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Silencing of cancer-germline genes in human preimplantation embryos: Evidence for active *de novo* DNA methylation in stem cells

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ABSTRACT

Several human germline-specific genes rely principally on DNA methylation for repression in somatic tissues. Many of these genes, including *MAGEA1*, were qualified as cancer-germline (CG), as they become activated in tumors, where losses of DNA methylation are common. The developmental stage at which CG genes acquire DNA methylation marks is unknown. Here, we show that in human preimplantation embryos, transcription of CG genes increases up to the morula stage, and then decreases dramatically in blastocysts, suggesting that CG gene silencing occurs in blastocyst stem cells. Consistently, transfection studies with *MAGEA1* constructs in embryonal carcinoma (EC) cells, which represent a malignant surrogate of blastocyst-derived stem cells, revealed active repression and marked *de novo* methylation of *MAGEA1* transgenes in these cells. Active repression of the endogenous *MAGEA1* gene in human EC cells was evidenced by its rapid re-silencing following prior induction with a DNA methylation inhibitor. Moreover, *de novo* DNA methyltransferases DNMT3A and DNMT3B appeared to contribute to the silencing of *MAGEA1* and other CG genes in EC cells. Altogether our data indicate that CG genes like *MAGEA1* are programmed for repression in the blastocyst, and suggest that *de novo* DNA methylation is a key event in this process.

1. Introduction

DNA methylation, occurring mainly at CpG dinucleotides in mammalian genomes, has been associated with long-term transcriptional repression. The role of DNA methylation in regulating tissue-specific gene expression has however been a matter of debate [1,2]. Evidence indicates that most tissue-specific genes use other regulatory mechanisms (e.g. histone modifications), and that DNA methylation, when present, serves merely as an additional layer of repression [3]. More recently however, it was reported that several genes with germline-specific expression use DNA methylation as a primary mechanism for selective repression in somatic tissues [4,5]. Many of these germline-specific genes were found to become aberrantly activated in tumors of somatic origin, where losses of DNA methylation are often observed [4,6]. Genes with this particular expression pattern include the human *MAGEA1* gene, and were grouped under the term cancer-germline (CG) genes.

How CG genes acquire differential DNA methylation states in somatic and germline cells is unclear. In mammalians, cell-type specific DNA methylation patterns are established during development by complex processes involving both DNA demethylation and *de novo* methylation [7]. Thus, soon after fertilization the genome undergoes a wave of DNA demethylation, which probably serves as an epigenetic resetting. Processes of *de novo* methylation subsequently occur either in stem cells at the blastocyst stage, or later during differentiation into more committed cells [8]. Some DNA sequences, which were methylated in early embryonic stages, may later become demethylated in specific cell types [8]. Central to the establishment and maintenance of DNA methylation marks are the DNA methyltransferases DNMT1, DNMT3A, and DNMT3B [9]. DNMT1 is essentially involved in DNA methylation maintenance, by copying pre-existing methylation sites onto the newly synthesized strand. DNMT3A and DNMT3B instead have *de novo* DNA methylation activity, and are responsible for the establishment of new DNA methylation marks in the developing embryo. Consistently, these two enzymes are highly expressed in embryonic stem (ES) cells, whereas they are expressed at low levels in differentiated somatic cells [10].

A recent study showed that *MAGEA1* and other CG genes are silent and methylated in human ES cells [11], which derive from the inner cell mass of blastocyst embryos. Similarly, DNA methylation and silencing of CG genes were observed in human embryonal carcinoma (EC) cells [11], which represent the tumoral counterpart of ES cells [12]. These observations provided a first indication that CG gene promoters might be programmed to gain CpG methylation marks at the blastocyst stage. In the present study, we exploited

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gene expression profiling data obtained from human preimplantation embryos to determine if CG genes become repressed specifically at the blastocyst stage. Furthermore, we performed transfection studies in EC cells, to assess the ability of pluripotent stem cells to induce silencing and *de novo* methylation within the promoter of *MAGEA1*. The role of DNMT3A and DNMT3B in this process was investigated by analyzing the effect of their depletion.

2. Material and methods

2.1. CG gene expression trend analysis in human preimplantation embryos

Microarray datasets were obtained from the GEO database (NCBI; http://www.ncbi.nlm.nih.gov/geo/) under the accession number GSE18290. These datasets (Affymetrics Human Genome U133 Plus 2.0 platform) derived from six stages of human preimplantation development: 1-cell, 2-cell, 4-cell, 8-cell, morula, and blastocyst [13]. Two biological replicates were available for each stage. Signal intensities of the probes corresponding to the selected CG genes were normalized according to the relative signal of housekeeping gene probes in each sample. For each CG gene, relative expression levels across the different preimplantation stages (maximum level = 1.0), derived from the mean of the two biological replicates, and of the different probes (if multiple probes were available for a single CG gene).

2.2. Cell lines

Human melanoma cell lines MZ2-MEL and EB16-MEL were obtained from the Brussels Branch of the Ludwig Institute for Cancer Research, where they were derived as previously described [14]. Their culture conditions have been reported elsewhere [15,16]. TERA-1 human embryonal carcinoma cells were kindly provided by Dr. Wolfgang Schultz, and were cultured in Dulbecco's modified Eagle medium plus Glutamax (Invitrogen Ltd., Paisley, UK), supplemented with 1X MEM non-essential amino acids (Invitrogen), 10% fetal bovine serum (HyClone-Perbio, Brackley, UK), 100 U/ml penicillin, and 100 μg/ml streptomycin (Invitrogen), in a humidified atmosphere of 5% CO_2.

2.3. Conventional and quantitative RT-PCR

Total RNA was extracted using the Tripure isolation reagent (Roche Diagnostics GmbH, Manheim, Germany), and was treated with Turbo DNA-Free DNase (Ambion, Austin, TX). Conventional RT-PCR was performed as previously described [17]. Primers for *MAGEA2*, and *CTAG2* (*LAGE-1*) have been described elsewhere [18,19]. Quantitative RT-PCR of *ACTINB*, *MAGEA1/EGFP*, *Npt* and *MAGEA1* was performed as previously described [20,21].

2.4. Transfections

Construction of the pMAGEA1/EGFP plasmid has been previously described [20]. For transfection of the plasmid, we used the Lipofectamine transfection reagent (Invitrogen) for MZ2-MEL cells, the Exgen500 reagent (Fermentas GmbH, Leon-Rot, Germany) for EB16-MEL cells, and the TurboFect reagent (Fermentas) for TERA-1 cells, according to the manufacturer's instructions. For transient transfections, triplicate experiments were performed in 25 cm^2 flasks containing cells at about 50% confluency, and with 3 μg of plasmid per flask. Stable transfections were performed in 75 cm^2 flask containing cells at about 70% confluency, and with a mixture of 5 μg plasmid DNA and 15 μg human genomic DNA, in order to avoid high copy number integration. Two days after transfection, the cells were transferred into medium containing 2 mg/ml (MZ2-MEL), 1 mg/ml (EB16-MEL), or 400 μg/ml (TERA-1) of geneticin. Pools of transfectants, representing several hundreds of clones, were used for subsequent analysis. The number of integrated *MAGEA1/EGFP* copies in the transfected populations was evaluated according to the ratio of *Npt* DNA sequences over *FTHL17* DNA sequences (used as an endogenous reference gene), which was determined by quantitative PCR on genomic DNA samples, as described previously [21,22]. The mean transgene copy number per cell was one in MZ2-MEL, five in EB16-MEL, and seven in TERA-1 transfectants.

Transfection of TERA-1 cells with anti-sense oligonucleotides targeting the different DNMTs was performed with Lipofectin (Invitrogen), essentially as described previously [20]. Cells were transfected every other day with 25 nM of the indicated anti-DNMT oligonucleotide(s), plus 50 nM scramble oligonucleotide where indicated. Control cells were transfected with 75 nM scramble oligonucleotide. After 9 days of this treatment, either RNA or DNA was extracted from the cells for further analysis. Proper down-regulation of *DNMT* genes (between 61% and 88%) was verified by Q-RT-PCR (see Supplemental Fig. S1).

2.5. Bisulfite sequencing and MS-PCR

Methylation analyses of the *MAGEA1/EGFP* transgene were performed by bisulfite sequencing, as described previously [20]. For the analysis of the methylation level of the endogenous *MAGEA1* gene, we resorted to methylation-specific PCR (MS-PCR). Reaction conditions and validation of the MAGEA1 MS-PCR are shown in Supplemental Fig. S2.

3. Results

3.1. CG gene expression trends across human preimplantation embryonic stages

To define the expression profile of CG genes in human preimplantation embryos, we exploited gene expression datasets previously produced by Xie et al. and deposited in the GEO database [13]. These datasets derived from six preimplantation embryonic stages: 1-cell, 2-cell, 4-cell, 8-cell, morula, and blastocyst. It is worth mentioning that gene transcription is atypical during these early stages. Between fertilization and the 4-cell stage, the human embryonic genome is globally untranscribed (regardless of its methylation status), with most gene transcripts originating from the maternal oocyte [23]. Between the 4-cell and 8-cell stages however, maternal transcripts become degraded and transcription from the embryonic genome is initiated, a process known as zygote genome activation (ZGA).

Interestingly, we observed that transcription of eight representative CG genes increased between the 4-cell and 8-cell stages, and reached a peak level at the morula stage (Fig. 1). This suggested that CG gene promoters are competent for transcriptional activation at these early stages of development, and become therefore activated along with the ZGA. Most remarkably, transcription of all tested CG genes showed an abrupt decline in blastocysts (Fig. 1). Together, these observations suggested the existence of a mechanism of transcriptional repression of CG genes taking place in blastocyst cells.

3.2. Silencing of MAGEA1 transgenes in human EC cells (TERA-1)

The above data suggested that pluripotent stem cells might have the ability to actively repress CG genes. To address this issue, we decided to check if *MAGEA1* transgenes would become silenced

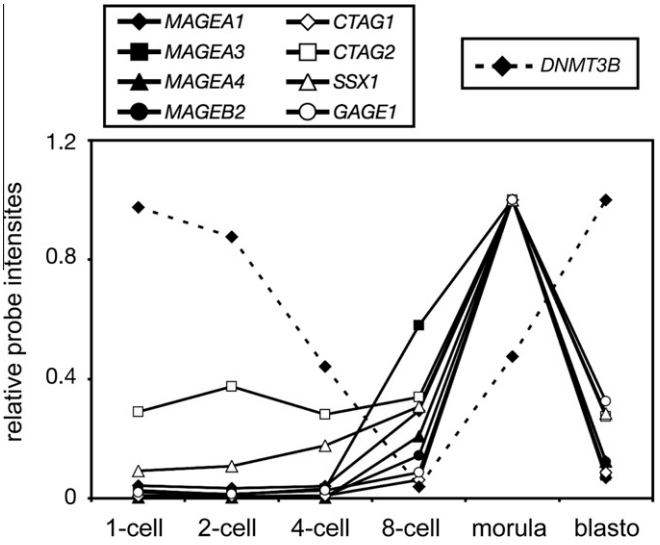

Fig. 1. Trends in mRNA expression of human CG genes during preimplantation embryonic development. Expression trends of eight CG genes are depicted (plain lines). Curves represent the relative transcript abundance (maximum level = 1), as deduced from previous microarray datasets [13]. For comparison, the expression trend of *DNMT3B* (dotted line), is also shown. Consistent with previous reports, *DNMT3B* transcripts appeared maternally deposited, decreased up to the 8-cell stage, and showed a subsequent marked increase peaking at the blastocyst stage.

following transfection into such cells. Because human ES cells are refractory to transfection, we used the human EC cell line TERA-1 as a recipient. EC cells, where CG genes are repressed and methylated [11], are commonly considered as a malignant surrogate for embryo-derived ES cells. Both cell types share indeed many common features [12], including expression of *OCT4* and *SOX2*, as well as over-expression of *DNMT3A* and *DNMT3B de novo* methyltransferases (Supplemental Fig. S3).

In a first set of experiments, we performed transient transfections of the pMAGEA1/EGFP plasmid (Fig. 2A) in TERA-1 cells, and in two recipients cell lines of somatic origin: the EB16-MEL melanoma cell line, which does not express *MAGEA1*; and the MZ2-MEL melanoma cell line, which expresses *MAGEA1*. The level of expression of the *MAGEA1/EGFP* transgene was evaluated at day 2 following transfection, and was reported that of the *Npt* gene carried on the same plasmid and driven by the ubiquitously active *SV40* enhancer/promoter. Remarkably, the level of transcription of the *MAGEA1/EGFP* transgene in TERA-1 cells was similar to that in MZ2-MEL cells (Fig. 2B). In the EB16-MEL cell line, the transgene displayed a reduced level of expression (about 20% of that in MZ2-MEL; Fig. 2B). These data indicate that, shortly after transfection, the exogenous *MAGEA1* promoter is permissive to the action of transcriptional activators, which appear to be abundant in TERA-1 cells.

We then resorted to stable transfection experiments, permitting evaluation of the expression of *MAGEA1/EGFP* transgenes at extended time points following integration into the genome of recipient cells. RT-PCR experiments performed at day 35 following transfection indicated that the *MAGEA1/EGFP* transgene maintained a high level of transcription in MZ2-MEL cells, and remained at a reduced, albeit consistent, level in EB16-MEL cells (Fig. 2C). In TERA-1 cells instead, transcription of the *MAGEA1/EGFP* transgene was markedly reduced, reaching nearly background levels (0.09% compared to MZ2-MEL). Analysis of *MAGEA1/EGFP* expression levels at different time points in TERA-1 transfectants revealed a sharp decline within the first 15 days following transfection, a further slight reduction up to day 35, and maintenance of this repressed state thereafter (Fig. 2D). Importantly, this transcriptional repression appeared to specifically affect the *MAGEA1* promoter within the transgene, since the *Npt* gene driven by a *SV40* promoter maintained

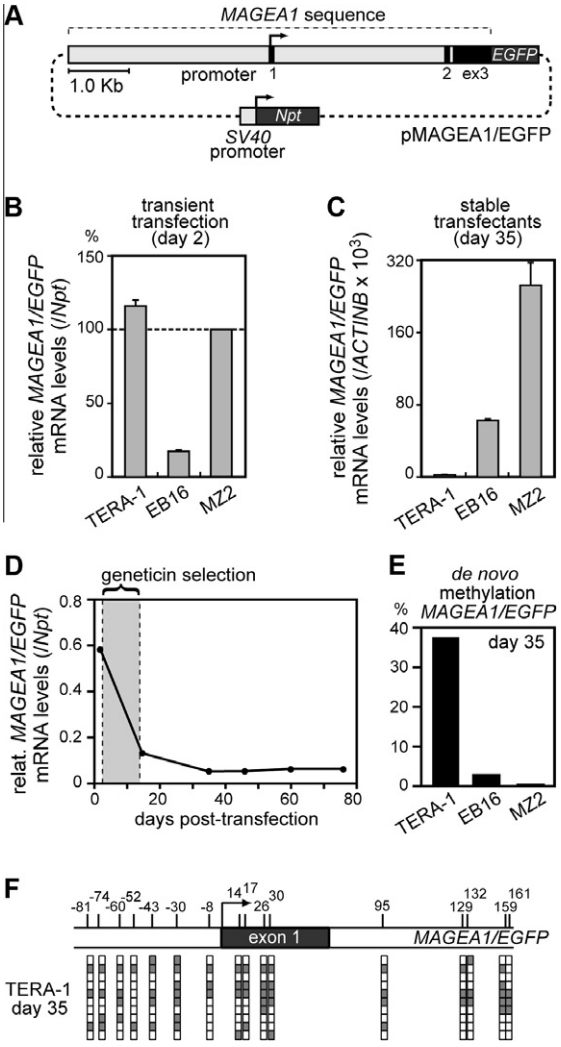

Fig. 2. Initial transcription, and later *de novo* DNA methylation-associated repression of *MAGEA1* transgenes in TERA-1 cells. (A) Schematic representation of the pMAGEA1/EGFP plasmid. The *MAGEA1* fragment is represented with dark boxes indicating exons 1, 2 and part of exon 3. The plasmid also carries the *Npt* coding region (conferring geneticin resistance, dark gray box below) driven by the *SV40* enhancer/promoter (light gray box below). Broken arrows correspond to transcription start sites. (B) *MAGEA1/EGFP* expression levels reported to that of *Npt* were evaluated 2 days after transfection into TERA-1, EB16-MEL (*MAGEA1*-negative) and MZ2-MEL (*MAGEA1*-positive). The data are expressed relative to the value in MZ2-MEL (100% reference), and derive from at least three independent transfections (bars are ±SEM). (C) Cells transfected with pMAGEA1/EGFP were selected in geneticin for 11–17 days, further cultured in the absence of the drug, and the relative expression of *MAGEA1/EGFP* was determined at day 35 post transfection by Q-RT-PCR. Data, which are expressed as a ratio to *ACTINB* $\times 10^4$, were normalized by the number of integrated transgene copies, and derive from at least three independent measurements (bars are ±SEM). (D) Q-RT-PCR was used to assess the relative expression of *MAGEA1/EGFP* (reported to the *Npt* level) at different time points post-transfection in TERA-1 cells, either before or after selection with geneticin (shaded area). (E) The level of *de novo* methylation within the 5′ region of the *MAGEA1/EGFP* transgene (16 CpG sites between positions −81 to +161) was evaluated by bisulfite sequencing in each group of transfectants at day 35: percentage of methylated CpGs derive from the sequencing of 10–13 clones in each group. (F) Detailed bisulfite sequencing results in TERA-1 transfectants. Vertical bars correspond to the location of CpG sites, with their position. Below, each line of boxes corresponds to a clone sequence (unmethylated CpG: empty box; methylated CpG: filled box).

a significant level of expression over time in TERA-1 transfectants (mean level: 163 ± 53 *Npt*/*ACTINB* $\times 10^4$ ratio).

The methylation status of the 5′-region of *MAGEA1* within the transgene was assessed by bisulfite sequencing (Fig. 2E). Consistent with our previous studies [17], CpG sites within the transfected *MAGEA1* sequence remained mostly unmethylated in the expressing

MZ2-MEL cell line. A weak level of *de novo* methylation (3.4%) was detected in EB16-MEL, which is consistent with the low, yet sustained, level of *MAGEA1/EGFP* expression in these cells. In TERA-1 cells instead, the transfected *MAGEA1* sequence underwent marked *de novo* methylation, reaching a mean level of 38% methylated CpG sites. This *de novo* methylation appeared to affect all CpG sites within the analyzed region of *MAGEA1* (Fig. 2F). Together these data indicate that TERA-1 cells have the ability to counteract the initial effect of transcriptional activators on the promoter of *MAGEA1*, and to impose its silencing by a process involving *de novo* DNA methylation.

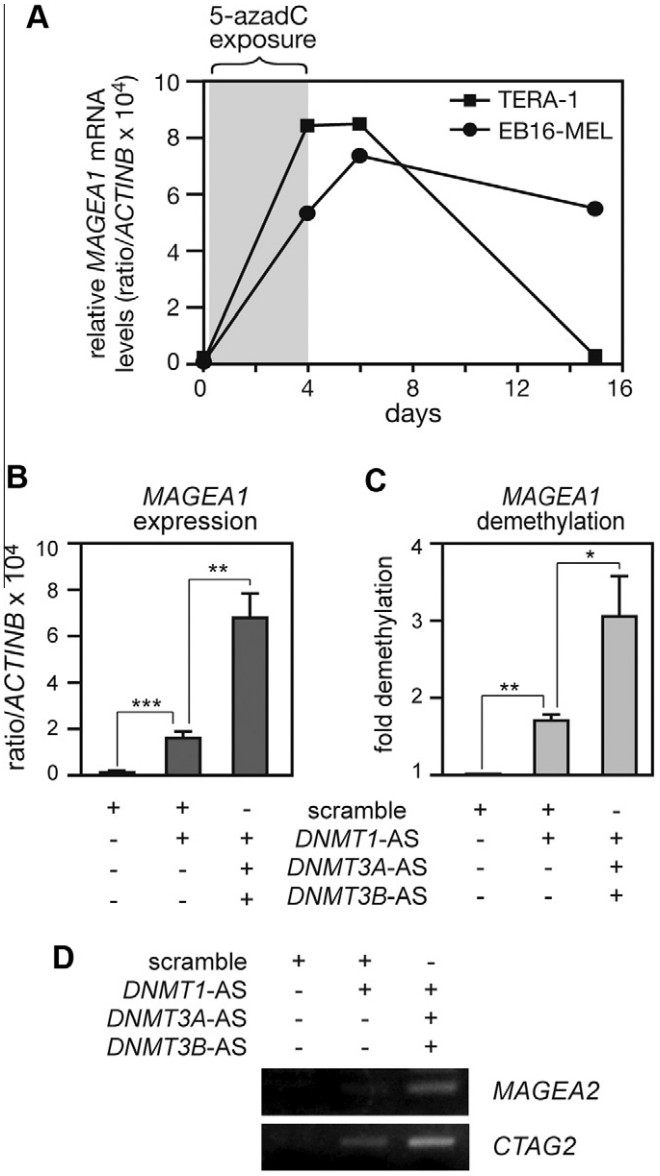

Fig. 3. Effect of DNMT depletion on the expression of the endogenous *MAGEA1* gene in TERA-1 and EB16-MEL cells. (A) Cells were exposed to 2 μM 5-azadC during 4 days. *MAGEA1* induction was evaluated by quantitative RT-Q-PCR right after treatment, or at different intervals in cells that were further cultured in the absence of 5-azadC. Untreated cells served as control. Data represent the mean of two Q-PCR experiments, each in triplicate. (B) TERA-1 cells were transfected every other day with the indicated antisense oligonucleotides, and their RNA was extracted after 9 days of treatment. Q-RT-PCR was performed to evaluate transcription of the *MAGEA1* gene. Data are expressed as a ratio to *ACTINB* $\times 10^4$, and derive from at least four independent transfection experiments (bars are ±SEM). (C) The methylation status of the 5′ region of *MAGEA1* was evaluated by MS-PCR in TERA-1 cells that had been treated during 9 days with the indicated antisense oligonucleotides. Data represent the fold demethylation relative to the scramble condition, and are the mean (±SEM) of three measurements in two independent transfection experiments. (B and C) *P* values were determined by Student's *t* tests: *<0.05; **<0.001; ***<0.0001. (D) Conventional RT-PCR was performed to check the activation of two other CG genes, *MAGEA2* and *CTAG2*, following transfection of the indicated antisense oligonucleotides.

3.3. Role of de novo DNMTs in MAGEA1 silencing in EC cells

To confirm the role of DNA methylation in the repression of the endogenous *MAGEA1* gene in EC cells, we assessed induction of this gene in TERA-1 cells that were exposed to the DNA methylation inhibitor 5-aza-2′-deoxycytidine (5-azadC). Consistently, the *MAGEA1* gene was induced in TERA-1 cells after 4 days of treatment with 2 μM 5-azadC (Fig. 3A). Interestingly, *MAGEA1* expression in TERA-1 cells disappeared as soon as day 11 following removal of the DNA methylation inhibitor (Fig. 3A). In contrast, *MAGEA1* induction was still present at this time point in similarly treated EB16-MEL cells (Fig. 3A). The rapid re-silencing of *MAGEA1* in 5-aza-dC treated TERA-1 cells suggested that the active repression mechanism, which we found to be directed against transgenic *MAGEA1* sequences in these cells, also acts on the endogenous *MAGEA1* gene.

We also performed experiments using previously validated antisense oligonucleotides directed against DNMT1, DNMT3A, or DNMT3B [24,25]. Previous studies using these antisense oligonucleotides in cells of somatic origin revealed a predominant role of DNMT1 in maintaining *MAGEA1* silencing [20]. In TERA-1 cells, the additional contribution of DNMT3A and DNMT3B to the silencing of *MAGEA1* was evidenced by the increased induction of *MAGEA1* in cells depleted of all three DNMTs, as compared with cells depleted of only DNMT1 (Fig. 3B). Consistently, when we evaluated DNA methylation levels within the 5′-region of *MAGEA1* by quantitative methylation-specific PCR, we observed that TERA-1 cells depleted of all three DNA methyltransferases displayed a higher degree of DNA demethylation, as compared with cells where only DNMT1 had been depleted (Fig. 3C). Similarly to *MAGEA1*, other CG genes (*MAGEA2* and *CTAG2*) showed increased induction upon depletion of all three DNA methyltransferases (Fig. 3D). These data indicate that DNMT3A and DNMT3B *de novo* methyltransferases contribute to the silencing of CG genes in TERA-1 cells.

4. Discussion

Human CG genes represent a particular group of genes, which rely principally on the acquisition of DNA methylation marks in somatic tissues to ensure germline-specific expression [26]. In the present study, we provide evidence that epigenetic repression of CG genes occurs at the blastocyst stage, and that pluripotent stem cells display a distinct property to induce *de novo* DNA methylation of such genes. It is likely that while CG genes maintain this repressed state in somatic lineages, they undergo a process of DNA demethylation and re-activation during maturation of germline cells in the developing embryo. Consistently, immunohistochemical studies showed that proteins encoded by the *MAGEA1* gene and by another CG gene (*CTAG1/NY-ESO-1*) are initially absent in germ cell progenitors, and become detectable at week 9 and 13 in the gonads of human male and female fetuses, respectively [27].

Interestingly, an increase in the expression of human CG genes was observed between the 4-cell and the morula stage. This may be attributed to the transcriptional competency of CG promoters, which are likely unmethylated at these stages, and the process of ZGA whereby many genes become activated. Whether CG genes exert a functional role in the very early embryo remains to be determined.

Elevated *MAGEA1* promoter activity was initially detected in transiently transfected TERA-1 cells, indicating the presence of potent transcriptional activators. The transgene nevertheless became efficiently silenced at later time points, upon integration into the

genome of stable EC transfectants. This delayed repression may be due to the fact that exclusion of transcriptional activators from the *MAGEA1* promoter is a time-dependent process, or that it requires the acquisition of an accomplished chromatin structure. It is known indeed that un-integrated plasmids, which are most abundant in transiently transfected cells, adopt incompletely organized chromatin structures [28]. The chromatin-dependency of *MAGEA1* silencing in EC cells was supported by our observation that it involves DNA methylation, which requires proper chromatin structure for both being established and exerting its negative effect on transcription [3,29,30].

The initial transcription of *MAGEA1/EGFP* in transiently transfected TERA-1 cells indicates that *de novo* DNA methylation within *MAGEA1* sequences in EC cells does not represent on a compliant process that adapts to a previously repressed state, but rather involves a proactive process capable of reversing the transcriptional status of the gene. In this regard, EC stem cells appeared different from somatic tumor cells, where similar transfection studies showed that *de novo* methylation of *MAGEA1* transgenes occurred only when the promoter activity was reduced beforehand, either because of lack of appropriate activators in the recipient cells or because of the introduction of inactivating mutations in the transgenic *MAGEA1* promoter [17]. Several tumor cell lines of somatic origin display indeed slow but consistent *de novo* methylation activities. In our previous experiments however, *de novo* methylation within the 5′-region of *MAGEA1* transgenes in somatic tumor cells never reached the level we observed in TERA-1 cells in the present study. Moreover, we never observed complete silencing of *MAGEA1* transgenes in somatic tumor cells, as we did here in TERA-1 cells. It appears therefore that TERA-1 cells display unique properties to induce efficient methylation and silencing of the *MAGEA1* promoter. Not surprisingly, the *de novo* methyltransferases DNMT3A and DNMT3B appeared to be involved.

In conclusion, our study revealed that human CG genes are programmed for repression in embryonic pluripotent stem cells by a mechanism that appears to involve proactive epigenetic silencing. Further studies are required to identify the epigenetic factors and DNA binding proteins that contribute to this process.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2011.11.120.

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