Published in final edited form as:

Stem Cells. 2013 August ; 31(8): 1477–1487. doi:10.1002/stem.1410.

Geminin restrains mesendodermal fate acquisition of embryonic stem cells and is associated with antagonism of Wnt signaling and enhanced Polycomb-mediated repression

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Abstract

Embryonic cells utilize both growth factor signaling and cell intrinsic transcriptional and epigenetic regulation to acquire early cell fates. Underlying mechanisms that integrate these cues are poorly understood. Here we investigated the role of Geminin, a nucleoprotein that interacts with both transcription factors and epigenetic regulatory complexes, during fate acquisition of mouse embryonic stem cells. In order to determine Geminin's role in mesendoderm formation, a process which occurs during embryonic gastrulation, we selectively over-expressed or knocked down Geminin in an *in vitro* model of differentiating mouse embryonic stem cells. We found that Geminin antagonizes mesendodermal fate acquisition, while these cells instead maintain elevated expression of genes associated with pluripotency of embryonic stem cells. During mesendodermal fate acquisition, Geminin knockdown promotes Wnt signaling, while Bmp, Fgf, and Nodal signaling are not affected. Moreover, we showed that Geminin facilitates the repression of mesendodermal genes that are regulated by the Polycomb repressor complex. Geminin directly binds several of these genes, while Geminin knockdown in mesendodermal cells reduces Polycomb repressor complex occupancy at these loci and increases trimethylation of histone H3 lysine 4, which correlates with active gene expression. Together, these results indicate that Geminin is required to restrain mesendodermal fate acquisition of early embryonic cells and that this is associated with both decreased Wnt signaling and enhanced Polycomb repressor complex retention at mesendodermal genes.

Keywords

mesendoderm; Geminin; Wnt; embryonic stem cell; Polycomb complex

Introduction

During embryonic development, the primary germ layers, consisting of mesoderm, endoderm, and ectoderm, give rise to all somatic cell types in the body. These germ layers form during gastrulation, as some epiblast cells undergo an epithelial to mesenchymal transition (EMT) and ingress through a structure called the primitive streak to form

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Conflict of Interest The authors declare no conflict of interest.

mesendoderm, a bipotent precursor to mesoderm and definitive endoderm¹⁻⁴. Integration of growth factor signaling pathways is required for mesendoderm induction, with interplay between Nodal, Bmp4, and Wnt3 at the posterior side of the gastrulating embryo and their antagonists, such as Cer1 and Dkk1, at the anterior side^{5, 6}.

This growth factor signaling activates expression of transcription factors required for mesendodermal specification. These include Brachyury, Eomes, Goosecoid, and Mixl1, which are directly induced by Nodal signaling through Smad2/3 activity⁷⁻¹². Brachyury expression in the primitive streak also requires Fgf and Wnt signaling via Wnt3¹³⁻¹⁷. Mutation of either β -catenin or Lrp5/6 Wnt co-receptors blocks primitive streak formation, supporting a requirement for β -catenin-dependent Wnt signaling^{18, 19}. In vitro experiments performed in human or mouse embryonic stem cells (ESCs) also defined a Wnt signaling requirement for mesendodermal fate acquisition^{20, 21, 22}, and for subsequent mesodermal and endodermal gene expression^{20, 23, 24}. Activin/Nodal signaling is likewise required for formation of Brachyury- and Foxa2-positive primitive streak populations in differentiating ESCs²³, while BMP signaling is dispensable for this induction²⁴. Together, these findings indicate a central role for growth factor signaling in activating expression of transcription factors that specify mesendodermal fates.

In addition to extrinsic signaling requirements, the Polycomb repressor complex is a cell intrinsic epigenetic regulator that controls cell fate transitions in embryonic cells²⁵. Polycomb consists of two multiprotein complexes, Polycomb Repressive Complex 1 (PRC1) and 2 (PRC2). PRC2 contains the core subunits Suz12, Eed, and Ezh2, while PRC1 has a more complex subunit composition. In ESCs, Polycomb (PcG) occupies and prevents premature expression of genes that regulate cell fate transitions by catalyzing placement of a repressive histone modification, tri-methylation of histone H3 lysine 27 (H3K27me3)²⁶. In ESCs, many developmental regulatory genes carry both repressive H3K27me3 and a modification associated with gene transcription, tri-methylation of histone H3 lysine 4 (H3K4me3). This 'bivalent' modification status maintains genes in a poised-but-repressed transcriptional state²⁷. During differentiation, this bivalency is resolved as developmental genes are trans-activated or -repressed. H3K27me3 is lost at activated genes, which become exclusively enriched for H3K4me3²⁷.

Many of the molecular mechanisms that integrate growth factor signaling with intracellular responses to control mesendodermal fate acquisition remain to be elucidated. Among the potential regulators is the small nucleoprotein Geminin (Gmnn or Gem), initially described both for its ability to neutralize non-neural ectoderm in *Xenopus* embryos²⁸ and as a protein that underwent proteasomal degradation during mitosis^{28, 29}. Gem is highly expressed in early embryonic cells and in neural progenitors, while being subsequently down-regulated upon neuronal differentiation^{28, 30, 31}. In different contexts, Geminin interacts with distinct transcription factors and epigenetic regulators, including the Polycomb complex and Hox transcription factors to restrain Hox gene expression and function during embryonic rostrocaudal patterning³² and Six3 to regulate eye development³³. Geminin also interacts with the SWI/SNF chromatin remodeler to regulate *Xenopus* primary neurogenesis³¹.

Geminin deficiency in mouse embryos results in developmental arrest at pre-implantation stages upon failure to form the inner cell mass, from which mouse ESCs are derived, precluding further study³⁴. In this work, Geminin was required to prevent embryonic cells from acquiring an extra-embryonic trophoblast giant cell fate^{34, 35}. Recently, we performed Geminin knockdown in undifferentiated mouse ESCs and during neural fate acquisition³⁶. Geminin knockdown to ~20% of endogenous levels did not affect ESC proliferation, survival, or self-renewal. However, Geminin knockdown during neural fate acquisition strongly decreased expression of early neural markers, while increasing expression of genes

involved in formation of the mouse node in vivo (Pitx2, Lefty1, Mid1, Kif3b and Cited2)³⁶. These results suggested that Geminin may play a broader role in restraining mesendodermal gene expression and cell specification. To explore this hypothesis, we utilized an in vitro model of mesendodermal specification in order to study Geminin's role in controlling this developmental process. Differentiating ESCs form three-dimensional embryoid bodies (EBs), which are used as a proxy for the early gastrula embryo and contain cells of the three major germ layers. Using this approach we demonstrate that Geminin represses mesendodermal fate acquisition, while promoting retention of pluripotency-associated gene expression. Repression of mesendodermal fate acquisition by Geminin is associated with both reduced Wnt signaling and with enhanced Polycomb repressor complex binding to mesendodermal genes, which in turn facilitates their transcriptional repression.

Materials and Methods

Inducible mESC lines

Multiple clonal mouse ESC lines for inducible Geminin over-expression (OE) and knockdown (KD) were established as described previously (Fig. S1A)^{36,37}. GemOE lines enable Dox-inducible over-expression of Flag-tagged Geminin (Fig. S1B). For GemKD, Dox-inducible miR30-based shRNAmir expression cassettes were used (Fig. S1A, B). GemKD lines inducibly express GFP with shRNAmir expression cassettes inserted into the GFP 3'-UTR (Fig. S1C).

ESC to embryoid body commitment/differentiation scheme

ESCs were maintained as described previously³⁶. For EB formation, ESCs were feeder subtracted and plated at 6,000/ml in IMDM (Invitrogen) with 15% fetal calf serum (HyClone), 1% L-glutamine, 50µg/ml ascorbic acid and 4.5×10^{-4} M MTG on 10cm petri dishes treated with 5% polyheme to prevent cell adhesion^{38, 39}. Dox was added at 0.5µg/ml. Controls included comparisons: 1. Between clones plus versus minus Dox (endogenous Gem levels appear unperturbed by GemOE), 2. to our control (GFP-expressing) ESC line, differentiated in parallel, and, 3. for KD lines, between two clones carrying independent shRNAmir targeting sequences.

Microarray analysis

EBs were generated with versus without GemOE (by Dox addition from day 3-5) or GemKD (by Dox addition from day 0-4). RNA from three experiments was collected at day 5 (OE) or day 4 (KD) and subjected to Affymetrix Mouse Gene 1.0ST microarray analysis. Raw CEL and DAT files were analyzed with dChip software (<http://biosun1.harvard.edu/complab/dchip/>)³⁸ after normalization to exclude probe sets that did not meet preliminary cutoff values: (1) expression, represented by model-based expression indices (MBEI), changed more than 1.2-fold in treated versus untreated samples ($E/B > 1.2$), (2) MBEI differences were > 30 ($E-B > 30$). Probe sets were considered putatively Geminin-regulated if they met cutoff values in at least 2 of 3 experiments (Table S1). These data were deposited into the Gene Expression Omnibus (GSE39676).

Conditioned media

Conditioned media (CM) was collected from day 4 GemKD EBs generated with (+Dox) or without (-Dox) Gem knockdown. CM was filtered through a 0.22µm filter and added to day 4 EBs generated from parental A2lox ESCs. Factors added with CM-treatment included Dkk1 (R&D-5439; 50ng/ml), noggin (R&D-1967; 50ng/ml), SB431542 (R&D-1614; 5µM), or SU5402 (Santa Cruz-sc204308; 5µM).

Reverse transcription and quantitative PCR (qRT-PCR)

RNA was prepped (Qiagen RNeasy), reverse transcribed using random primers and SuperscriptII, and qRT-PCR was performed with FastSYBR mix (Applied Biosystems) on the ABI7500 Cyclor. Gene specific primers (Table S2) were designed using Primer3, with stringent settings for Tm/avoidance of secondary structure, and tested by melting curve. Samples were run in triplicate with negative controls and normalization to Rpl19 mRNA. For each gene, relative expression was calculated by comparison with a 3-point standard curve, generated using serial 10X dilutions of a cDNA sample where the gene is highly expressed.

Flow cytometry

EBs were disaggregated in 0.05% trypsin (5 minutes, 37°C), washed twice in phosphate buffered saline (PBS), fixed in 4% paraformaldehyde for 20 min on ice, and washed twice in PBS. Cells were permeabilized in 1% bovine serum albumin (BSA)/0.5% Saponin/PBS (20 min, room temp). Immunostaining was done for 40 min at room temp with primary antibody (Table S2) diluted in permeabilization buffer, washed twice, stained with secondary antibody (Table S2) for 30 min, and washed twice again. Cells were resuspended in FACS buffer (3% BSA/1mM EDTA/PBS). Cells were stained in parallel with secondary antibody alone as a control. For analysis of DNA content, EBs were dissociated, and 10^6 cells were resuspended in FACS buffer and fixed for 20 min in 70% ethanol, added dropwise while vortexing. Cells were washed in FACS buffer, stained with 100µl propidium iodide (PI) solution (400µl of 1.0mg/ml PI, 500µl of 10mg/mL RNaseA in 10ml PBS), and analyzed in FACS buffer. Flow cytometry was performed on the FACScalibur and analyzed using Cell Quest (BD Pharmingen).

Chromatin immunoprecipitation (ChIP)

Disaggregated cells were cross-linked (1% formaldehyde, 10 minutes, room temperature), and then treated with 125mM glycine (5 minutes). Nuclei were sonicated to generate chromatin fragments of ~250bp-1kb (Branson sonifier). Antibodies (5µg, Table S2) bound to Protein G-conjugated magnetic beads (Dynabeads) were added to chromatin from 10^6 cells (for histone modifications) or 5×10^6 cells for non-histone proteins (e.g. Suz12). Immunoprecipitated chromatin was washed with low salt buffer, high salt buffer, and LiCl buffer followed by TE after (<http://farnham.genomecenter.ucdavis.edu/protocol.html>)³⁹. Bound chromatin was eluted from beads and crosslink reversal performed overnight at 67°C. DNA was extracted with phenol/chloroform and analyzed by FastSYBR Green qPCR using primers indicated (Table S2). Samples were compared to 10% total input by qPCR using the delta-delta Ct method and expressed as % input, with IgG negative control ChIP for comparison.

Western blot

EBs were lysed with extraction buffer (for phospho-specific antibodies, phosphatase inhibitors 1mM sodium fluoride, 1mM sodium vanadate, 2.5mM sodium pyrophosphate, and 1mM -glycerophosphate were added) and 40µg lysate was analyzed by western blot using antibodies indicated (Table S2).

Results

Geminin restrains mesendodermal gene expression during embryoid body formation

To manipulate Geminin levels during cell fate acquisition, we utilized lines of mouse ESCs enabling Dox-inducible over-expression (OE) or knockdown (KD) of Geminin (Fig. S1). We used an ESC to EB differentiation scheme to generate cells that express markers of the

primitive streak by approximately differentiation day 4 (Fig. S2A). To comprehensively define the genes regulated by Geminin in EBs, microarray analyses were performed on day 4 (for GemKD) and day 5 (for GemOE). Three independent experiments were conducted and analyzed each for GemOE and KD: GemOE resulted in down-regulation of 90 and up-regulation of 79 genes (169 total), while GemKD resulted in up-regulation of 111 and down-regulation of 487 genes (598 total).

We next examined the relative gene expression profile during days 1 to 7 of EB formation for each Geminin-regulated gene⁴⁰. This analysis demonstrated that ~60% of Geminin-repressed genes (e.g. down-regulated upon GemOE or up-regulated upon GemKD) increase in expression during fate acquisition of EBs (Fig. 1A, Fig. S2B). By contrast, 67% of genes whose expression was promoted by Geminin (e.g. up-regulated upon GemOE) decline in expression during fate acquisition of EBs (Fig. 1A, Fig. S2B). As a baseline comparison, genes represented on the microarray are equally distributed between genes that are up- (51%) and down- (49%) regulated during ESC-EB formation (Fig. S2B). Together, these data indicate that Geminin negatively regulates genes that are associated with EB differentiation, while positively regulating genes that are associated with pluripotent ESCs.

The Metacore Suite was utilized to analyze enrichment for Gene Ontology (GO) terms among the Geminin-regulated gene sets, to define over-represented biological themes (Table S3). Among genes that were negatively regulated by Geminin, mesoderm formation, mesoderm morphogenesis, and gastrulation were top GO processes. Molecular functions of Geminin down-regulated genes included regulatory region DNA binding/transcription factors, and enriched gene maps included the top terms Wnt signaling and regulation of EMT (Table S3). Metacore network analysis defined significant functional interactions for a group of Geminin down-regulated transcription factors that control mesendoderm formation, including Eomes, Gsc, Lef1, T/Brachyury, and Mixl1 (Table S3). Geminin down-regulates expression of genes that mark primitive streak mesendoderm in vivo (Cxcr4, Eomes, Gsc, Lhx1, Mixl1, Wnt3), including genes encoding activities critical for formation, migration, and patterning of mesoderm (Eomes, Fgf8, Mixl1, T, Wnt3) and definitive endoderm (Eomes, Foxa2, Lhx1, Mixl1)(Fig. 1B). Expression of many of these genes peaks on day 4 in EBs, while declining by day 5 (Fig. S2A).

Geminin promoted the expression of a distinct set of genes that are most highly expressed in ESCs and decline in expression in EBs. This group of genes includes many molecules with roles in pluripotency maintenance and/or reprogramming of somatic cells to a pluripotent state (Esrrb, Fbxo15, Fgf4, Klf2/3/4, Dax1, Tbx3, Tcfcp2l1)(Fig. 1C). These data support a role for Geminin in blocking transcriptional activation of genes that mark or regulate mesendodermal fate acquisition, while pluripotency-associated genes continue to be expressed at elevated levels, rather than being down-regulated. Together, these data indicate that Geminin plays a role in restraining mesendodermal fate acquisition.

To validate the microarray analysis, some Geminin up-regulated or down-regulated genes were selected for further study. We inducibly over-expressed or knocked down Gem activity starting on day 0 and analyzed expression levels of these genes by reverse transcription and quantitative PCR (qRT-PCR) on days 3, 4 and 5 (Fig. 1B-C). These results were largely congruent with the microarray analyses. Geminin knockdown increased expression of mesendodermal fate-associated genes, relative to the uninduced control. This effect was most pronounced on day 4, when many mesendodermal genes are most highly expressed. Conversely, pluripotency-associated genes were diminished upon Gem knockdown (Fig. 1B-C). Largely reciprocal results were obtained upon Geminin over-expression: expression of genes that mark and regulate mesendodermal fate was repressed, while expression of

pluripotency-related genes was elevated. Therefore, these data further support the conclusions from our microarray work, described above.

Geminin knockdown increases mesendodermal cell fate acquisition

Geminin knockdown most strongly increased the expression of genes that are central regulators of mesendodermal specification and patterning during gastrulation. Therefore, we assessed whether these effects corresponded to a change in percentages of cells that acquired a mesendodermal fate. Geminin was knocked down from day 0, and day 3, 4, and 5 EBs were dissociated and analyzed by flow cytometry to define percentages of cells expressing Eomes, Mixl1, Brachyury, Foxa2, Gata4, and Sox17 (Fig. 2). Dox-mediated Geminin knockdown strikingly increased percentages of cells expressing each marker. These results indicate that Geminin knockdown leads to more cells acquiring a mesendodermal fate. In addition, when these EBs were further differentiated under conditions enabling formation of spontaneously beating EBs, Geminin knockdown increased the percentage of beating EBs, indicating increased propensity to differentiate into functional cardiomyocytes (Fig. S2C).

We next measured E-cadherin expression by flow cytometry, as mesendodermal cells lose E-cadherin expression and undergo EMT as they transit the primitive streak during gastrulation (Fig. 3A). Geminin knockdown resulted in downregulation of E-cadherin levels on days 4 and 5 and also increased mRNA expression of the EMT-promoting transcription factors Snail and Twist (Fig. 3A). These data supported the hypothesis that Geminin knockdown promotes some aspects of EMT in EBs, suggesting that Geminin may coordinately restrain mesendodermal fate acquisition and the EMT that accompanies it during gastrulation. We further examined the possibility that Geminin knockdown might promote EMT by conducting cell migration assays. However, Geminin knockdown instead inhibited migration of dissociated day 4 EB cells (Fig. S3). This indicates that despite the increase in Snail and Twist expression, Geminin knockdown results in a phenotype that includes reduced cell migration.

In addition to regulating gene expression, Geminin can control the fidelity of DNA replication, by binding the pre-replication protein Cdt1 during S phase and blocking re-initiation of DNA replication within a cell cycle⁴¹. To determine whether Geminin knockdown could affect cell cycle parameters in EBs, we performed cell cycle analysis on day 4 EBs, plus or minus Dox treatment, by flow cytometry. Both induced and uninduced cells showed very similar cell cycle distributions in G1, S and G2 phases, and cells with >4N DNA content were not observed (Fig. S4A). Likewise, flow cytometry analysis demonstrated that equal percentages of cells express the mitotic marker phosphorylated-histone H3 under baseline (–Dox) and Geminin knockdown conditions (Fig. S4B). Together, these data indicate that Geminin knockdown does not affect cell cycle progression or chromosomal ploidy in EBs.

Geminin knockdown promotes mesendodermal fate acquisition by increasing Wnt signaling

Geminin over-expression represses, while Geminin knockdown increases, the expression of key genes encoding activities critical for specifying mesendodermal lineages. A number of these genes are direct transcriptional targets of Activin/Nodal signaling in anterior mesendoderm or BMP signaling in the posterior primitive streak. This suggested that Geminin may regulate gene expression by altering activation of these signaling pathways. To test whether Geminin affected BMP or Activin/Nodal signaling activities, we assessed whether Geminin knockdown altered phosphorylation of Smad1 or Smad2, indicating activation of BMP and Nodal signaling, respectively. Geminin reduction did not affect levels of either phosphorylated Smad1 or Smad2 (Fig. S5). Since FGF signaling through

Erk1/2 regulates pluripotency exit and initiation of pan-commitment programs, we assessed whether Geminin could control the levels of phosphorylated (active)-Erk1/2. However, neither over-expressing nor reducing Geminin altered Erk1/2 phosphorylation during ESC-EB fate acquisition (Fig. S5). Together, these results suggest that Geminin's activity is either downstream of or unrelated to activation of Bmp, Activin/Nodal, and FGF signaling.

We found that Gem knockdown up-regulated expression of Wnt ligands found in the primitive streak, including Wnt3, Wnt3a, and Wnt5a, and of the Wnt effector Lef1 (Fig. 3B)^{42, 43}. Therefore, we hypothesized that Gem knockdown may increase Wnt signaling levels. To test this hypothesis, we used the GemKD line to form EBs for 4 days, with or without Dox addition. We collected conditioned medium under conditions of Gem knockdown (+DoxCM) and control medium (–DoxCM) on day 4. We then treated day 4 EBs made using the parental A2lox line (which is not engineered for GemOE or KD) with +DoxCM or –DoxCM for 24 hours (Fig. 4). Following treatment with conditioned medium, mesendodermal marker expression in the A2lox EBs was analyzed by flow cytometry. We found that +DoxCM was sufficient to increase the Brachyury, Mixl1, and Eomes positive cell populations, while –DoxCM was not, by comparison with untreated control EBs (Fig. 4). This result suggests that Gem knockdown promotes mesendodermal fate through an extracellular mechanism. Additionally, 3 hour treatment with GemKD +DoxCM was sufficient to induce Brachyury, Mixl1, Eomes, and Wnt3a RNA expression, while –DoxCM was not (Fig. S6). These results indicate that Gem knockdown conditioned medium stimulates mesendodermal gene expression.

Gem knockdown increased gene expression levels of secreted Wnt ligands (Fig. 3B). The activity of Wnt3 and Wnt3a ligands is inhibited by binding of the Wnt inhibitor Dkk1 to the Wnt co-receptors, Lrp5/6. Therefore, we tested whether the inhibitory effect of Dkk1 on mesendoderm formation could be modified by treatment with conditioned medium from Gem knockdown EBs. Day 4 EBs from the A2lox line were treated concurrently with Dkk1 and with day 4 GemKD +DoxCM or –DoxCM for 24 hours (Fig. 4). As expected, the percentage of cells expressing mesendodermal markers was markedly reduced among EBs treated with Dkk1 and –DoxCM. However, when EBs were treated with Dkk1 and +DoxCM, the +DoxCM rescued Dkk1-induced inhibition of the mesendoderm population (Fig. 4), indicating that Gem knockdown conditioned medium (+DoxCM) could counteract the inhibitory effects of Dkk1 and promote mesendoderm formation. Together, these results demonstrate that Gem knockdown promotes mesendodermal fate by enhancing Wnt signaling. Since expression of both Wnt3 and Wnt3a is upregulated upon Gem knockdown (Fig. 3B), these secreted ligands may mediate this effect.

We next tested the possibility that Gem knockdown could promote other signaling pathways important in mesendodermal fate acquisition, such as Activin/Nodal, Bmp, or Fgf. Day 4 EBs from the A2lox line were treated with the Bmp inhibitor Noggin (Nog) and with day 4 GemKD +DoxCM or –DoxCM. In this case, both treatments (Noggin with +DoxCM and Noggin with –DoxCM) inhibited mesendoderm formation (Fig. S7A). Similarly, +DoxCM could not rescue the inhibitory activity of the Activin/Nodal inhibitor SB431542 (SB) or the Fgf inhibitor SU5402 (SU)(Fig. S7B-C). Together, these results indicate that Geminin knockdown does not promote mesendodermal fate by increasing extracellular Bmp, Activin/Nodal, or Fgf signaling activities.

Our observations thus far indicate that Geminin knockdown can promote mesendoderm formation through an increase in Wnt signaling activity. However, this mechanism does not appear to fully account for increased Brachyury, Mixl, and Eomes expression, because treatment with Gem knockdown conditioned medium alone did not promote mesendodermal fate acquisition to the extent seen upon cell-autonomous Gem knockdown (compare Figs. 2

and 4). Additionally, treating EBs with Dkk1 (from day 3-4) was insufficient to inhibit mesendoderm induction upon Geminin knockdown (Fig. 5). Therefore, a mechanism distinct from effects on Wnt signaling may also contribute to Geminin's ability to antagonize mesendodermal fate acquisition.

Polycomb-bound and bivalent genes are enriched among Geminin down-regulated genes

Prior work has demonstrated that Geminin interacts with the Polycomb complex³², which is required for the repression of expression of developmental genes in ESCs²⁶. We compared the Gem-regulated gene sets defined by our microarray analysis to genes bound by four Polycomb complex proteins in mouse ESCs²⁶. While 8% of genes represented on the Gene 1.0 microarray are Polycomb-bound in ESCs (dark blue bars, Fig. S8A), the Gem down-regulated gene sets were enriched 3-4-fold for Polycomb binding (30% of KD up-regulated and 22% of GemOE down-regulated genes; light blue bars, Fig. S8A). Therefore, enrichment for Polycomb-bound loci among Gem down-regulated genes, which tend to increase in expression during ESC-EB commitment, is congruent with a potential role for Gem-Polycomb cooperative repression in restraining lineage commitment.

We also surveyed the histone modification status of Gem-regulated genes, using chromatin state maps generated for mouse ESCs⁴⁴. Gem regulated genes were enriched for the 'bivalent' H3K4me3 plus H3K27me3 modification signature in ESCs: 17% of all genes on the array are bivalent in ESCs, compared with 36% and 46% of GemOE down- and up-regulated genes and 42% and 26% of KD up- and down-regulated genes respectively (Fig. S8B). In most cases, the same genes are both Polycomb-bound and bivalently marked as expected and these genes include the mesendodermal specifier genes characterized in our work above (examples, Fig. S8C). By contrast, genes marked by H3K4me3-only in ESCs, usually denoting constitutively active genes, were generally underrepresented among all Gem-regulated gene sets (Fig. S8B). Together, these data suggest that Gem down-regulates a group of target genes that are frequently Polycomb-bound and bivalently marked in ESCs and that specify mesendodermal fate.

Based on these data, we reasoned that Gem knockdown may alter Polycomb complex binding at Gem down-regulated genes. To examine Polycomb complex occupancy during EB formation, we measured enrichment of the PRC2 member Suz12 by chromatin immunoprecipitation (ChIP). We compared Suz12 ChIP enrichment in control (-Dox) versus Gem knockdown (+Dox) EBs at day 4. For all three mesendodermal genes examined (Mixl1, Gsc, Brachyury), Suz12 enrichment was markedly reduced upon Gem knockdown (Fig. 6A). In contrast, H3K4me3 enrichment increased at these loci upon Gem knockdown (Fig. 6A). We also assessed enrichment of Smad2, H3K27me3, and acetylation of H3K9 (H3K9ac) at these locations, but these were not significantly changed by Gem knockdown (Fig. S9). By day 4, H3K27me3 was not highly enriched at the locations tested (compared with H3K4me3 and H3K9ac enrichment), consistent with active expression of these genes by that time (Fig. S9, Fig. S2A). To test whether Gem could directly bind some mesendodermal genes, we also conducted Geminin ChIP in ESCs and in day 4 EBs. We found that Gem directly binds the Lef1 and Eomes genes in ESCs, in the same promoter location where Polycomb complex binding occurs²⁶. Gem binding at the Eomes promoter was lost by day 4, coincident with target gene transactivation, while Gem enrichment at the Lef1 promoter was still detected in day 4 EBs (Fig. 6B). Together with the data indicating that Gem knockdown promotes expression of these mesendodermal genes, these data suggest that Gem can bind to the promoters of and may directly inhibit transcription of Lef1 and Eomes in ESCs and/or EBs, which may contribute to Gem's ability to antagonize mesendodermal fate acquisition.

Discussion

Using inducible knockdown and overexpression of Geminin in an in vitro model of mesendodermal fate specification, we found that Geminin inhibits mesendodermal fate acquisition suppresses mesendodermal gene expression programs, while promoting maintenance of pluripotency-associated gene expression. Geminin represses the expression of mesendodermal transcription factors that are directly regulated by Wnt and/or Nodal signaling. We investigated the molecular basis of this activity and found that Geminin inhibits Wnt signaling, while Bmp, Activin/Nodal, and Fgf signaling are not affected. Additionally, Geminin bound to two genes at locations bound by Polycomb, and Geminin-repressed genes were frequently enriched for Polycomb repressor complex binding, while Gem knockdown reduced Polycomb complex occupancy (as measured by Suz12 enrichment) at several mesendodermal genes. Together, these data support roles for Geminin in repressing mesendodermal fate acquisition, through effects on both Wnt signaling and on Polycomb occupancy or activity at mesendodermal genes.

Similar to observations made upon Gem knockdown in these ESCs and during neural fate acquisition, we found that Gem knockdown in EBs did not affect cell cycle progression or ploidy³⁶. This indicates that the observed effects of Gem knockdown on cell fate are not a consequence of cell cycle alteration. Most cells can utilize alternate mechanisms for maintaining the fidelity of DNA replication, including SCF/skp2- or CDR/Ddb1-mediated Cdt1 proteolysis⁴⁵⁻⁴⁷. Therefore, in this context sufficient Gem activity may remain to perform replication-associated functions and/or these alternative mechanisms may be sufficient to maintain the fidelity of DNA replication.

Geminin antagonized the expression of many genes whose activities specify primitive streak mesendoderm. Correspondingly, Geminin knockdown increased expression both of Goosecoid, Foxa2, and Sox17 (which are expressed in the anterior primitive streak in the embryo) and of the posterior primitive streak markers Brachyury, Mixl1, and Eomes. Geminin thus contributes to the repression of both anterior and posterior primitive streak fates at gastrulation. Geminin knockdown also induced several features of EMT, including downregulation of E-cadherin and upregulation of Snail1 and Twist1 expression. However, Geminin knockdown functionally reduced migration of dissociated EB cells through an unknown mechanism. This result agrees with a recent report published while this work was under review⁴⁸, and suggests that Geminin may positively regulate genes that control mesodermal migration at primitive streak stages in the embryo. Amongst genes that were up-regulated by Geminin, the Metacore Suite analysis returned cell adhesion, cell-matrix interactions, and extracellular matrix remodeling as top GO networks, possibly indicating that Geminin promotes cell migration through effects on cell adhesion.

Geminin maintained the expression in EBs of many genes characteristic of undifferentiated ESCs. These observations are consistent with findings made in *Xenopus* embryos, where Geminin has broad roles in restraining multi-lineage commitment, some of which require intact Polycomb complex activity⁴⁹. Recently, shRNA-mediated Gem knockdown in mouse embryos was reported to result in gastrulation defects⁵⁰. Although high variability in the degree of knockdown and the absence of littermate controls complicate interpretation of some of these results, this manipulation expanded the primitive streak, consistent with our findings. However, while we found that Gem knockdown in EBs promoted down-regulation of E-cadherin, this study instead reported a negative correlation between Geminin and E-cadherin expression at post-gastrula stages. These distinctions may reflect differences in the methodologies, models, or biological processes examined, as prior work supports multiple, context dependent roles for Geminin in embryonic development.

Here we observed that Geminin knockdown increased levels of extracellular Wnt signaling activity. Wnt3 and Wnt3a, which were up-regulated upon Gem knockdown, may mediate this effect. Wnt signaling is required to form the Brachyury-expressing posterior primitive streak, as reducing Wnt signaling in embryos preferentially expands the anterior and reduces the posterior primitive streak¹⁹, while Wnt3a also preferentially induces posterior primitive streak-like populations in vitro²³. Wnt3a and Brachyury form an autoregulatory loop to promote mesodermal fates, with Brachyury promoting Wnt3a expression, while loss of this Wnt-dependent Brachyury expression causes failure to maintain mesodermal progenitors^{51, 52}. Here, increased Wnt signaling resulting from Geminin knockdown would be expected to promote this Wnt3a-Brachyury autoregulatory loop.

Two genes negatively regulated by Geminin, Eomes and Lef1, were directly bound by Geminin around the promoter. Eomes critically regulates mesoderm formation at gastrulation, with Eomes knockout in epiblast resulting in defects in fate acquisition and EMT at gastrulation^{53, 8}. Eomes may also transactivate expression of Mixl1, another mesendoderm specifier strongly repressed by Geminin in EBs^{53, 54}. Lef1, a Wnt pathway effector, also controls primitive streak formation and is required to maintain Brachyury expression during gastrulation⁴³. We are currently identifying Geminin bound locations in ESCs at a genome-wide level, which will comprehensively define locations at which gene regulation by Geminin could occur.

The mesendodermal gene programs repressed by Geminin in EBs are enriched for genes that are Polycomb-bound in ESCs, to restrain their expression. We further found that Gem and Polycomb bind two of these genes at the same location, proximal to the promoter. Additionally, at several mesendodermal genes, Geminin knockdown diminished Suz12 binding (marking Polycomb complex occupancy), while increasing H3K4me3 enrichment, which correlates with activation of gene expression. Together, these observations suggest that Geminin could repress mesendodermal gene expression by enhancing either retention or activity of Polycomb repressor complexes. This hypothesis is congruent with prior work demonstrating that Geminin can directly interact with Polycomb complexes via the PRC1 protein Scmh1 and that Gem and Polycomb cooperatively repress Hox expression during embryonic anterior-posterior patterning³². Further work will be needed to determine whether Geminin's ability to repress mesendodermal fate acquisition involves direct control of Polycomb retention or alteration of its activity at mesendodermal specifier genes.

In summary, our work here demonstrates a requirement for Geminin to restrain mesendodermal fate and gene expression programs and shows that this is associated with alterations in extracellular signaling and epigenetic regulation. These findings describe a new role for Geminin in modulating the complex interplay between extrinsic signaling and intrinsic transcriptional responses that directs the initial fate acquisition of pluripotent embryonic cells.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Dr. Lorraine Robb and the Walter and Eliza Hall Institute for the Mixl1 antibody, Dr. Michael Kyba for A2lox ESCs, Yaming Wang for assistance with flow cytometry analysis, Dr. Nicolas Christoforou and Yaming Wang for critical review of the manuscript, and the Alvin J. Siteman Cancer Center at Washington University School of Medicine for use of the Flow Cytometry Core. The Siteman Cancer Center is supported in part by Grant #P30 CA91842. This work was supported by grants from the March of Dimes (FY10-381) and NIH (GM66815) to K.L.K. The WUSTL Markey Pathway and NIH T32 Training Grant in Cellular and Molecular Biology (GM7067-35) provided partial salary support for E.C.

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Fig. 1. Gem represses mesendodermal gene expression and promotes pluripotency-associated gene expression

(A) Genes (y-axis) that decreased (green) or increased (red) in expression upon GemOE or GemKD by microarray analysis were compared with their expression change without Gem manipulation during days 1-7 of EB formation (normalized fold changes, relative to ESC expression levels). Data was expressed as a heat map. (B-C) Gene expression changes were validated by qRT-PCR in day 3-5 EBs, after Geminin KD or OE from day 0. For each gene a representative experiment +/-S.D. is shown as fold change +/-Dox. Gem suppresses mesendodermal (B) and promotes pluripotency-associated (C) gene expression.

Fig. 2. Gem antagonizes mesendodermal cell fate acquisition during EB formation

Control (–Dox) and Gem knockdown (+Dox from day 0) EBs were analyzed by flow cytometry on days 3, 4, and 5 to define percentages of cells expressing the mesendodermal markers Eomes, Mixl1, Brachyury, Foxa2, and Gata4, and the endodermal marker Sox17. Negative control cells were stained with secondary antibody alone.

Fig. 3. Gem knockdown promotes features of EMT and enhances expression of Wnt ligands and effectors

(A) Control (–Dox) and Gem knockdown (+Dox from day 0) EBs were analyzed by flow cytometry for E-cadherin expression on days 3, 4, and 5. Gene expression changes in these EBs were also defined by qRT-PCR for: (A) the EMT-associated transcription factors Snail and Twist, and (B) the Wnt ligands and effectors Wnt3/3a/5a, and Lef1. Gene expression levels were normalized to expression in day 3 EBs (–Dox). Paired t-test, *p value < .05, n = 3.

Fig. 4. Gem knockdown promotes mesendodermal fate acquisition by increasing extracellular Wnt signaling

Conditioned media (CM) was collected from day 4 control (–DoxCM) or Gem knockdown (+DoxCM) EBs. Day 4 EBs from the parental A2lox line were treated with –DoxCM or +DoxCM for 24 hours and analyzed by flow cytometry for percentages of cells expressing Brachyury (T), Mixl1, and Eomes. Recombinant Dkk1 and either –DoxCM or +DoxCM were added to day 4 EBs for 24 hours and analyzed by flow cytometry. Error bars represent S.D. from the average of three experiments. Paired t-test, *p value < .05, n = 5.

Fig. 5. Gem knockdown also promotes mesendodermal fate acquisition by a Wnt-independent mechanism

EBs generated without (–Dox) versus with (+Dox) Gem knockdown were treated with Dkk1 from day 3 to 4. Percentages of cells expressing Mixl1, Brachyury, Eomes, and Foxa2 on day 4 were defined by FACS analysis, and average results of three experiments $\pm$ S.D. are shown in the graphs. Paired t-test, *p-value < .05 or n.s. is not significant, n = 3.

Fig. 6. Gem knockdown reduces Polycomb complex enrichment and increases H3K4 tri-methylation at mesendodermal genes, and Gem directly binds to the promoter of the early mesendodermal gene *Eomes* and the Wnt signaling effector *Lef1*

(A) Day 4 EBs were generated without (–Dox) or with (+Dox) Gem knockdown and were analyzed by ChIP. ChIP enrichment for Suz12 and H3K4me3 at the *Mixl1*, *Brachyury* and *Goosecoid* genes at locations –500, +1, and +500 (relative to transcription start site) was determined by qPCR and is expressed as % input for a representative experiment (of at least three, with single qPCRs performed at each position indicated). Paired t-test was performed for each position, *p value < .05, n = 4. (B) GemKD ESCs and day 4 EBs were analyzed by ChIP for Geminin enrichment. Enrichment was defined by qPCR (in triplicate $\pm$ S.D) and expressed as % input for a representative experiment with IgG ChIP as a control. Negative control primers amplify a region 500 base pairs upstream of the *Wnt3a* transcription start site. Paired t-test, *p value < .05, n = 5.