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A stem cell–like chromatin pattern may predispose tumor suppressor genes to DNA hypermethylation and heritable silencing

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Adult cancers may derive from stem or early progenitor cells[1,2]. Epigenetic modulation of gene expression is essential for normal function of these early cells but is highly abnormal in cancers, which often show aberrant promoter CpG island hypermethylation and transcriptional silencing of tumor suppressor genes and pro-differentiation factors[3–5]. We find that for such genes, both normal and malignant embryonic cells generally lack the hypermethylation of DNA found in adult cancers. In embryonic stem cells, these genes are held in a 'transcription-ready' state mediated by a 'bivalent' promoter chromatin pattern consisting of the repressive mark, histone H3 methylated at Lys27 (H3K27) by Polycomb group proteins, plus the active mark, methylated H3K4. However, embryonic carcinoma cells add two key repressive marks, dimethylated H3K9 and trimethylated H3K9, both associated with DNA hypermethylation in adult cancers[6–8]. We hypothesize that cell chromatin patterns and transient silencing of these important regulatory genes in stem or progenitor cells may leave these genes vulnerable to aberrant DNA hypermethylation and heritable gene silencing during tumor initiation and progression.

Epigenetic gene silencing and associated promoter CpG island DNA hypermethylation are prevalent in all cancer types and are an alternative mechanism to mutations that inactivate tumor suppressor genes within a cancer cell[3–5]. These epigenetic changes may precede genetic changes in premalignant cells and foster the accumulation of additional genetic and epigenetic hits[9]. Adult cancers may derive from stem or early progenitor cells[1,2], and epigenetic modulation of gene expression is essential for the normal functioning of these early cells. We now explore whether DNA hypermethylation and heritable silencing of groups of genes in adult tumor initiation and progression might reflect chromatin properties of these genes associated with stem or precursor cells.

We compared the epigenetic status of a group of genes frequently hypermethylated and silenced in adult cancers (**Fig. 1**; associated references listed in **Supplementary Table 1** online) in both normal embryonic stem (ES) cells and malignant counterparts of these cells, embryonal carcinoma (EC) cells[10]. Notably, we found that the genes frequently undergoing promoter CpG island DNA hypermethylation in adult human cancer cells generally remained unmethylated in both ES and EC cells (**Fig. 1**). Among the genes studied, 13 of 29 (45%) were hypermethylated in a single adult colon cancer line, HCT-116, but none was hypermethylated in ES cells, and only 3% and 7% were completely methylated in the Tera-1 and Tera-2 EC lines, respectively. Thus, the key epigenetic parameter of promoter CpG island hypermethylation, which is common in a large group of genes in adult cancer cells, does not seem to be a common feature of EC cells.

In mouse ES cells, many developmental genes are maintained in a state of low transcriptional activity and are available for transcriptional increases or decreases when differentiation cues are received[11]. The genes we studied in EC cells retained this plasticity of expression, which is lacking in adult cancers when these same genes are hypermethylated. Both ES and EC cells can be induced to differentiate toward a neural lineage with all-*trans* retinoic acid (ATRA) *in vitro*[10]. The genes we studied with a high basal transcription state in EC cells (**Fig. 2a**) generally showed lower expression after ATRA treatment. However, a second, larger cluster of genes showed low to medium expression in undifferentiated Tera-2 cells (**Fig. 2a**), and the majority of these (9 of 11) showed a distinct activation pattern with increased expression upon or during differentiation (**Fig. 2a**).

Both ES and EC cells, when grown as xenografts in NOD/SCID mice, form teratomas and teratocarcinomas, respectively, in which there is spontaneous differentiation and multilineage commitment to varying degrees[10] (**Fig. 2b**). The changes in expression of the genes we studied in these tumors were similar to the changes induced by ATRA in EC cells (**Fig. 2c**; for example, see *CDKN2A* (also known as *p16*), *GATA4* and *GATA5*). E-cadherin and *SFRP5* are notable

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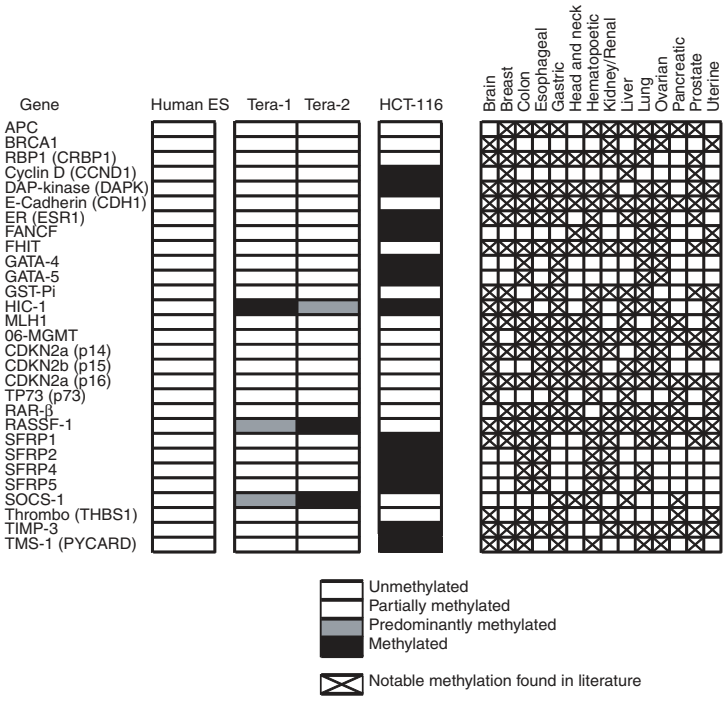

Figure 1 Genes showing frequent DNA hypermethylation and frequently silenced in adult cancers remain unmethylated in embryonal carcinoma (EC) and embryonic stem (ES) cells. We selected a panel of 29 tumor suppressor and candidate tumor suppressor genes that are known to be frequently hypermethylated in various cancer cell lines and primary tumor samples (right) from a review of the literature and from studies in our own laboratories (see **Supplementary Table 1** for a list of references used; methylation was considered notable when >5% of the human cell lines or patient samples surveyed were methylated). Methylation-specific PCR was used to determine the promoter DNA methylation status of these genes in colon cancer HCT-116 cells, in WA01 human ES cells and in two EC cell lines: Tera-1 and Tera-2 cells. Genes were characterized as unmethylated (empty boxes), fully methylated (filled black boxes) or partially methylated (gray boxes).

exceptions, and differences probably reflect multilineage commitment *in vivo* versus single-lineage differentiation *in vitro*.

We found that the promoter regions of the genes we studied each contained a combination of active (dimethylated H3K4) and repressive (trimethylated H3K27) histone modifications in ES cells (**Fig. 3a**). This 'bivalent' state has been recently described in mouse ES cells[11,12] for a subset of developmental genes that are maintained in a low expression state; here, we extend these observations to genes frequently epigenetically silenced in cancer. We have previously found trimethylated H3K27 to be enriched at each of the of the promoters of a small panel of DNA-hypermethylated genes we studied in adult cancer cells[6]. This histone modification is catalyzed by EZH2, a key component of the Polycomb group (PcG) complexes (PRC1, PRC2/3 and PRC4) (**Fig. 3b**) that maintain long-term gene silencing in diverse organisms and are essential for the normal state of stem and progenitor cells and their commitment to various tissue types[13,14]. Recent studies have linked EZH2 with DNA methyltransferases and have established a role for this protein during the induction and targeting of DNA methylation[15]. These complexes are present in stem and progenitor cells as well as in tumor cells that have similar properties[14,16].

We next matched our list of genes showing frequent DNA hypermethylation in adult cancers to the full list of PcG-targeted genes identified by

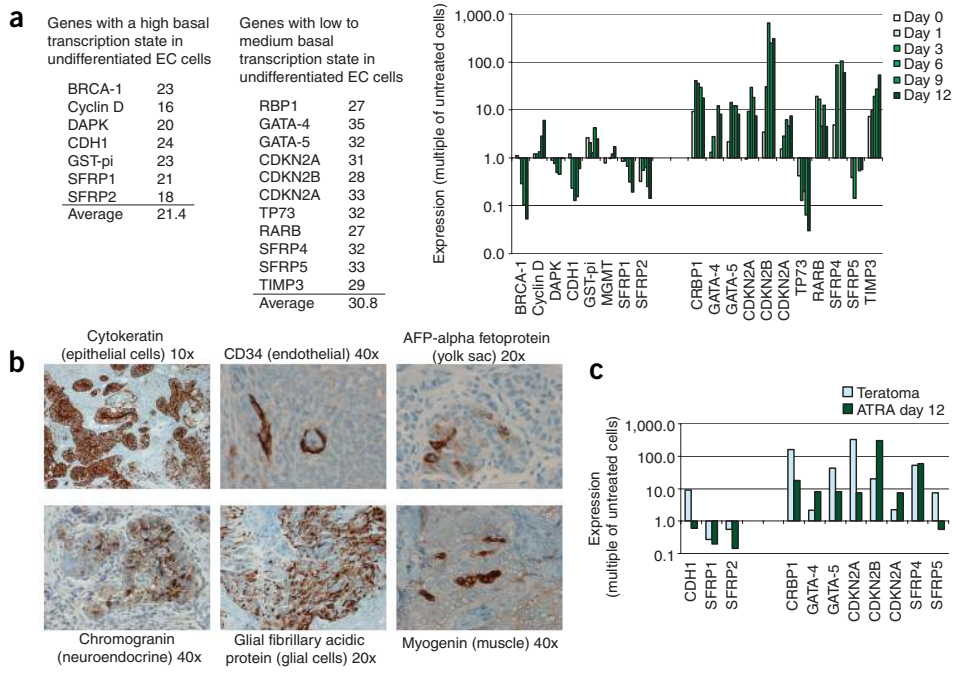

Figure 2 EC cells retain a plasticity of expression that is lacking in adult cancer cells. (**a**) Real-time quantitative RT-PCR analysis of genes frequently hypermethylated in adult cancers after treating EC cells with ATRA (2 μM) for 0 (untreated), 1, 3, 6, 9 and 12 d. PCRs were performed in triplicate, and mean threshold cycle for altered gene expression with ATRA treatment was normalized to GAPDH. The change in expression (log scale) for each gene as a multiple of expression in untreated Tera-2 cells was calculated using the formula $-\log(c^{t(\text{treatment})} - c^{t(\text{untreated})})$. Representative results of two or more independent experiments are shown. Genes are divided into two groups by threshold cycle: low to medium expression (top) (note high cycle threshold number for the PCR; mean = 30.8) and genes with high basal expression (note low threshold cycle number; mean = 21.4). (**b**) Immunohistochemistry of teratocarcinoma tumors grown in NOD/SCID mice. Immunostaining was performed by the Johns Hopkins Immunopathology Department according to established protocols on paraffin-embedded section using antibodies to CD34 (a marker for endothelial cells), chromogranin (a marker for neuroendocrine cells), cytokeratin (a marker for epithelial cells), alpha fetoprotein (AFP; a marker for yolk sac development), glial fibrillary acidic protein (GFAP; a marker for glial cells) and myogenin (a marker for muscle). (**c**) Quantitative RT-PCR was performed for RNA from 5×10^6 Tera-2 cells grown as xenographs in NOD/SCID mice until tumors reached approximately 1.5 cm in diameter. Change in expression was calculated as described above, and results from Tera2 cells treated with ATRA (2 μM) for 12 d are shown for comparison.

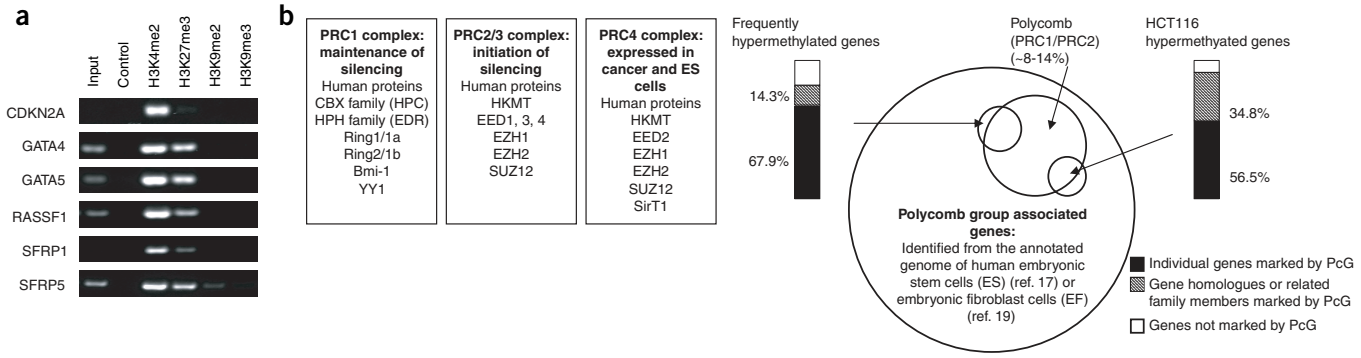

Figure 3 Genes showing frequent DNA hypermethylation in cancer are marked by 'bivalent' chromatin in ES cells, including the Polycomb group (PcG) protein–associated histone modification, methylated H3K27 (H3K27me). (**a**) Chromatin immunoprecipitation (ChIP) was performed using antibodies to dimethylated H3K4 (H3K4me2), trimethylated H3K27 (H3K27me3), dimethylated H3K9 (H3K9me2) and trimethylated H3K9 (H3K9me3) on WA09 human ES cells. PCR was performed for regions within the CpG islands and near the transcription start sites of *CDKN2A*, *GATA4*, *GATA5*, *RASSF1*, *SFRP1* and *SFRP5*. Representative results of two independent experiments are shown. A negative control without antibodies is included for comparison. (**b**) Left panel: Polycomb repressive complexes 1, 2/3 and 4 are shown. Right panel: genes showing frequent DNA hypermethylation in adult cancers are targeted by PcG target genes in human ES cells and EF cells. We compiled a list of genes marked by PcG proteins and estimated in two recent studies to represent between ~8% (PRC2; ES cells)[17] and ~14% (PRC1/PRC2; EF cells)[19] of the annotated genome. In these lists, we identified our studied genes showing frequent DNA hypermethylation in adult cancers (top left); 68% were associated with PcG in either ES or EF cells in the above studies. An additional 14% had related proteins under Polycomb control. Similar results were seen using 23 genes newly identified by microarray studies (data not shown) to be DNA hypermethylated in HCT-116 cells (top right).

recent studies of human and mouse ES[17,18] and embryonic fibroblast (EF) cells[19]. These genome-wide tiling studies identified between ~8% (PRC2; ES cells)[17] and ~14% (PRC1/PRC2; EF cells)[19] of the annotated genome to be PcG regulated in these cell types. Notably, we found that 68% of the genes in **Figure 1** were associated with PcG in either ES or EF cells, as were 56.5% of 23 genes (**Fig. 3b**) newly identified as hypermethylated in HCT-116 cells (K.E.S. *et al.*, data not shown).

Among embryonic cells, the degree of lineage commitment may determine the balance of chromatin modifications at gene promoters and the genes' basal expression levels[11]. Similarly, this may be true for compartments of precursor cells in adult renewing cell systems from which adult cancers derive. We found it interesting that of the genes from **Figure 1** that were identified as PcG targets, approximately 50% were listed as PcG targets in both ES and EF cells. However, the remaining 50% were listed as unique PcG targets in either ES or EF cells. This might explain how different patterns of hypermethylated genes in adult cancers might, then, reflect their chromatin status in cells from which cancers develop.

We found that although the genes studied in EC cells shared the bivalent chromatin pattern seen in ES cells (**Fig. 4a**,**b**), they also acquired two additional key repressive marks characteristic of adult cancers in EC cells. Thus, trimethylated H3K9, which is characteristic of silenced transcription in pericentromeric regions[20], and to a lesser and more variable extent, dimethylated H3K9, were enriched at the

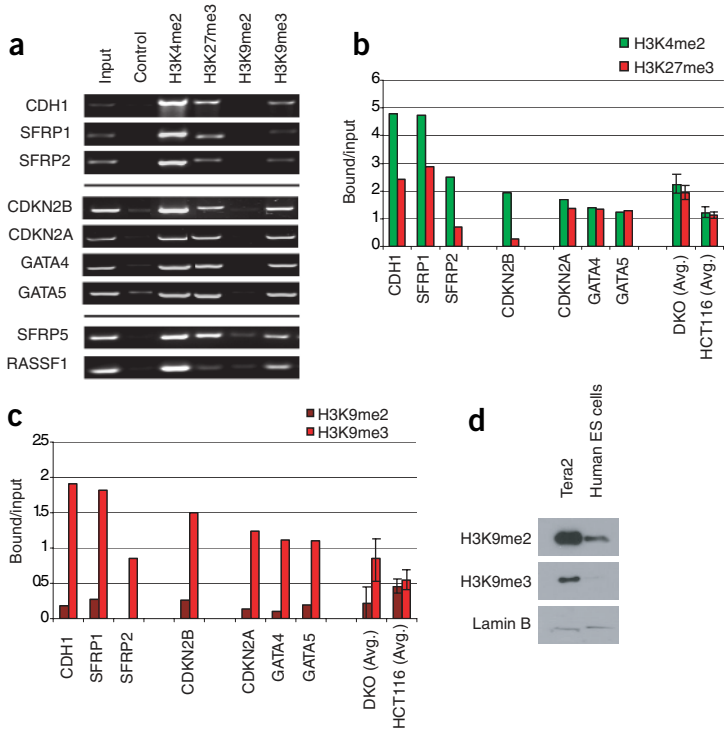

Figure 4 Genes studied in EC cells share the bivalent chromatin pattern seen in ES cells but add additional repressive marks characteristic of these same genes when DNA demethylation is induced in adult cancers. (**a**) ChIP was performed using antibodies to H3K4me2, H3K27me3, H3K9me2 and H3K9me3 on Tera-2 cells. PCR was performed for regions within the CpG islands and near the transcription start sites of *CDH1*, *SFRP1*, *SFRP2*, *CDKN2B*, *CKDN2A*, *GATA4*, *GATA5*, *SFRP5* and *RASSF1*. Representative results of two independent experiments are shown. A negative control without antibodies is included for comparison. (**b**) Quantification of representative gels shown in **a** showing the ratio of H3K4me2 (active mark) and H3K27me3 (repressive mark) to input DNA in Tera-2 cells for *CDH1*, *SFRP1*, *SFRP2*, *CDKN2A*, *CDKN2B*, *GATA4* and *GATA5*, and as a mean for four of these genes (*SFRP2*, *SFRP5*, *GATA4* and *GATA5*) from previous studies[6] of HCT-116 DKO and wild-type cells (far right). Error bars indicate s.e.m. (**c**) Ratio of H3K9me2 and H3K9me3 to input DNA in Tera-2 cells for *CDH1*, *SFRP1*, *SFRP2*, *CDKN2B*, *CDKN2A*, *GATA4* and *GATA5*, and as an average for four of these genes (*SFRP2*, *SFRP5*, *GATA4*, *GATA5*) from previous studies[6] in HCT116 wild-type cells, where each of the genes shows DNA hypermethylation and lacks any basal transcription, and in HCT116 DKO cells, where each gene has become fully DNA demethylated and is re-expressed[6] (far right). (**d**) Protein blot analysis for the repressive histone modifications, dimethylated H3K9 and trimethylated H3K9 in Tera2 cells and the human ES cell line WA01. Lamin B was used as a loading control.

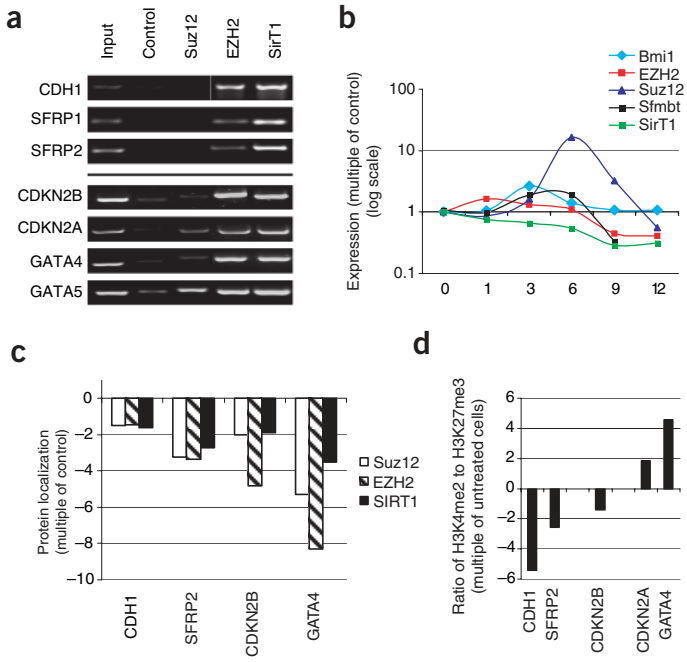

Figure 5 Changes in histone modifications and localization of known Polycomb group (PcG) proteins to the gene promoters in Tera-2 cells with ATRA-induced differentiation. (**a**) ChIP was performed using antibodies against Suz12, EZH2 and SIRT1 in Tera-2 cells. We studied genes having low to medium basal expression in undifferentiated Tera-2 cells that are upregulated with differentiation (*CDKN2A*, *GATA4*, *GATA5* and *CDKN2B*) and genes with high basal expression in Tera-2 cells that are downregulated with differentiation (*CDH1*, *SFRP1* and *SFRP2*). Representative results of two independent experiments are shown. A negative control without antibodies is included for comparison. (**b**) RT-PCR was performed as described in **Figure 2** for Bmi1, Suz12, EZH2, Sfmbt and SIRT1 during ATRA-induced differentiation of Tera-2 cells. Change in expression as a multiple of expression in untreated cells (log scale) is shown after 0, 1, 3, 6, 9 and 12 d of differentiation. (**c**) Real-time ChIP PCR shows a reduction in PcG protein localization to the promoters of *CDH1*, *SFRP2*, *CDKN2B* and *GATA4* after ATRA-induced differentiation (2 μM, 10 d) of Tera-2 cells. Protein localization is shown as a multiple of that in untreated cells is shown. A representative experiment from two or more PCRs is shown. (**d**) Quantitative ChIP PCR showing change in the H3K4me2/H3K27me3 ratio after 10 d of ATRA treatment in genes that are downregulated after ATRA treatment (*CDH1* and *SFRP2*) and genes that are upregulated after ATRA treatment (*CDKN2B*, *CDKN2A* and *GATA4*). A representative experiment from two or more PCRs is shown.

promoters (**Fig. 4a**,**c**). Both of these H3K9 marks are characteristic of DNA-hypermethylated genes in adult cancers[6]. In both Neurospora and *Arabidopsis thaliana*, mutations in genes encoding histone methyltransferases that catalyze H3K9 methylation cause significant loss of genomic DNA methylation[21–23]. We found it interesting that in the EC cells, global levels of both of the H3K9 repressive marks were considerably higher than in ES cells (**Fig. 4d**), suggesting a permissive background for the promoter changes in the neoplastic cells.

Although the chromatin pattern identified above in EC cells is similar to that for DNA-hypermethylated genes in adult cancers, several important differences exist. First, in adult cancer cells, the activating mark, methylated H3K4, is diminished, and these genes generally have fully repressive rather than bivalent chromatin[6,24]. However, this activating mark is enriched again when expression and DNA demethylation of these genes in adult cancer cells are induced, either by genetic knockout of DNA methyltransferases or treatment with 5-aza-2′-deoxycytidine (DAC)[6,24]. Second, in adult cancers, the repressive chromatin present for DNA-hypermethylated genes is initially more enriched for dimethylated H3K9 (refs. 6,24) than is seen in the EC cells. The ratio of trimethylated H3K9 to dimethylated H3K9 in EC for unmethylated genes is greater than 5:1 in Tera2 cells, compared with a mean of 1.2:1 (ref. 6) for DNA-hypermethylated promoters in adult cancers (**Fig. 4d**). Notably, in EC cells, the fully methylated *RASSF1* gene and the minimally methylated *SFRP5* gene both demonstrated an increased presence of the dimethylated H3K9 mark at their promoters (**Fig. 4a**). Most importantly, when DNA-hypermethylated genes are demethylated in adult cancer cells, dimethylated H3K9 is the only repressive mark uniformly reduced[6,24], and the ratio of trimethylated H3K9 to dimethylated H3K9 increases to 4:1 (see the mean for previously published results in **Fig. 4d**), a value virtually identical to that for unmethylated genes in EC cells[6,24] (**Fig. 4d**).

The chromatin findings for genes in EC cells present an opportunity to study how the various repressive histone modifications and the proteins that maintain them are altered with differentiation of these cells. This is a key question, as DNA-hypermethylated genes in adult cancers are very deeply, heritably repressed and thus are difficult to reactivate unless the DNA methylation is removed. We first observed that the key PcG proteins SUZ12, EZH2 and SirT1 were enriched at the promoters of the genes in EC cells (**Fig. 5a**). The steady-state levels of these proteins fell (**Fig. 5b**), as did their levels at the above promoters, with ATRA treatment (**Fig. 5c**). Additionally, several PcG proteins, including Bmi1, Suz12 and Sfmbt, showed a transient increase in expression at various points during the differentiation process, followed by a lowering of expression as cells entered a more differentiated state. These data support the extensive work of others in discerning a role for this family during normal differentiation[16].

In mouse ES cells, many developmental genes marked by the above bivalent chromatin states are maintained in a low expression state but demonstrate a plasticity of chromatin and expression by increasing transcription and shifting to a more monovalent active chromatin pattern when differentiation cues are received[11]. We see this for *CDKN2A* (also known as *p16*) and *GATA4*, which have a moderate to low basal expression state in EC cells and an equivalent initial ratio of dimethylated H3K4 to trimethylated H3K27 (**Fig. 4a**,**b**) but adopt a more active, monovalent state as their expression is distinctly increased by ATRA (**Fig. 5d**). A second subset of genes (*CDH1*, *SFRP1* and *SFRP2*) showing frequent DNA hypermethylation in adult cancers and with a higher basal expression level in EC cells and a higher initial ratio of active to repressive marks (**Fig. 4a**,**b**), generally showed lower expression after ATRA treatment (**Fig. 2a**) and showed a decrease in the ratio of dimethylated H3K4 to trimethylated H3K27 (**Fig. 5d**). An exception is *CDKN2B* (also known as *p15*), which is expressed at an intermediate level in undifferentiated cells and demonstrates significant upregulation with differentiation (**Fig. 2a**) but already shows an active, monovalent chromatin state in EC cells (**Fig. 4a**,**b**) that is not significantly altered with differentiation (**Fig. 5d**).

If a stem cell gene promoter chromatin pattern, including PcG-mediated repressive histone modifications, might help render certain genes vulnerable to DNA hypermethylation, can one perturb the system in embryonic cells to further test this hypothesis? We tested this by forcing overexpression of Bmi1, a central component of PRC1. PRC1 is involved in recognition of the H3K27 mark established by EZH2

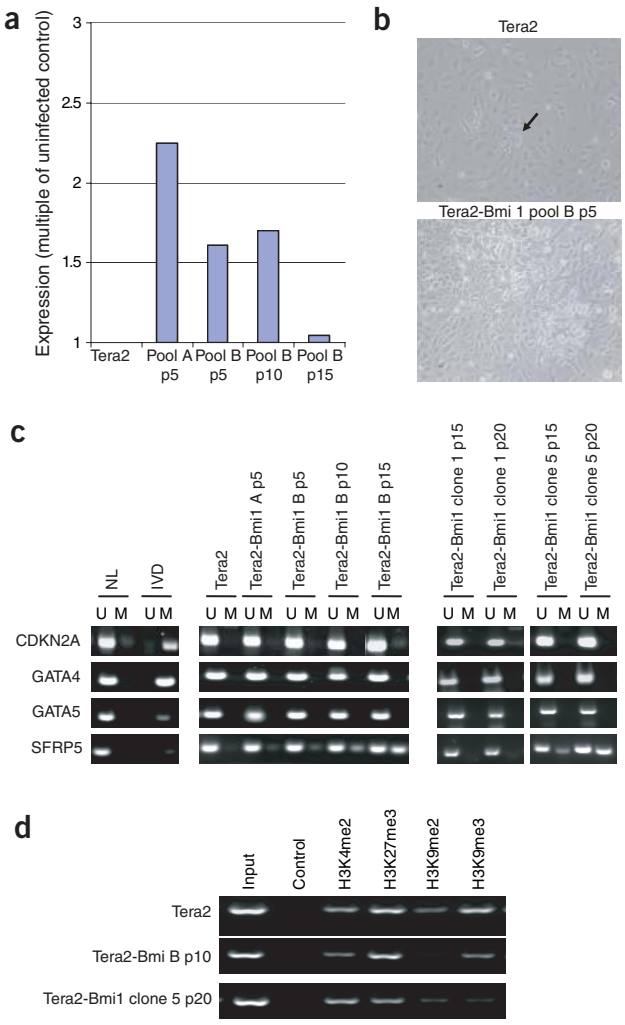

Figure 6 Overexpression of Bmi1 can cause progressive promoter DNA methylation of the *SFRP5* gene in EC cells. (**a**) Tera2 cells were stably infected with a Bmi1-expressing lentivirus, and quantitative RT-PCR for Bmi1 transcript is shown in Bmi1-infected pools of cells at passages 5 (p5), 10 and 15 post-infection. A representative experiment from two PCRs is shown. (**b**) Wild-type Tera2 (top) and Bmi1-overexpressing Tera2 cells (bottom) in culture (10× magnification). Arrow indicates small, infrequent cluster of cells in Tera2 showing increased proliferation and loss of contact inhibition. (**c**) Methylation analysis by methylation-specific PCR (MSP) for *CDKN2A*, *GATA4*, *GATA5* and *SFRP5* for Tera2 and Bmi1-overexpressing pools at passages 5, 10 and 15 and five individual clones at passages 15 and 20. For clones, representative results for two of five clones are shown. For MSP, representative results of two independent experiments are shown. M = methylated signal; U = unmethylated. Normal lymphocytes (NL) and *in vitro*–methylated DNA (IVD) are included as positive and negative controls for methylated DNA. (**d**) ChIP was performed at the *SFRP5* promoter in Tera2 cells, in pooled Bmi1 infected cells at passage 10 post-infection and in a single clone of Bmi1-infected cells at passage 20. ChIP was performed using antibodies to H3K4me2, H3K27me3, H3K9me2 and H3K9me3 as described in **Figure 4**.

in the PRC2 complex and has a role in subsequent maintenance of PcG-mediated long-term gene silencing[13,14]. Bmi1 is endogenously expressed in the wild-type Tera2 cells and shows a transient increase and subsequent decline during ATRA-induced differentiation (**Fig. 5b**). Forced, stable overexpression of Bmi1 in these cells resulted in an initial increase in *BMI1* mRNA that was sustained for more than ten passages and then returned to baseline (**Fig. 6a**). This was accompanied in pooled cells, and multiple cloned populations studied, by an overall increase in cell proliferation, cell number and loss of contact inhibition of subsets of cells *in vitro* that was seen only infrequently in wild-type cells (**Fig. 6b**). The overexpression of Bmi1 did not acutely induce methylation of most unmethylated tumor-suppressor genes examined, including *CDKN2A* (also known as *p16*), E-cadherin, *GATA4* or *GATA5*. However, in the pooled cells and in two of five separate clones studied, increased DNA methylation of the Wnt antagonist gene *SFRP5* occurred over time (**Fig. 6c**), a result never observed in wild-type cells over chronic passages. The histone modifications at the *SFRP5* promoter remained unchanged in the pooled Bmi1-overexpressing cells through passage 10 (**Fig. 6d**). However, as DNA methylation increased at passage 20 for one clone examined, the ratio of dimethylated H3K9 to trimethylated H3K9 became approximately equal (**Fig. 6d**) and thus was similar to that for DNA-hypermethylated genes in adult cancers[6] (**Fig. 4c**).

In summary, our studies demonstrate that genes showing frequent DNA hypermethylation and deep transcriptional silencing in adult cancers usually lack such DNA methylation in normal and neoplastic embryonic cells. However, the chromatin of these genes is virtually identical in embryonic cancer cells to that of the genes in adult cancers, especially when the DNA methylation in the latter cells is reduced and the involved genes are re-expressed. The repressive pattern for the EC cells may, then, represent a 'transition' state somewhere between that for genes in ES cells and that for fully DNA-hypermethylated and tightly silenced genes in adult cancers that facilitate neoplastic progression. When DNA demethylation is transiently induced by drugs in DNA-hypermethylated genes in adult cancers, they retain a promoter chromatin pattern virtually identical to what we now show in EC cells; this may be important in explaining why these same genes reacquire the DNA methylation and silencing once the drug is removed[6,25].

In terms of human cancer biology, our findings suggest that a stem cell–like promoter 'ground state' involving the key PcG mark, trimethylated H3K27, may be indicative of the contribution of stem cell and/or progenitor cells to the derivation of adult cancers (**Supplementary Fig. 1** online). We and others have suggested that stem and progenitor cells are especially at risk for cancer initiation owing to their continued expansion during states such as chronic wound healing and inflammation[9,26]. If further repressive marks for H3K9 are added to the stem cell chromatin state described here, the combination of H3K27 and H3K9 methylation may provide a program for making key tumor suppressor genes vulnerable for imposition of promoter CpG island DNA methylation during such expansion (**Supplementary Fig. 1**). These changes may enhance the likelihood of tumor initiation and progression from cell transformation as it renders a transient 'transcription-ready' state to one of heritable, permanent gene silencing.

METHODS

Cell culture. Tera-1, Tera-2 and HCT-116 (ATCC) cells were maintained in McCoy's 5A medium supplemented with 10% FBS and grown at 37 °C under 5% CO_2, as were HCT 116 cells in which both DNA methyltransferases 1 and 3b are genetically deleted (DKO cells)[27]. DNA for methylation-specific PCR (MSP) was isolated in the laboratory of S.J.S. (Johns Hopkins University) from the human ES cell line WA01 (WiCell Research Institute) according to established protocols. Cross-linking for human ES cell chromatin immunoprecipitation (ChIP) and subsequent DNA isolation was performed in the laboratory of L. Cheng (Johns Hopkins University) using the human ES cell lines WA01 and WA09 (WiCell Research Institute).

Bisulfite treatment and MSP. Bisulfite treatment and MSP were performed as described previously[28]. DNA was extracted following a standard phenol-chloroform

extraction method. Primer sequences and cycling conditions are included in **Supplementary Table 2** online. Any information not included is available by request from the authors.

RNA purification and real-time RT-PCR analysis. RNA was isolated with TRIzol Reagent (Invitrogen) according to the manufacturer's instructions. For RT-PCR, 1 μg of total RNA was reverse transcribed using the Superscript First-Strand Synthesis System (Invitrogen). Quantitative real-time RT-PCR was performed as described previously[29]. PCR primers and amplification conditions are included in **Supplementary Table 2**. Any information not included is available by request from the authors.

ChIP. ChIP assays were performed as described previously[6], with the modification that immunoprecipitation was performed using Dynal Magnetic beads purchased from Invitrogen (Protein A beads (100-02D) and Protein G beads (100-04D)). Antibodies to trimethylated H3K27, dimethylated H3K9 and trimethylated H3K9 were produced as previously described[6]. Antibodies to Suz12 (Abcam); H3K4me2, EZH2 (Upstate) and SIRT1 (Delta Biolabs) were purchased from commercial sources. Primers and amplification conditions are included in **Supplementary Table 2**. Any information not included is available by request from the authors.

Lentiviral vector preparation, viral production and infection. An untagged Bmi1 lentiviral expression clone was generated using the full-length cDNA from a pBABE-puro retroviral construct (see Acknowledgments) and using the ViraPower Promoterless Lentiviral Gateway System (Invitrogen). Briefly, a Bmi1 entry clone with an intact stop codon was incorporated along with a second UbC promoter–containing entry clone obtained with this kit (pENTR5′-UbCp) into the pLenti6/R4R2/V5-DEST vector from Invitrogen. The expression clone was transformed into Stbl3-competent cells (Invitrogen), and Bmi1-containing recombinants were selected using ampicillin and blasticidin resistance and were confirmed by restriction digest. Plasmid DNA was purified using GenElute Plasmid Maxiprep Kit (Sigma-Aldrich), and lentivirus was packaged in 293FT cells and infected in Tera2 cells using the manufacturer's recommended protocols. Blasticidin (0.5–1 μg/ml) was added to complete medium 48 h post-infection, and stable expressing pools and clones were maintained in 0.5 μg/ml blasticidin for the duration of the experiments.

Note: Supplementary information is available on the Nature Genetics website.

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AUTHOR CONTRIBUTIONS

J.E.O. and S.B.B. designed this study. J.O., K.M.M., X.Y., K.E.S., H.P.M., W.C., V.C.D. and K.P. contributed collaborative experimental results. L.C. and S.G.S. managed the growth and manipulation of ES cells. D.M.B. assisted with characterization of the teratocarcinoma cell explants. D.N.W. and J.G.H. assisted J.E.O. and S.B.B. with data analysis and preparation of the manuscript. L.C. and W.Y. helped with performance and interpretation of microarray results that contributed to the data. T.J. provided antibodies generated in his laboratory for most of the histone modifications studied by ChIP analysis.

COMPETING INTERESTS STATEMENT

The authors declare competing financial interests (see the *Nature Genetics* website for details).

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