Published in final edited form as:
Epigenetics. 2010 April 1; 5(3): .

Autonomous silencing of the imprinted *Cdkn1c* gene in stem cells

Michelle D. Wood[1], **Hitoshi Hiura**[2], **Simon Tunster**[1], **Takahiro Arima**[2], **Jong-Yeon Shin**[3], **Michael Higgins**[3], and **Rosalind M. John**[1,*]

[1]Cardiff School of Biosciences, Cardiff University, Cardiff, UK

[2]Innovation of New Biomedical Engineering Center, University of Tohoku, Aoba-ku, Sendai Japan

[3]Department of Molecular and Cellular Biology, Roswell Park Cancer Institute, New York, NY USA

Abstract

Parent-of-origin specific expression of imprinted genes relies on the differential DNA methylation of specific genomic regions. Differentially methylated regions (DMRs) acquire DNA methylation either during gametogenesis (primary DMR) or after fertilization when allele-specific expression is established (secondary DMR). Little is known about the function of these secondary DMRs. We investigated the DMR spanning *Cdkn1c* in mouse embryonic stem cells, androgenetic stem cells and embryonic germ stem cells. In all cases, expression of *Cdkn1c* was appropriately repressed in in vitro differentiated cells. However, stem cells failed to de novo methylate the silenced gene even after sustained differentiation. In the absence of maintained DNA methylation (*Dnmt1*−/−), *Cdkn1c* escapes silencing demonstrating the requirement for DNA methylation in long term silencing in vivo. We propose that post-fertilization differential methylation reflects the importance of retaining single gene dosage of a subset of imprinted loci in the adult.

Keywords

DNA methylation; imprinted; secondary DMR; stem cells

Introduction

Studies on the DNA methyl-transferases (Dnmts) Dnmt3a and Dnmt3b and the accessory protein Dnmt3L demonstrate the necessity of de novo DNA methylation for the establishment of allele-specific gene expression.1–5 Maintenance of imprinting by Dnmt1 is only essential for a subset of imprinted genes.6–8 DNA methylation is established in one parental germline at specific cis-acting regulatory elements termed “imprinting centres” (IC).9 Both differentially methylated regions (DMRs) and imprinted regions lacking differential DNA methylation show allele-specific histone modifications suggesting a key

*Correspondence to: John R.M., JohnRM@cf.ac.uk.

role for these modifications in both the establishment and the maintenance of allele-specific gene expression.8,10–17

Mouse chromosome 7 (human chromosome 11p15) contains two IC controlling mechanistically distinct sub domains.7,18,19 One of these domains has a genetically defined IC, known as *KvDMR1*, which regulates several maternally expressed genes including *Cdkn1c*.19 The absence of DNA methylation at *KvDMR1*/IC2 is linked to repression of the normally maternally expressed genes including *Cdkn1c*.2–4,20–24 Conversely, a targeted deletion of this locus or premature termination of the non-coding, paternally expressed RNA *Kcnq1ot1* results in expression of the normally paternally silenced genes.19,25,26

Some DMRs are methylated only after fertilization and are known as secondary, somatic or post-fertilization DMRs.27–32 The maternally expressed *Cdkn1c* gene is spanned by one such DMR that is DNA methylated on the paternal allele. DNA methylation is present at the *Cdkn1c* gene only after monoallelic expression of *Cdkn1c* has been established.28 While DNA methylation catalysed by Dnmt3a and 3L is required to activate *Cdkn1c* expression,2–4 DNA methylation catalysed by the maintenance Dnmt1 is required for *Cdkn1c* repression.7,28 The histone methyltransferase, Eed, is required for complete repression of *Cdkn1c* but not the adjacent imprinted gene, *Slc22a18*.27 Silencing and DNA methylation of *Cdkn1c* also requires the SNF2-like protein, lymphoid-specific helicase-1 (Lsh/Hells) that binds to the 5' promoter DMR.33 These data indicate that permanent, heritable silencing of *Cdkn1c* occurs as a temporal sequence of events involving both histone modification and DNA methylation and requires several trans-acting factors.

Embryonic stem (ES) cell lines are established from blastocyst stage embryos and represent an excellent system for studying epigenetic events in vitro. In biparental ES cell lines, DNA methylation is present at primary DMRs, including *KvDMR1*, and only expression from the maternal *Cdkn1c* allele can be detected in in vitro differentiated cells.14 Pluripotent stem cells in the mouse can also be derived from primordial germ cells and these show many similar properties to ES cells.21,34–42 As these cells are epigenetically modified during development, removing DNA methylation and erasing parental imprints, EG cell lines can be derived that lack DNA methylation at all DMRs. In these “imprint-erased” cells, some imprinted genes are released from silencing while others are biallelically repressed. *Cdkn1c* falls into the latter category. After in vivo differentiation of imprint-erased EG cells in chimeras and derivation as primary embryonic fibroblasts (PEFs), expression of *Cdkn1c* is biallelically repressed and the gene acquires de novo DNA methylation on both alleles.21 Here, we sought to establish whether these cells would provide an opportunity to study the sequence of events that lead to silencing the *Cdkn1c* gene and to further understand the processes that result in de novo methylation at a secondary DMR. Contrary to our expectations, the silent *Cdkn1c* gene did not attract direct DNA methylation even after prolonged differentiation but, nonetheless, silencing of *Cdkn1c* was maintained.

Results

Silencing of paternal *Cdkn1c* allele during in vitro differentiation

The maternally expressed *Cdkn1c* gene is located within the IC2 domain on mouse chromosome 7 and is regulated by an imprinting control region, *KvDMR1*, located more than 200 kb away (Fig. 1A). In somatic cells, *Cdkn1c* is expressed from the maternal allele and silenced and DNA methylated on the paternal allele. Previous studies demonstrated a similar parental-specific expression pattern in ES cells differentiated in vitro for seven days.14 We used the same hybrid ES cell line, SF1-1 (*M. domesticus* × *M. spretus*), to demonstrate that parental-specific expression was apparent as early as day five of in vitro differentiation (Fig. 1B).

Silencing of non-imprinted *Cdkn1c* gene during in vitro differentiation

Imprint-erased EG stem cell lines silence *Cdkn1c* expression when they are differentiated in vivo in chimaeras and selected as PEFs.21 We sought to determine whether this silencing also occurred in vitro in EG stem cells by comparing the relative level of expression of *Cdkn1c* between two biparental embryonic stem cell lines, SF1-1 and CES3, and two imprint-erased EG stem cell lines, TMAS21G21 and Sv6.1,41 before and after differentiation. To study the initial stages of *Cdkn1c* regulation, two biparental ES cell lines, CES3 (129/Sv) and SF1-1 (*Mus domesticus* (BL6/CBA) × *Mus spretus*), were differentiated in vitro and quantitative real time PCR (QPCR) was used to compare the expression level of *Cdkn1c* in the undifferentiated cells with expression level in cells differentiated for 14 days. The level of expression of *Cdkn1c* in the biparental differentiated cells was, respectively, 8.7-fold and 13-fold higher than the level in these cells when they were undifferentiated (Fig. 1C). In a similar experiment using the EG cell lines Sv6.1 and TMAS21G, the level of expression of *Cdkn1c* was 3-fold higher and 0.3-fold higher, respectively, in the differentiated cells in comparison to undifferentiated cells suggesting relative repression of *Cdkn1c* (Fig. 1C). A comparison of *Cdkn1c* expression over 21 days of differentiation between CES3 (129/Sv) and Sv6.1 (129/Sv) was also performed. Very low expression was detected at day five of differentiation, a time point when expression of *Cdkn1c* from the paternal allele was already known to be restricted (Fig. 1B). In the biparental cells, *Cdkn1c* expression rose steadily over time to reach a maximum of 18-fold higher than in undifferentiated level, after 21-days of differentiation. Although changes in expression of *Cdkn1c* in the imprint-erased EG stem cell line followed the same profile, the maximum differentiated level reached was 3.3-fold higher than in the undifferentiated cells (Fig. 1D). Similarly low levels were detected for *Phlda2,* an adjacent imprinted gene that shares the same imprint control region as *Cdkn1c*, in differentiated EG cells (data not shown). These data demonstrate that, as in vivo, expression of the maternally expressed genes in the IC2 domain was relatively repressed in the absence of germline DNA methylation.

The *Kcnq1ot1* transcript was readily detectable in both undifferentiated and differentiated ES and EG cells (Fig. 2A and B). Expression in both EG cell lines was consistently higher than in the biparental ES cell line. The *Kcnq1ot1* transcript was also detectable in differentiated EG cells using an *RNase* protection assay, analogous to a somatic tissue, suggesting that the gene was actively expressed (Fig. 2C).

Hypomethylation of *Cdkn1c* after silencing

In somatic cells, DNA methylation of the paternal *Cdkn1c* allele extends from the promoter into the body of the gene as far as intron II.28,43 ES cells are generally derived from E3.5 blastocysts but DNA methylation is not detected at the *Cdkn1c* gene in vivo until E7.5 suggesting that undifferentiated ES cells should lack DNA methylation at *Cdkn1c*. We examined the predicted *Cdkn1c* promoter region in somatic cells by bisulphite sequencing 50 CpG sites just upstream of the predicted transcriptional start site, a region which spans the *EagI/Not1* restriction enzyme site described in our previous study.44 In neonatal brain and kidney, *Cdkn1c* was partially methylated, a pattern consistent with allele-specific DNA methylation (Fig. 3A and data not shown). An unmethylated pattern was present in two independent, undifferentiated (D0) biparental ES cell lines, CES3 and KES1, consistent with timing of derivation of ES cells. The *Cdkn1c* gene was also unmethylated in the EG stem cell line, Sv6.1 (Fig. 3B) and, as previously reported,21 in TMAS21G cells (data not shown). Southern blotting was used to demonstrate that two additional biparental ES cell lines, 129/1 and Pgk, also lacked detectable DNA methylation at *Cdkn1c* when undifferentiated (Fig. 3C). We also examined androgenetic stem cells. This type of stem cell is derived from blastocysts that have been engineered to carry two paternal genomes (monoparental).44 We found that the androgenetic AKR1 cell line also contained a hypomethylated *Cdkn1c* gene when undifferentiated (Fig. 3C).

In vivo, the *Cdkn1c* gene acquires paternal allele-specific DNA methylation within two days of establishing of allele-specific expression at E7.5.28 ES cells carry both parental alleles and show allele-specific expression of *Cdkn1c* after five days of differentiation in vitro (Fig. 1B). However, when we examined DNA methylation at the *Cdkn1c* gene after 14 days of in vitro differentiation as a monolayer, we found no evidence for differential de novo DNA methylation (Fig. 3D). EG stem cells, which are imprint-erased and lack DNA methylation at ICs, acquire de novo methylation at the *Cdkn1c* gene when these cells are differentiated in chimaeras and then isolated as PEFs.21 When we differentiated the imprint-erased EG cell line, Sv6.1, the *Cdkn1c* gene remained predominantly hypomethylated (Fig. 3D). In AKR1 cells, both *Cdkn1c* alleles are paternal in origin (predicted biallelic DNA methylation) but these cells showed a hypomethylated pattern (Fig. 3D). A region within the second intron of the gene was examined and was also found to be hypomethylated after differentiation (data not shown). There was small region of DNA methylation within the 50 CpG scanned that showed a degree of DNA methylation in some samples. However, in the SF1-1 cells, this methylation was present on both parental alleles indicating that it was not an allele-specific modification. To explore the possibility that the absence of methylation at the gene was due to the method of differentiation, the EG cell line Sv6.1 and the androgenetic cell line AKR1 were in vitro differentiated for 21 days exclusively by the embryoid body method with similar results (Fig. 3C and D).

H3K27 trimethylation at *Cdkn1c* during in vitro differentiation of EG stem cells

Differential histone modifications have been reported in somatic cells and in SF1-1 ES cells at the *Cdkn1c* gene. In particular, the silent paternal *Cdkn1c* allele is enriched for histone H3 trimethylation at lysine 27 (H3K27me3) while the active allele is enriched for histone H3 lysine 4 (H3K4Me3).14 We quantified the relative level of these marks between the *Cdkn1c*

promoter region and the *Kcnq1ot1* promoter region in AKR1 cells, where both alleles carry the paternal imprint, and in the two EG cells lines, Sv6.1 and TMAS21G, where both alleles are imprint-erased. The *Kcnq1ot1* promoter region was relatively enriched for the active mark, H3K4Me3, in both undifferentiated and differentiated cells (Fig. 4) consistent with expression of *Kcnqtot1* (Fig. 2). Conversely, the *Cdkn1c* promoter region was relatively enriched for H3K27me3 in differentiated AKR1 and EG cells (Fig. 4). However, only undifferentiated AKR1 cells were enriched for this repressive mark at *Cdkn1c*. This suggested that H3K27me3 was acquired as the EG cells differentiated and that imprint-erased EG cells might represent a more rudimentary silent state for *Cdkn1c* than AKR1 cells.

In summary, we have shown that stem cells cultured in vitro are able to repress *Cdkn1c* expression until at least 21 days of differentiation but do not acquire DNA methylation at the *Cdkn1c* secondary DMR.

Discussion

In this study we demonstrate that expression of *Cdkn1c* is suppressed in in vitro differentiated stem cells in the absence of DNA methylation. The absence of direct DNA methylation at the *Cdkn1c* gene in ES cells is a novel finding but consistent with the time point at which ES cells are derived. The *Cdkn1c* genes acquires differential DNA methylation from E7.5, four days after ES cells are normally derived.28 The absence of significant DNA methylation at the *Cdkn1c* gene after differentiation in vitro of biparental ES cells was unexpected.21 EG cells (two imprint-erased genomes, unmethylated *KvDMR1*) and androgenetic stem cells (two paternal genomes, unmethylated *KvDMR1*) also silenced *Cdkn1c* expression but failed to directly de novo methylate the *Cdkn1c* gene.

Role of direct DNA methylation in regulating *Cdkn1c* expression

We have shown that silencing of *Cdkn1c* can be maintained in vitro without DNA methylation for at least 21 days. In vivo, the maintenance DNA methylase, Dnmt1, is required to keep the paternal *Cdkn1c* allele repressed in E9.5 embryos and the ectoplacental cone.7,28 This loss of imprinted expression is apparent three days after imprinted expression is detectable in wild type embryos and two days after the silent allele normally acquires DNA methylation.28 This demonstrates that DNA methylation is required to keep the paternal *Cdkn1c* allele silent in vivo.

Role of histone modification in regulating *Cdkn1c* expression

Cdkn1c is located more than 200 kb from the imprinting centre that regulates its imprinted expression. Termination of *Kcnq1ot1* leads to inappropriate activation of the paternal *Cdkn1c* allele suggesting that the *Kcnq1ot1* transcript participates in the long range silencing of *Cdkn1c*. Bhogal et al.28 demonstrated that silencing of *Cdkn1c* takes place prior to direct DNA methylation at the *Cdkn1c* gene in vivo. Our finding that there is a relative enrichment of the H3K27me3 mark in stem cells after five days of differentiation demonstrates that this modification also precedes de novo methylation at *Cdkn1c*. The low abundance of

H3K27me3 at *Cdkn1c* in undifferentiated EG cells suggests that this mark is recruited to *Cdkn1c* at the very earliest stages of silencing.

Other genes

Cdkn1c is not the only imprinted gene spanned by a secondary DMR. Similar DMRs are located over the transcriptional start sites for *Igf2r, Nesp55* and *Gtl2*.29,30,45 These all acquire methylation on the paternal allele after fertilization and, at least in the case of *Gtl2*, within a similar time frame to *Cdkn1c*. Paternal repression of *Gtl2* and *Cdkn1c* is lost in *Eed*-deficient embryos whereas *Igf2r* expression appears to be unaffected.27 Another scenario in which there is altered imprinted expression of the *Cdkn1c* gene is in *lymphoid specific helicase (Lsh)*-deficient embryos.33 Lsh (official symbol Hells) is involved in reinforcing DNA methylation and silencing of polycomb repressive complex targets and *Lsh*-deficiency leads to loss of repression of *Cdkn1c* but not *H19, Igf2, Igf2r, Zac1* or *Meg9/Mirg*.33 The fact that *Cdkn1c* and *Igf2r* do not respond in a similar way in either of these models argues against a common mechanism involving either Lsh (Hells) or Eed. However, it will be important to determine the expression status of *Nesp55* in *Eed*-deficient embryos and both *Nesp55* and *Gtl2* in *Hells*-deficient embryos and also to identify any commonalties with other genes encompassed by secondary DMRs as this could provide support for a common mechanism for their establishment and maintenance.

Long term silencing

Once differential methylation is established at the IC, histone modifications appear to be sufficient to transmit the imprint signal to adjacent genes within an imprinted domain and to maintain this imprint, at least within the placenta.8 Lewis and colleagues suggested that the differences in imprinted gene expression between the placenta and the embryo might reflect the existence of an evolutionarily older imprinting mechanism based on histone modifications with DNA methylation recruited in the embryo as a more stable epigenetic mark for use in specifically in embryonic lineages. Our data suggest that *Cdkn1c* can also be effectively silenced in the short term through just the action of histone modifications in stem cells. One possibility is that signalling from the IC and the recruitment of histone modifications is a continual process in placental lineages and in stem cells and that direct DNA methylation is only required for the long term silencing of genes that lie at a distance from their IC. This in turn suggests that the dosage of genes spanned by post fertilization DMRs, which include *Cdkn1c, Igf2r, Gtl2* and *Nesp55*, is critical not just during embryonic development but also during the life span of the organism. Identifying any commonalities in the postnatal function of the imprinted genes in this category may provide insight into the rational for regulating gene dosage in the adult mammal.

CDKN1C in humans

The human *CDKN1C* gene also exhibits imprinted expression but, in contrast to the mouse gene, there is no evidence for direct DNA methylation of the paternal allele and this allele is not fully silenced.24,43,46–48 In some respects, this scenario is similar to our in vitro differentiated stem cells. Either humans have lost the ability to directly methylate the *CDKN1C* locus or mice have specifically acquired a secondary DMR. Either way, this could

suggest that the evolutionary necessity to fully repress paternal *Cdkn1c* expression differs between the two species.

Summary

We have shown here that the silencing of *Cdkn1c* can be established and maintained in differentiated stem cells without direct DNA methylation. However, the requirement for Dnmt1 in vivo demonstrates that DNA methylation is critical for the long term silencing of *Cdkn1c*. We suggest that this reflects the importance of the controlled dosage of this gene in the adult animal as well as during embryogenesis. In addition, the failure of stem cells to complete their full silencing program could have bearing on their usefulness in in vitro differentiation studies and stem cell-based therapies.

Materials and Methods

Stem cell lines

Biparental ES cell stem lines KES1 and CES3 were derived from 129/Sv embryos and were a kind gift from M. A. Surani. SF1-1, AKR1, TMAS21G and Sv6.1 were described previously.21,41,44 Cells were maintained in the undifferentiated state on a SNL (mouse fibroblast STO cell line transformed with neomycin resistance and murine LIF genes) feeder layer as described previously.43 Stem cell lines were all XY and were reconfirmed to be Oct4 positive with a full chromosomal compliment when undifferentiated at the end of the study. Feeders were removed from the undifferentiated cells prior to RNA, DNA or chromatin preparation by panning for 20 minutes. Cells were differentiated by plating at low density in the absence of LIF and feeders on non adherent (embryoid bodies) or adherent (monolayer) plates as indicated. Medium was changed every 1–2 days.

RNA analysis

Expression levels were determined using real-time quantitative RT-PCR as described previously49 on cDNA from three independent differentiations for CES3, Sv6.1 and TMAS21G and a single differentiation for SF1-1. Primers *Cdkn1c* 5'-AGA GAA CTG CGC AGG AGA AC-3' and 5'-TCT GGC CGT TAG CCT CTA AA-3' and *Kcnq1ot1* 5'-TCC AAT CGG GTA GAG ATT CG-3' and 5'-AGA CCA TCG GAA AAC ACA GG-3'. For the ribonuclease protection assay, the *Kcnq1ot1* RPA probe was located approximately 1,000 bp downstream of the *Kcnq1ot1* promoter (nucleotides 144,718 to 145,568 from sequence AJ271885). RNase protection was performed as previously described.25 Essentially, radioactive RNA probes were synthesized using the MaxiScript T7/T6 kit (Ambion) and ^{32}PUTP (800 Ci/mmol). RPA was performed using the RPAIII kit (Ambion), with hybridization at 45°C (*Kcnq1ot1* plus *Cyclophillin*). For restriction fragment length polymorphism analysis, 35 cycles of PCR was performed using the RED Genomic Template PCR system (Sigma) at an annealing temperature of 64.5°C using the published primers that span a polymorphic *AvaI* restriction site within exon 3 of the *Cdkn1c* gene.50

DNA analysis

Genomic DNA was prepared as described previously.43 Bisulphite sequencing was performed as described51 using primers 5'-TGG GTG TAG AGG GTG GAT TTA GTT

A-3's and 5'-CCC ACA AAA ACC CTA CCC CC-3' and hemi-nested primer 5'-GTA TTG TTA GGA TTA GGA TTT AGT TGG TAG TAG TAG. Southern blotting and hybridisation was performed with a 0.53 kb *XhoI-EagI* probe fragment from the *Cdkn1c* cDNA as described.43

Chromatin preparation and analysis

Chromatin immunoprecipitation (ChIP) assays were carried out using the Orange ChIP assay kit (Diagenode) according to the manufacturer's instructions, with a few modifications. For undifferentiated stem cells, the cells were separated from the feeder layer and then resuspended in cold phosphate-buffered saline supplemented with protease inhibitors (Sigma). Cross-linking for all cells was performed with 0.8% formaldehyde (Sigma) for 10 minutes at room temperature and the reactions were quenched with 0.125 M glycine for 5 minutes at room temperature. Cells were resuspended in lysis buffer at 10,000 cells/μl and incubated on ice for 5 minutes. Following lysis, sonication was carried out with a Diagenode Bioruptor. For each ChIP, 10 μl of the sonicated cell supernatant (equivalent to 100,000 cells) was diluted 10-fold in ChIP dilution buffer (Diagenode). 100 μl of the diluted material was removed prior to the addition of antibodies (input). 100 μl of diluted chromatin was used for each IP reaction with antibodies against H3K4me3 (Kch-403-020, Diagenode) and H3K27me3 (Ab6002, Abcam). IgG (Ab6697, Abcam) was used as the negative control. For the final step, samples and input chromatin were heat treated to reverse the crosslinks and the DNA was precipitated and resuspended in 50 μl of Tris-EDTA. 5 μl was used for each qPCR reaction. Percentage enrichment was calculated using the formula A^(Input CT − IP CT) × 100 where A is the amplification efficiency of the qPCR. Two independent differentiations were performed. *Cdkn1c* promoter region primers 5'-GCG GTG TTG TTG AAA CTG AA-3' and 5'-GTC TGG ATC GCT TGT CCT GT and *Kcnq1* promoter region primers 5'-AAG CTC ACC CAA TCC AAA TG and 5'-CTC CTA GCG ACA ACG GGT AG.

Acknowledgments

M. W. was supported by a BBSRC Epigenetic Initiative studentship. We would like to thank Dr. Nick Allen, Professor Azim Surani and Dr. Gabriella Durcova-Hills for providing the stem cell lines and Dr. Tatyana Nesterova for help with Southern blotting.

Abbreviations

IC	imprinting centre
DMR	differentially methylated region
PEFs	primary embryonic fibroblasts

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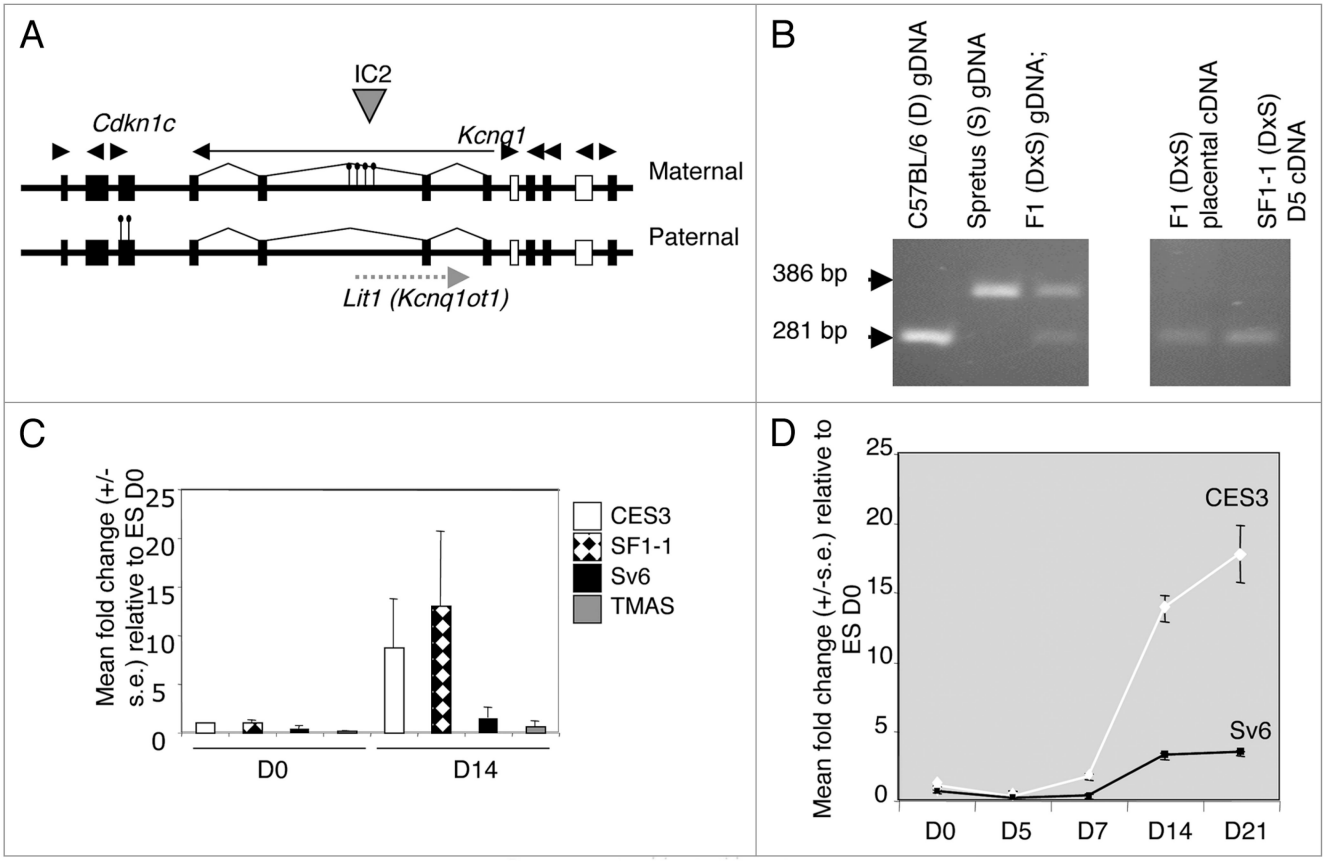

Figure 1.

Cdkn1c expression in in vitro differentiated stem cells. (A) 800-kb IC2 domain on mouse distal chromosome 7 showing regions of differential DNA methylation in somatic cells as indicated by lollypops. (B) Imprinting of *Cdkn1c* in stem cells as assessed by the presence or absence of an *AvaI* restriction enzyme site in *Cdkn1c* PCR products amplified from cDNA samples as indicated. (C) Relative expression level of *Cdkn1c* before (D0) and after (D14) differentiation in biparental ES cell lines CES3 and SF1-1 and the EG cell lines Sv6.1 and TMAS21G. Combined results from three independent differentiation using the embryoid bodies protocol (2) or two experiments using the monolayer protocol (1). (D) Expression profile of *Cdkn1c* over 21 days of in vitro differentiation by the embryoid bodies protocol.

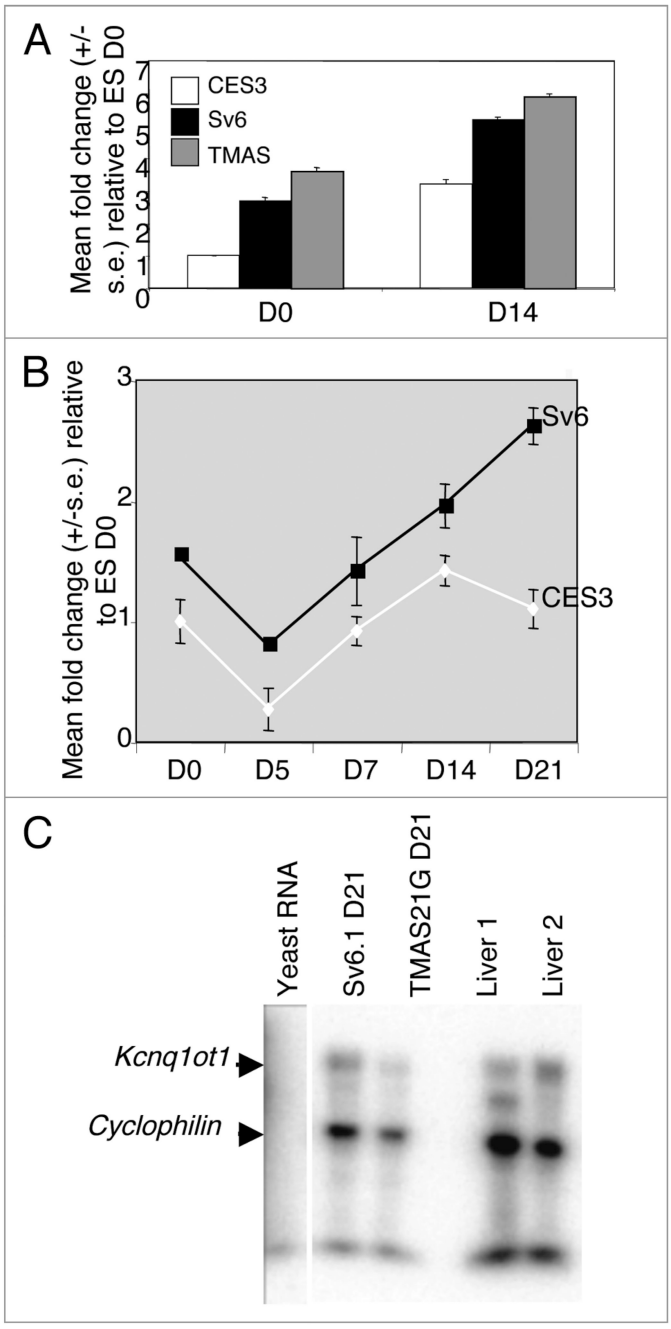

Figure 2.

Kcnq1ot1 expression. (A) Relative expression level of *Kcnq1ot1* before (D0) and after (D14) differentiation in biparental ES cell line CES3 and the EG cell lines Sv6.1 and TMAS21G. (B) Expression profile of *Kcnq1ot1* over 21 days of differentiation demonstrating consistently higher *Kcnq1ot1* expression in the EG cell line. (C) RNase protection assay against the *Kcnq1ot1* transcript. Yeast RNA included as a negative control and riboprobe to *cyclophilin* included to control for RNA integrity and loading. Differentiation by the embryoid bodies protocol.

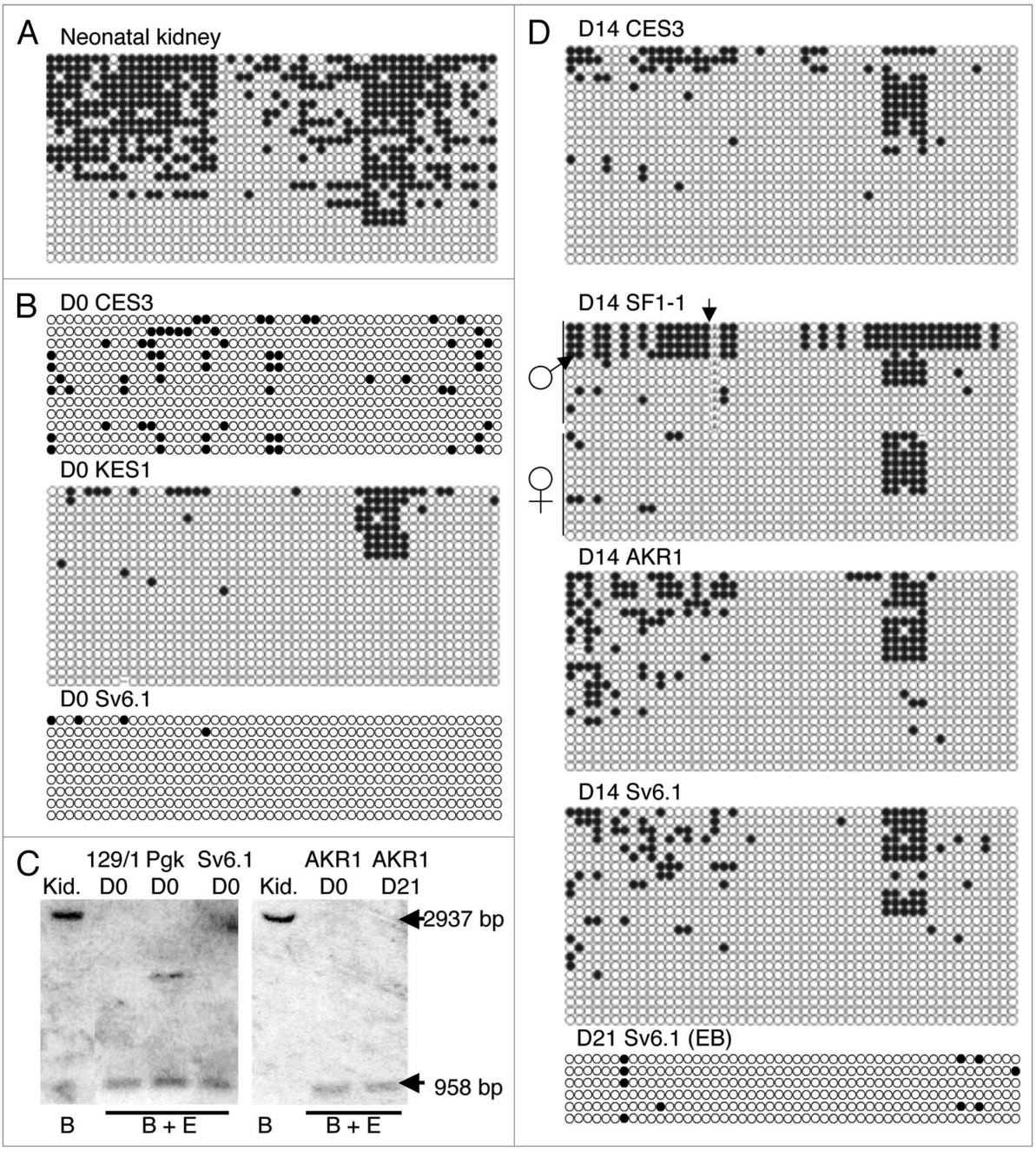

Figure 3.

Methylation analysis of the secondary *Cdkn1c*-DMR in undifferentiated and differentiated stem cells. (A) Bisulphite sequence data for neonatal kidney. Each row corresponds to an individual sequenced DNA clone. Each circle represents a CpG on the strand, filled circles and open circles indicate methylated and unmethylated sites, respectively. (B) Bisulphite sequence data for the undifferentiated biparental ES stem cell lines, CES3 and KES1 and the undifferentiated, imprint-erased EG stem cell line, Sv6.1. (C) Southern blot data for undifferentiated biparental stem cell lines 129/1 and Pgk, undifferentiated EG stem cell line

Sv6.1 and undifferentiated and D21 differentiated androgenetic stem cell line AKR1. DNAs were digested with *BamHI* and *EagI*. (D) Bisulphite sequence data for stem cell lines CES3, SF1-1, AKR1 and Sv6.1 differentiated for 14 days by the monolayer protocol and data for Sv6.1 differentiated for 21 days by the embryoid bodies protocol. Arrow marks the position of a polymorphism between *M. domesticus* (C57BL/6) and *M. spretus*.

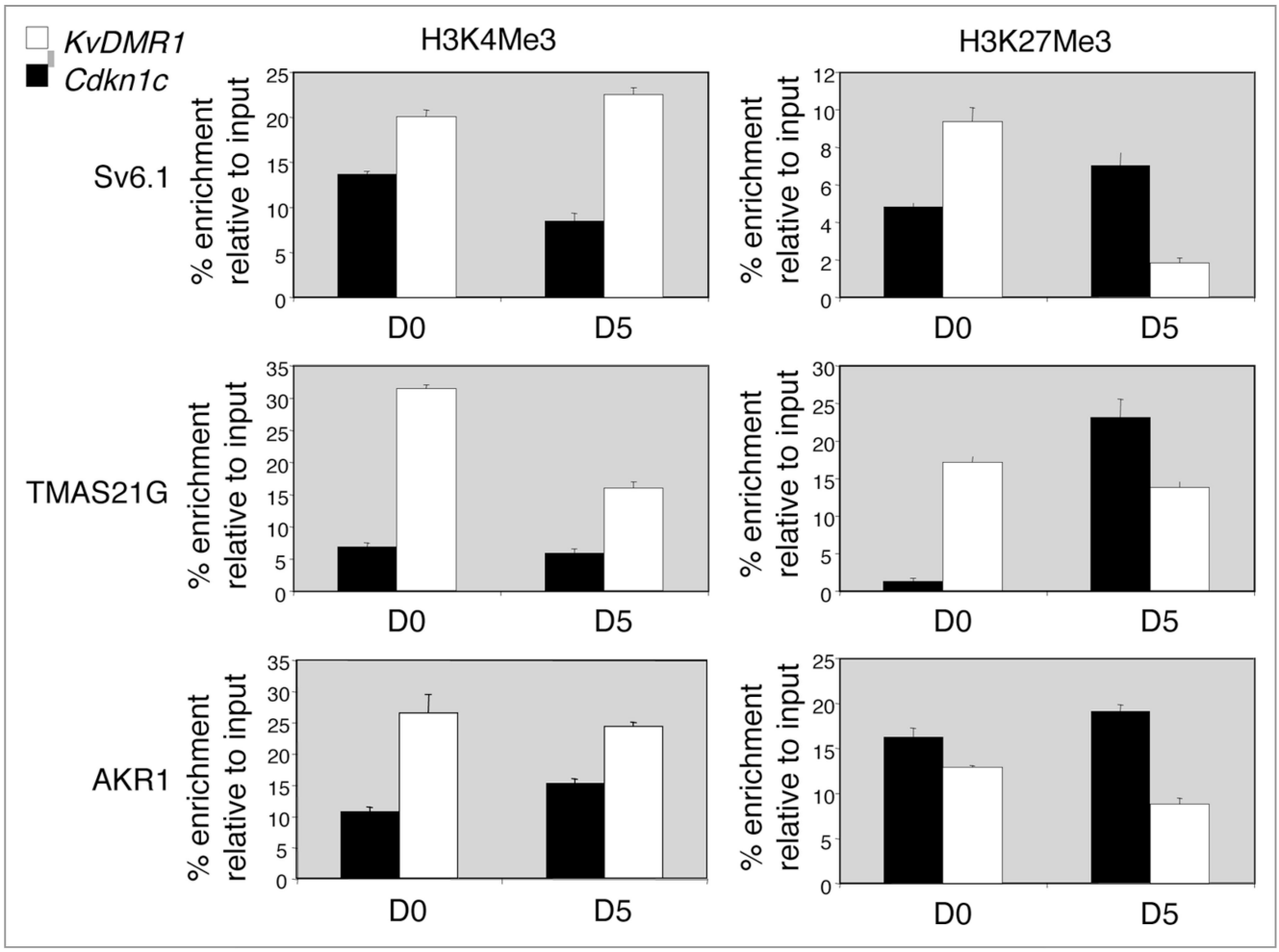

Figure 4.

ChIP analysis of the *Kcnq1ot1* and *Cdkn1c* promoter regions in undifferentiated and differentiated stem cells. Chromatin immunoprecipitation (ChIP) was performed using antibodies to detect trimethylated H3K4 (H3K4me3), a mark associated with active chromatin and trimethylated H3K27 (H3K27me3), a mark associated with silent chromatin, in undifferentiated and differentiated AKR1 and EG stem cells. Results are expressed as the % enrichment relative to input chromatin. Quantitative PCR was performed for regions within 600 bp of the transcription start sites of *Cdkn1c* and *Kcnq1ot1*. Embryoid bodies protocol.