SNAI1, SNAI2, ZEB1, and GAPDH proteins in A549 cells. Each immunoblotting was performed at least three times, and the quantified protein expression data are presented in a graphical form alongside the representative blot. p values are based on two-way ANOVA with Bonferroni post-test (**, p < 0.01; ***, p < 0.001; ns, not significant). Error bars represent S.D. C, altered expression of EMT-related genes induced by MEG8 was cancelled with the introduction of exogenous miR-34a and miR-203. QRT-PCR analysis was performed to detect the expression of CDH1/E-cadherin, SNAI1, and SNAI2 in A549 cells infected with control retrovirus or retrovirus expressing MEG8 with or without transfection of the precursors of miR-34a and miR-203. p values are based on one-way ANOVA with Bonferroni post-test (**, p < 0.01; ***, p < 0.001; ns, not significant). Error bars represent S.D. WB, Western blotting.

indicated that MEG8 overexpression caused the repression of CDH1 gene (Fig. 2A and Figs. S2A and S3A), confirming E-cadherin down-regulation observed in Fig. 1. In contrast, MEG8 did not affect the expression of mesenchymal marker genes such as fibronectin and vimentin (Fig. 2A and Figs. S2A and S3A), although Panc1 cells do not originally express fibronectin gene. We next examined the expression of EMT-TFs such as SNAI1, SNAI2, ZEB1, and ZEB2 in the MEG8-overexpressing cells. MEG8 itself significantly increased the expression of SNAI1 and SNAI2 but had no effect on the expression of the ZEB family (ZEB1 and ZEB2) (Fig. 2A and Figs. S2A and S3A), suggesting the specificity of MEG8 in the control of gene expression. The expression of TWIST was extremely low and not detected in these cells as described previously (29). These results led us to investigate the possibility that the MEG8 effects on EMT-TFs might be due to the regulation of microRNAs because we previously found that MEG3 enhanced ZEB family expression through the repression of the miR-200 family of microRNAs (miR-200a and miR-200c) (19). It was reported that the SNAIL family (SNAI1 and SNAI2) was specifically induced by miR-34a and miR-203 during EMT (30–33). We examined the effects of MEG8 overexpression on the expression of these miRNAs. The expression of miR-34a, miR-203, miR-200a, and miR-200c was down-regulated by TGF-β treatment in control A549, LC-2/ad, and Panc1 cells (Fig. 2A and Figs. S2A and S3A). MEG8 itself decreased the expression of miR-34a and miR-203 but did not influence the expression of the miR-200 family (Fig. 2A and Figs. S2A and S3A). These effects of MEG8 on the microRNAs were correlated with the observed expression changes of the target EMT-TFs. These results indicated that MEG8 could partially affect the EMT-inducing gene expression program, at least enough to cause E-cadherin down-regulation.

We further analyzed several other known TGF-β–responsive genes to prove the specificity of MEG8 effects in gene expression (19). MEG8 overexpression did not affect the expressions of PMEPA1, SMAD7, ID1, and ID2 genes in the absence or presence of TGF-β (Fig. 2A and Figs. S2A and S3A). These
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results suggested that MEG8 lncRNA could not regulate all of the TGFB-β-responsive genes but only a subset of genes, which includes some genes that are important for the EMT process.

We also confirmed the changes in protein expression for some of the EMT-related gene products in A549, LC-2/ad, and Panc1 cells. MEG8 by itself could decrease the expression of E-cadherin protein and increase SNAI1 and SNAI2 but had no effect on fibronectin, vimentin, and ZEB1 (Fig. 2B and Figs. S2B and S3B), which was consistent with the QRT-PCR results (Fig. 2A and Figs. S2A and S3A).

To investigate the role of miR-34a and miR-203 down-regulation by MEG8 in the EMT program, we transfected the precursors of miR-34a and miR-203 into control or MEG8-expressing A549 cells. In control cells, QRT-PCR revealed that the precursors of miR-34a and miR-203 resulted in significant up-regulation of CDH1/E-cadherin and down-regulation of SNAI1 and SNAI2 (Fig. 2C), suggesting that these miRNA precursors could inhibit the endogenous target EMT-TFs and enhance the epithelial phenotype of A549 cells. As described above, MEG8 overexpression decreased CDH1 expression, but simultaneous introduction of miR-34a and miR-203 precursors prevented its down-regulation (Fig. 2C). Transfection of miR-34a and miR-203 also inhibited the elevated expression of SNAI1 and SNAI2 caused by MEG8 (Fig. 2C). These results indicated that the precursors of miR-34a and miR-203 cancelled the gene expression changes induced by MEG8. Therefore, it was suggested that suppression of miR-34a and miR-203 expression by MEG8 was primarily responsible for MEG8-dependent up-regulation of the SNAIL family and down-regulation of CDH1/E-cadherin in cancer cells.

MEG8 affected the recruitment of EZH2 and histone H3 methylation on the regulatory regions of miR-34a and miR-203 genes

In a previous study, we discovered that MEG3 lncRNA regulates PRC2 recruitment and histone H3 methylation on the regulatory regions of miR-200 family genes for gene repression (19). Thus, we hypothesized that MEG8 overexpression would change the status of histone H3 methylation on the regulatory regions of miR-34a and miR-203 genes, whose expressions were suppressed by MEG8. Chromatin immunoprecipitation (ChIP) was performed to analyze the methylation of H3K27 and H3K4 and EZH2 occupancies on these genes in A549, LC-2/ad, and Panc1 cells overexpressing MEG8 (Fig. 3 and Fig. S4). TGF-β treatment resulted in a significant increase of trimethylated H3K27 (H3K27me3), enhanced recruitment of EZH2, and a substantial decrease of transcriptionally active H3K4me3 marks on the regulatory regions of miR-34a and miR-203 genes (Fig. 3, A and B, and Fig. S4, A, B, D, and E). These findings strongly suggested that TGF-β-induced EZH2 recruitment on these regulatory regions might be responsible for the transcriptional repression of these genes (Fig. 2A and Figs. S2A and S3A). On these regions, MEG8 overexpression resulted in a significant increase of H3K27me3, EZH2 recruitment, and a decrease of H3K4me3 compared with the control cells (Fig. 3, A and B, and Fig. S4, A, B, D, and E), which was correlated with the decreased expression of these genes by MEG8 (Fig. 2A and Figs. S2A and S3A). We did not observe any changes in EZH2 signals and H3 methylation on the regulatory region of unrelated GAPDH gene by MEG8 (Fig. 3C and Fig. S4, C and F). These results suggested that MEG8 lncRNA could mediate EZH2 recruitment and histone H3 methylation on the specific target loci such as miR-34a and miR-203 genes for transcriptional repression during the EMT process.

MEG8 could interact with EZH2 and associate with the regulatory regions of miR-34a and miR-203 genes

To investigate the molecular mechanism by which MEG8 lncRNA induces the recruitment of EZH2 for H3K27 methylation on the specific loci, we first examined whether MEG8 could interact with EZH2 or PRC2 complex. Because we identified the interaction between MEG3 lncRNA and JARID2 protein by RNA immunoprecipitation (RIP) assay in the previous study (19), we performed a similar RIP assay by using MEG3 and JARID2 as a positive control. A549 cells were infected with various combinations of the retroviruses expressing MEG3, MEG8, and FLAG-tagged JARID2, and the cross-linked cell lysates were prepared. The cell lysates were immunoprecipitated with control antibody (mouse IgG), anti-EZH2 antibody, or anti-FLAG antibody. QRT-PCR analysis was performed to detect the presence of MEG3 or MEG8 in the coprecipitated RNAs (Fig. 4, A and B). The amplified signal for MEG3 lncRNA was strongly detected when the cell lysate expressing both MEG3 and JARID2 was immunoprecipitated with anti-FLAG antibody as reported previously (19) (Fig. 4A). Additionally, MEG3 could be detected in the immunocomplex with anti-EZH2 antibody (Fig. 4A), suggesting the possibility that MEG3 could bind to endogenous EZH2 protein directly and/or indirectly through JARID2 protein. These results were basically consistent with the previous reports (19, 34). In the case of MEG8, its strong signals were observed in the anti-EZH2 antibody immunoprecipitates of MEG8-overexpressing cells (Fig. 4B), indicating that MEG8 could interact with endogenous EZH2 protein preferentially, which was in contrast to the interaction between MEG3 and JARID2. A weak signal for MEG8 was also detected when the cell lysate expressing MEG8 and JARID2 was immunoprecipitated with anti-FLAG antibody (Fig. 4B), but we could not distinguish whether MEG8 bound to JARID2 protein directly or indirectly through EZH2. Next we tried to detect the interaction between endogenous MEG8 or MEG3 lncRNA and EZH2 protein or FLAG-tagged JARID2 protein in A549 cells. However, we failed to detect the interaction by RIP assay (data not shown), probably because of the low expression of both lncRNAs. Instead, we used Panc1 cells for this experiment because endogenous expressions of MEG3 and MEG8 are 6–7-fold greater than those in A549 cells (as shown in Fig. S6). We could observe a strong signal derived from endogenous MEG3 when the cell lysate expressing FLAG-tagged JARID2 was immunoprecipitated with anti-FLAG antibody (Fig. 4C). Endogenous MEG8 was also detected in the anti-EZH2 antibody immunoprecipitates (Fig. 4D). These results indicated that endogenous MEG8 lncRNA could interact with endogenous EZH2 protein in cancer cells.

Previously, we demonstrated that JARID2 was required for MEG3 function in EMT-related gene expression by the JARID2 knockdown experiment (19). To further clarify the mechanism of MEG8 regulation of the expression of the target EMT-related
genes, we examined the requirement of EZH2 or JARID2 for MEG8 function by a knockdown experiment. A549 cells with or without MEG8 overexpression were infected with the control lentivirus, EZH2, and JARID2 shRNA-expressing lentivirus, and the expression of representative EMT-related genes was analyzed by QRT-PCR (Fig. 4E).

Efficient knockdown of EZH2 or JARID2 expression was confirmed by QRT-PCR (Fig. S5). In control cells, EZH2 knockdown resulted in significant up-regulation of CDH1/E-cadherin, miR-34a, and miR-203 and down-regulation of SNAI1 and SNAI2 (Fig. 4E), confirming our previous results showing that a decrease of EZH2 or PRC2 activity strengthened the epithelial phenotype of A549 cells (15, 16).

MEG8 overexpression caused down-regulation of CDH1, miR-34a, and miR-203 and up-regulation of SNAI1 and SNAI2 as described (Fig. 2A). Interestingly, EZH2 knockdown completely inhibited MEG8-induced repression of CDH1, miR-34a, and miR-203 and activation of the SNAIL family (Fig. 4E). However, JARID2 knockdown had no influence on the expression changes induced by MEG8 (Fig. 4E), although its knockdown could antagonize the MEG3-induced effects as described previously (19). These results indicated that knockdown of EZH2 but not of JARID2 prevented the ability of overexpressed MEG8 to regulate a subset of EMT-related genes. Therefore, we concluded that EZH2 was essential for MEG8 function in EMT-related gene expression, but JARID2 was dispensable. This difference between MEG8 and MEG3 might explain why MEG8 and MEG3 mediated the recruitment of EZH2-containing PRC2 complex to the different target gene loci.

We next aimed to examine whether MEG8 lncRNA could associate with the chromatin of the regulatory regions of the target genes. A chromatin isolation by RNA purification (ChIRP) assay was performed to detect the enrichment of the specific regulatory regions that associated with MEG8 lncRNA. MEG8 overexpression led to a significant increase of the amplified signals for the regulatory regions of miR-34a and miR-203 genes but not of miR-200b/200a/429, miR-200c/141, and GAPDH genes (Fig. 4, F–J). These results indicated that TGF-β signal was not necessary for the specific association of MEG8 with the target loci. Moreover, the observed association of MEG8 with the specific target sites was correlated with the EZH2 recruitment, histone H3K27 methylation, and transcrip-

Figure 3. MEG8 influenced the regulation of histone H3 methylation and EZH2 recruitment on the regulatory regions of miR-34a and miR-203 genes in A549 cells. A549 cells were infected with control retrovirus or retrovirus expressing MEG8 without or with TGF-β treatment. ChIP analyses of H3K27me3, EZH2, and H3K4me3 on the regulatory regions of miR-34a (A), miR-203 (B), and GAPDH genes (C) in A549 cells are shown. p values are based on two-way ANOVA with Bonferroni post-test (*, p < 0.05; **, p < 0.01; ns, not significant) (see Table S2). Error bars represent S.D.
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Figure 4. MEG8 could mainly interact with EZH2 and associate with the specific regulatory regions of miR-34a and miR-203 genes for transcriptional regulation. A and B, interaction of overexpressed MEG3 or MEG8 lncRNA with JARID2 or EZH2 protein. A549 cells were infected with the various combinations (as indicated) of retroviruses expressing MEG3, MEG8, and FLAG-tagged JARID2. The cross-linked cell lysates were immunoprecipitated with control antibody (mouse IgG; C), anti-EZH2 antibody (E), or anti-FLAG antibody (F), and the coprecipitated RNA was transcribed to cDNA. QPCR was performed to detect the enrichment of MEG3 (A) or MEG8 (B) in the precipitates. n.d. means not detected. p values are based on one-way ANOVA with Bonferroni post-test (*, p < 0.05; ***, p < 0.001) (see Table S2). Error bars represent S.D. C and D, interaction of endogenous MEG8 and EZH2 detected by RIP. Panc1 cells were infected with the control or the retroviruses expressing FLAG-tagged JARID2. The cell lysates were immunoprecipitated with the same antibodies as shown above, and QPCR was performed to detect MEG3 (C) or MEG8 (D), p values are based on one-way ANOVA with Bonferroni post-test (***, p < 0.01; ***, p < 0.001; ns, not significant). Error bars represent S.D. E, the effects of MEG8 on gene expression were dependent on EZH2 but not JARID2. QRT-PCR analysis was performed to detect the expression of CDH1/E-cadherin, SNAI1, SNAI2, miR-34a, and miR-203 in A549 cells infected with control retrovirus or retrovirus expressing MEG8 and/or lentivirus expressing EZH2 shRNA 1 or JARID2 shRNA 1, p values are based on two-way ANOVA with Bonferroni post-test (*, p < 0.05; **, p < 0.01; ***, p < 0.001; ns, not significant). Error bars represent S.D. F–J, ChiRP analyses of MEG8 on the regulatory regions of miR-34a (F), miR-203 (G), miR-200b/200a/429 (H), miR-200c/141 (I), and GAPDH genes (J) in A549 cells are shown. The cells were infected with the control or the retrovirus expressing MEG8 with or without TGF-β treatment. MEG8 lncRNA–binding complexes were recovered by magnetic beads, and QPCR was performed to detect the enrichment of specific regulatory regions that associated with MEG8 lncRNA. p values are based on two-way ANOVA with Bonferroni post-test (*, p < 0.05; ***, p < 0.001; ns, not significant). Error bars represent S.D. KD, knockdown.

Figures 2 and 3. Based on these results, we conclude that MEG8 lncRNA associated with EZH2 and recruited EZH2-containing PRC2 complex on the regulatory regions of miR-34a and miR-203 genes for histone H3K27 methylation and transcriptional repression during EMT.

Coexpression of MEG3 and MEG8 induced EMT phenotypes in the absence of TGF-β in lung and pancreatic cancer cells

We previously found that MEG3 induced the expression of ZEB family transcription factors (ZEB1 and ZEB2) by suppressing miR-200 family expression (19). In this study, we showed that MEG8 activated SNAIL family (SNAI1 and SNAI2) expression through suppressing miR-34a and miR-203. Because the ZEB family and SNAIL family consist of important EMT-TFs in A549, LC-2/ad, and Panc1 cells, we tried to examine the effects of coexpression of MEG3 and MEG8 on the EMT process. Overexpression of MEG3 and MEG8 was confirmed by qRT-PCR in these cells (Fig. S6). We first observed the cell morphologies and the status of E-cadherin and actin. Expression of either MEG3 or MEG8 revealed E-cadherin down-regulation but not actin remodeling in A549, LC-2/ad, and Panc1 cells as described (19) (Fig. 5, A, B, and C). However, coexpression of MEG3 and MEG8 further induced actin stress fiber formation...
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As shown in Fig. 7A and Fig. 5D, knockdown of MEG8 by itself showed no significant effects on cell shapes and staining of E-cadherin and actin. However, MEG8 knockdown inhibited EMT-related changes of cell morphology, E-cadherin, and actin status after TGF-β treatment (Fig. 7A and Fig. 5D). These results indicated that MEG8 knockdown antagonized TGF-β–induced EMT phenotypes in lung and pancreatic cancer cells. For cell migration, TGF-β treatment dramatically increased the number of migrated A549 cells per field (Fig. 7B). MEG8 knockdown did not change cell migratory activity by itself but counteracted the TGF-β–dependent increase (Fig. 7B). Taken together, these results suggested that endogenous MEG8 IncRNA was required for TGF-β–dependent induction of EMT-related cellular phenotypes in A549 lung cancer and Panc1 pancreatic cancer cells.

We next analyzed the expression level of epithelial and mesenchymal marker genes in the MEG8 knockdown cells with or without TGF-β. QRT-PCR showed that transcriptional repression of CDH1 and activation of mesenchymal markers such as fibronectin and vimentin induced by TGF-β were blocked by MEG8 knockdown in A549 and Panc1 cells (Fig. 7C and Fig. 5D). These results confirmed that MEG8 knockdown could antagonize the effect of TGF-β in EMT induction. For the EMT-TFs and the corresponding microRNAs, MEG8 knockdown by itself significantly reduced the expression of SNAI1 and SNAI2 and increased the expression of miR-34a and miR-203 (Fig. 7C and Fig. 5D). These findings strongly suggested the involvement of endogenous MEG8 in the transcriptional regulation of SNAI1, SNAI2, miR-34a, and miR-203 genes. Furthermore, MEG8 knockdown clearly counteracted TGF-β–dependent changes of these genes (Fig. 7C and Fig. 5D), which might contribute to the inhibition of EMT induction. In contrast, MEG8 knockdown itself had no influence on the expression of ZEB1, ZEB2, miR-200a, and miR-200c, indicating the specificity of MEG8 effects (Fig. 7C and Fig. 5D). TGF-β–induced expression changes of these genes was significantly, but not completely, inhibited by MEG8 knockdown (Fig. 7C and Fig. 5D). Again, we confirmed that the effects of MEG8 knockdown on microRNA expression were closely correlated with the observed expression changes of the target EMT-TFs. For other TGF-β–responsive genes such as PMEPA1, SMAD7, ID1, and ID2, MEG8 knockdown had no effect on their expression changes as expected (Fig. 7C and Fig. 5D). Immunoblot analysis also revealed the changes in protein expression for several of the EMT-related gene products in A549 and Panc1 cells. TGF-β–dependent reduction of E-cadherin protein and induction of fibronectin, vimentin, SNAI1, and SNAI2 proteins were blocked by MEG8 knockdown (Fig. 7D and Fig. 5E). ZEB1 induction was decreased but was not completely inhibited by MEG8 knockdown. These results were consistent with the QRT-PCR results (Fig. 7C and Fig. 5D). To examine whether or not the TGF-β signaling pathway would be impaired by MEG8 knockdown, we analyzed the status of phosphorylated SMAD3 protein (2). The phosphorylation of SMAD3 proteins was observed in response to TGF-β and was not influenced by MEG8 knockdown (Fig. 7D and Fig. 5E). This result suggested that MEG8 knockdown might not affect the TGF-β signaling and signal-dependent activation of downstream transcription factors but could inhibit TGF-β–induced transcriptional regulation of EMT-related genes possibly through an epigenetic mechanism. Taken together, we concluded that endogenous MEG8 IncRNA was involved in and indispensable for the TGF-β–dependent EMT-inducing transcriptional program of A549 lung cancer and Panc1 pancreatic cancer cells.

Knockdown of MEG8 antagonized TGF-β–induced EMT phenotypes by inhibiting the expression changes of EMT-related genes

To gain further insight into the regulation of EMT by MEG8, we examined whether knockdown of MEG8 would affect the EMT process induced by TGF-β in cancer cells. We designed two different shRNAs for MEG8 (MEG8 shRNAs 1 and 2) and confirmed that both MEG8 shRNAs efficiently reduced MEG8 expression in A549 and Panc1 cells (Fig. 5D). For LC2/ad cells, we sometimes found that MEG8 expression was efficiently decreased by the two shRNAs and that the EMT phenotypes were significantly affected by MEG8 knockdown (data not shown). However, these results were not stable and reproducible. We speculated that it might be a consequence of the inefficient knockdown of MEG8 due to the low expression of endogenous MEG8 in this cell line (19) (Fig. 1). Therefore, for the MEG8 knockdown experiments, we used A549 and Panc1 cells. Because both MEG8 shRNAs caused the same effects in our EMT study (shown in Fig. 7), we sometimes present the data of MEG8 shRNA 1 as a representative result.

As shown in Fig. 7A and Fig. 5D, knockdown of MEG8 by itself showed no significant effects on cell shapes and staining of E-cadherin and actin. However, MEG8 knockdown inhibited EMT-related changes of cell morphology, E-cadherin, and actin status after TGF-β treatment (Fig. 7A and Fig. 5D). These results indicated that MEG8 knockdown antagonized TGF-β–induced EMT phenotypes in lung and pancreatic cancer cells. For cell migration, TGF-β treatment dramatically increased the...
Figure 5. Coexpression of MEG3 and MEG8 resulted in cell morphological changes characteristic of EMT and increased migratory activities of cancer cells. A–C, cell morphological changes of A549 (A), LC-2/ad (B), and Panc1 (C) cells after TGF-β treatment. The cells were infected with control retrovirus or retrovirus expressing MEG3 and/or MEG8 without or with 1 ng/ml TGF-β treatment for 72 h. Cells were stained with 0.4% crystal violet (upper panel), with anti-E-cadherin antibody and DAPI (middle panel), or with TRITC-phalloidin and DAPI (lower panel). Scale bars, 10 μm. D, expression of both MEG3 and MEG8 increased the cell migratory activity. A549 cells that migrated through the filter within 24 h were fixed, stained, and counted. p values are based on one-way ANOVA with Bonferroni post-test (***, p < 0.001) (see Table S2). Error bars represent S.D.
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Discussion

In this study, we uncovered a novel function of MEG8 lncRNA in the epigenetic regulation of EMT of A549 and LC2/ad lung cancer and Panc1 pancreatic cancer cell lines. MEG8 overexpression led to the down-regulation of an epithelial marker, E-cadherin, in these cells similarly to the case of MEG3. However, MEG8 influenced the expression of a different subset of EMT-related genes such as SNAI1, SNAI2, miR-34a, and miR-203 compared with MEG3. Mechanistic investigations suggested that MEG8 could specifically associate with EZH2 protein and the regulatory regions of miR-34a and miR-203 genes, resulting in EZH2 recruitment and histone H3K27 methylation on these regions for transcriptional repression. Our results revealed that MEG8 and MEG3 are essential for TGF-β–induced EMT in lung and pancreatic cancer cells, but ectopic expression of either MEG8 or MEG3 could not promote EMT completely. Remarkably, coexpression of MEG8 and MEG3 was shown to up-regulate mesenchymal marker genes and induce EMT-related cellular phenotypes, possibly through the activation of both SNAI1 and ZEB family EMT-TFs. This is the first study to demonstrate the functional significance of MEG8 lncRNA in the EMT process, which validates the importance of lncRNA-mediated epigenetic regulation for tumor progression.

PRC2 is indispensable for epigenetic silencing during development and cancer (17, 35). EZH2, an enzymatic subunit, catalyzes histone H3K27 methylation, a chromatin mark correlated with transcriptional repression. EZH2 overexpression has been found in a number of tumors and correlated with poor prognosis (36). Therefore, the activity of EZH2 or PRC2 is closely associated with tumor initiation and malignant progression. We also reported that PRC2 could specifically repress the expression of CDH1 and miR-200 family genes through H3K27 methylation, which was required for TGF-β–induced EMT in lung cancer cells (15, 16). Because none of the core components of PRC2 possess sequence-specific DNA binding activities, it would be important to clarify the mechanism by which PRC2 is recruited to its specific target loci. Multiple protein factors have been proposed for the PRC2 recruitment process, and JARID2 is one of the best known candidates, playing a crucial role in embryonic development and EMT of cancer cells (16, 37, 38). Recently, lncRNAs have emerged as potential “initiators,” being capable of tethering PRC2 to the specific target loci (26). It was reported that RepA and HOTAIR lncRNAs interacted with PRC2 and formed an assembling scaffold for PRC2 targeting to specific gene loci of the genome (24, 25). Several groups, including us, have revealed that MEG3 lncRNA associated with JARID2, stimulated the assembly of JARID2 and PRC2, and recruited them to the specific target loci in pluripotent stem cells or during EMT of cancer cells (19, 34, 39). It was also reported that MEG3 interacted with PRC2 and PRC2 recruitment only a subset of genes but not all of the PRC2–regulated loci (19, 34). Therefore, we should note that PRC2 can associate with different lncRNAs for its recruitment to the chromatin, and
these interactions may determine the specificity of target gene selection. In the current study, we discovered that MEG8 lncRNA could interact with EZH2 and stimulate its recruitment to a different subset of EMT-related genes compared with the case of MEG3 (19) (Figs. 3 and 4). As shown in Fig. 4, F–J, a ChIRP assay revealed that MEG8 associated with the regulatory regions of miR-34a and miR-203 genes but not of miR-200b/200a/429 and miR-200c/141 genes, which were the targets of
MEG3, and the unrelated GAPDH gene. A RIP assay also indicated that endogenous MEG8 formed a complex with EZH2 protein preferentially, which was in contrast to the interaction between MEG3 and JARID2 (Fig. 4, A–D). Moreover, the effect of MEG8 overexpression on specific EMT-related gene expression was shown to be dependent on the expression of EZH2 but not of JARID2 (Fig. 4E). These results suggested that the different interaction properties between MEG8 and MEG3 might be responsible for distinct gene specificities of PRC2 recruitment mediated by these lncRNAs. However, it is still difficult to establish a model for locus-specific recruitment of PRC2 by its interaction with lncRNAs based on the current findings. Further experiments will be necessary to clarify the mechanism for lncRNA-mediated targeted gene selection of PRC2.

EMT is a phenotypic conversion associated with cancer metastasis (1). The cellular plasticity of EMT is controlled by an epigenetic mechanism that assures reversible changes in gene expression (3, 4). It is a complicated process regulated by many factors such as EMT-TFs, chromatin remodelers, histone-modifying enzymes, and noncoding RNAs. Our experimental data provide an interesting clue for understanding the epigenetic regulation of EMT. Overexpression of MEG8 lncRNA induced transcriptional repression on mir-34a and mir-203 genes, thereby activating SNAI1 and SNAI2 EMT-TFs for E-cadherin down-regulation (Fig. 2). This was in contrast to the previous observation for MEG3 lncRNA, which activated ZEB1 and ZEB2 EMT-TFs by suppressing mir-200 family expression (19). In fact, overexpression of either MEG8 or MEG3 did not cause increased expression of mesenchymal marker genes, fibronectin and vimentin, and also did not induce the EMT phenotype completely (Figs. 5 and 6). Because the expression of all four EMT-TFs, SNAI1, SNAI2, ZEB1, and ZEB2, was upregulated during TGF-β–induced EMT in A549, LC-2/ad, and Panc1 cells, the activation of either the SNAI1 family or ZEB family might not be sufficient to proceed with the EMT program in these cells. In contrast, coexpression of MEG8 and MEG3 could induce the EMT phenotype, judging from cell morphology, actin reorganization, cell migration properties, and elevated expression of mesenchymal markers (Figs. 5 and 6). These results indicated that MEG8 and MEG3 might regulate the expression of a different subset of EMT-responsive genes, respectively, but could promote EMT when their effects were combined. This model is consistent with the fact that MEG8 and MEG3 are neighbor genes located at the DLK1–DIO3 locus and are transcriptionally induced simultaneously in response to TGF-β during EMT.

Accumulating studies have described the important roles of lncRNAs in multiple pathological steps of cancer, including cell proliferation, angiogenesis, and metastasis (20, 22, 23). The expressions of lncRNAs are frequently deregulated in tumors, and some lncRNAs have been reported to act as oncogenes or tumor suppressor genes. Although the function and biological relevance of the vast majority of lncRNAs remain enigmatic, some of them can interact with DNA, proteins, and other RNAs and function as guides, scaffolds, and decoys to regulate various cellular processes, including transcription, splicing, and intracellular trafficking (23, 40). MEG8 and MEG3 lncRNA genes are neighbor genes located at the DLK1–DIO3 locus in the human chromosome 14q32.3 region (27, 28). Many studies have reported on the expression and function of MEG3 especially in cancer cells; however, there are few reports on MEG8. MEG3 is highly expressed in normal tissues but is decreased in many human tumors and tumor-derived cell lines (27, 28). Thus, it is suggested that MEG3 is one of the lncRNAs with tumor suppressor activity. In contrast to these observations, our study clearly indicated that MEG3 and MEG8 were immediately up-regulated during TGF-β–induced EMT of lung and pancreatic cancer cells and played an important role in EMT, a hallmark of malignant tumor progression. In addition, we found that endogenous MEG8 expression was 4–5-fold lower than that of MEG3 in A549, LC-2/ad, and Panc1 cancer cells (Fig. S6). It is very interesting but currently we do not have any evidence indicating that the different expression levels of MEG3 and MEG8 might be implicated in their regulatory function. Further experiments will be required to understand the novel functional interaction of these two lncRNAs in detail. Recently, the integrated analysis of ovarian cancer molecular profiles has identified MEG3 overexpression associated with ovarian cancer EMT (41), which strongly supports our findings. Furthermore, based on the report that the expressions of lncRNAs from DLK1–DIO3 locus were essential for the establishment of stem cell activities such as pluripotency (34, 42), the observed function of MEG3 and MEG8 in EMT might help rationalize the connection between the EMT program and acquisition of stem cell traits.

In summary, we have found a novel functional relationship between MEG8 and MEG3 lncRNAs during TGF-β–induced EMT of lung and pancreatic cancer cells. MEG8 and MEG3 were shown to induce the recruitment of EZH2-containing PRC2 complex on a different subset of EMT-related genes for histone H3K27 methylation and transcriptional repression. These results suggested the important role of these lncRNAs in the decision of target specificity of PRC2 recruitment. In addition, we found that simultaneous induction of MEG8 and MEG3 from the DLK1–DIO3 locus by TGF-β might be responsible for activating the gene expression program, which was sufficient to promote EMT. Because plastic epigenetic control of gene expression plays a critical role in cancer malignancies, a better understanding of lncRNA-mediated epigenetic regulation will provide fresh insights into the development of novel therapeutic strategies for targeting cancer.

Experimental procedures

Plasmids, cell culture, and transfections

Lentiviral vectors expressing shRNAs were constructed as described previously (14). The sequences of oligonucleotides for shRNAs were described previously (16) and are listed in Table S1. For human MEG8 cDNA, the primer set was designed based on the reference sequence (ENST00000636052.1) in GENCODE database and used for PCR cloning (described in Table S1). The amplified MEG8 cDNA was cloned into pDON-5 Neo plasmid (Takara, Ohtsu, Japan) to produce retrovirus expressing MEG8. pMXs-puro plasmid that produces retrovirus expressing MEG3 was constructed similarly as described previously (16). Human lung cancer cell lines A549

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and LC-2/ad and human pancreatic cell line Panc1 were obtained from ATCC and RIKEN BioResource Research Center and maintained in culture medium at 37 °C in 5% CO₂ as described previously (15). A549, LC-2/ad, and Panc1 cells are a good model system for EMT, showing quick and clear changes for cell morphology and EMT-related gene expression induced by treatment with 1 ng/ml TGF-β (R&D Systems, Minneapolis, MN) for 24–72 h. The methods for the production and infection of shRNA-expressing lentiviruses or cDNA-expressing retroviruses were essentially the same as described previously (14). Transfection of microRNA precursors (Invitrogen) was performed with Lipofectamine RNAiMAX (Invitrogen) by a reverse transfection procedure according to the manufacturer’s protocol. Cells were transfected with pre-miR-34a (PM11030), pre-miR-203 (PM10152), or negative control 1 (AM17110) miRNA at a final concentration of 10 nM, further incubated for 3 days, and used for the experiments as described previously (7).

Quantitative PCR

RNA preparation and quantitative RT-PCR were carried out as described previously (14). To detect the target genes with very low expression such as MEG8 and MEG9, a highly efficient qPCR enzyme, KiQStart SYBR Green qPCR ReadyMix (Sigma), was used. The expression of human GAPDH was measured as an internal control to calculate the relative expression. The averages of expression data from at least three independent experiments are presented with the standard deviations. p values were determined by one-way or two-way ANOVA with Bonferroni’s post-test. The primer sets used for the quantitative PCR were described previously (7, 14, 16, 19) and are listed in Table S1. For microRNA analysis, real-time PCR was performed using TaqMan MicroRNA Assays (Applied Biosystems, Waltham, MA) for miR-34a (000426), miR-203 (005070), miR-200a (00502), and miR-200c (002300). All microRNA data were normalized with RNU6B (U6 small nuclear RNA; 001093) expression.

Cell staining, immunofluorescence, and immunoblotting

A549, LC-2/ad, or Panc1 cells were fixed in 4% paraformaldehyde and stained with 0.4% crystal violet to observe the cell morphology changes. For actin staining, fixed cells were permeabilized in 0.1% Triton X-100 and stained with 0.25 μg/ml phalloidin conjugated with tetramethylrhodamine isothiocyanate (TRITC) (Sigma). For indirect immunofluorescence, fixed and permeabilized cells were probed with anti-E-cadherin antibody (610181, BD Biosciences) and treated with Alexa Fluor 546–conjugated anti-mouse IgG secondary antibody (Invitrogen). To detect nuclei, cells were costained with 4',6-diamidino-2-phenylindole (DAPI). Immunoblotting was carried out as described previously (7). Briefly, 1 × 10⁶ cells were lysed in 200 μl of radioimmune precipitation assay buffer (50 mM Tris-HCl at pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 0.1% SDS, 1 mM EDTA supplemented with protease inhibitors (Nakarai, Kyoto, Japan)). The cell lysates were separated on a SuperSep Ace 10 or 5–20% running gel (Wako, Osaka, Japan) and transferred to Hybond™-LFP membrane (GE Healthcare Japan). Each membrane was probed once with each primary antibody followed by reaction with the horseradish peroxidase–conjugated secondary antibody (NA9310V or NA9340V, GE Healthcare Japan). The bands were detected by an enhanced chemiluminescence system (Immunostar LD, 290-69904, Wako) and a Fusion FX imaging system (Vilber Lourmat, France) and quantified by Fusion software. The quantified protein band data were normalized with GAPDH protein expression, and the band data in control cells were defined as 100 in each experiment. The averages of protein expression data from at least three independent experiments are presented with the standard deviations. The primary antibodies used in this study were anti-E-cadherin, anti-fibronectin (SAB4500974, Sigma), anti-vimentin (ab8069, Abcam, Cambridge, MA), anti-SNAI1 (3879, Cell Signaling Technology, Danvers, MA), anti-SNAI2 (9585, Cell Signaling Technology), anti-ZEB1 (3396, Cell Signaling Technology), anti-phosphorylated SMAD3 (ab51451, Abcam), and anti-GAPDH (6C5, Millipore, Billerica, MA).

Cell migration assay

Cell migration activities were measured by using Transwell migration chambers (3422, Corning) as described previously (19). Briefly, serum-starved cells (2 × 10⁵) were suspended on the upper chamber in Dulbecco’s modified Eagle’s medium containing 1 mg/ml BSA and 0.5% fetal bovine serum. In the lower chamber, Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum was used as a chemoattractant. The chambers were incubated for 24 h. For TGF-β–treated cells, cells were pretreated with 1 ng/ml TGF-β for 4 days and then allowed to migrate. Cells on the underside of the filter were fixed with 4% paraformaldehyde and stained with 0.4% crystal violet. Cells were counted under a microscope in at least five different fields and three independent experiments.

ChIP assays

ChIP experiments were performed essentially the same as described previously (14, 43). The cross-linked chromatin was sonicated, and the lysates were immunoprecipitated with antibodies (anti-H3K27me3 and anti-H3K4me3 (43) and anti-EZH2 (5246, Cell Signaling Technology)). Quantitative PCR was carried out to detect enrichment of specific amplified regions. Results are shown as the percentage of input chromatin. Primers used for the QPCR correspond to the proximal promoter regions of miR-34a gene and miR-203 gene and are listed in Table S1. Primers for region b of miR-200b/a/429, region b of miR-200c/141, and region a of GAPDH genes were described previously (7).

RNA immunoprecipitation

The RIP assay was carried out as described previously (19). Briefly, cross-linked cells were lysed with RIP buffer (50 mM Tris-Cl, pH 7.5, 150 mM NaCl, 10 mM EDTA, 0.5% Nonidet P-40) supplemented with protease inhibitors (Nakarai) and SUPERase-In (Thermo Fisher, Waltham, MA). The lysates were incubated with anti-EZH2 antibody, anti-FLAG antibody (M2, F1804, Sigma), or normal mouse IgG. Then the immunocomplexes were recovered with Protein G or anti-mouse IgG–coupled Dynabeads (Invitrogen). The coprecipitated RNAs were extracted with a High Pure RNA Tissue kit (Roche Applied Science) and quantified by quantitative RT-PCR.
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ChIRP assays

The ChIRP experiment was performed as described previously (19). The 3’-end biotin-triethylene glycol–modified probes were synthesized by Rikaken Co. (Nagoya, Japan). We used ChIRP Probe Designer to design the antisense DNA probes for MEG8 IncRNA (listed in Table S1). Briefly, cells were fixed with 1% glutaraldehyde for 10 min at room temperature. The cross-linked cells were suspended with lysis buffer (50 mM Tris-Cl, pH 7.5, 750 mM NaCl, 1% SDS, 1 mM EDTA, 75 mM formamide) overnight at 4°C. Then streptavidin magnetic C1 beads (Invitrogen) were added in the reaction to recover the binding complexes. The DNA was eluted from the beads with elution buffer (50 mM NaHCO3, 1% SDS) overnight at 4°C. The DNA was eluted from the beads with 15% formamide (50 mM Tris-Cl, pH 7.5, 1% SDS) supplemented with protease inhibitors and SUPEREase-In and subjected to sonication by Bioruptor II (Diagenode, Denville, NJ). The sheared chromatin lysates were hybridized with the mixture of biotinylated DNA probes against human MEG8 in hybridization buffer (50 mM Tris-Cl, pH 7.5, 750 mM NaCl, 1% SDS, 1 mM EDTA, 15% formamide) overnight at 4°C. Then streptavidin magnetic C1 beads (Invitrogen) were added in the reaction to recover the binding complexes. The DNA was eluted from the beads with elution buffer (50 mM NaHCO3, 1% SDS) and used for quantitative PCR analysis to detect the enrichment of specific regulatory loci associated with MEG8 IncRNA.

Statistical analysis

We performed statistical analyses using IBM SPSS Statistics version 25 software. The significance between means was determined by one-way or two-way ANOVA with Bonferroni’s post-test. If there was an interaction, Bonferroni post-test was performed for multiple comparisons. If not, Bonferroni post-test for planned comparisons was performed. Statistical analyses performed for each experiment are summarized in each figure legend, and the F statistics, degrees of freedom, and p values are listed in detail in Table S2. p values < 0.05 were considered statistically significant.

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