[Cell Cycle 3:12, 1645-1650; December 2004]; ©2004 Landes Bioscience

Report

A Dynamic Switch in the Replication Timing of Key Regulator Genes in Embryonic Stem Cells upon Neural Induction

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Received 11/02/04; Accepted 11/04/04

Previously published online as a *Cell Cycle* E-publication: http://www.landesbioscience.com/journals/cc/abstract.php?id=1346

KEY WORDS

epigenetic, DNA replication timing, chromatin structure, embryonic stem cells, neural induction

ABBREVIATIONS

BrdU	5'-bromodeoxyuridine
EB	embryoid bodies
ES cells	embryonic stem cells
LIF	leukaemia inhibitory factor
H3K4	histone H3 lysine 4
H3K9	histone H3 lysine 9
H4K20	histone H4 lysine 20
RA	retinoic acid

ACKNOWLEDGEMENTS

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ABSTRACT

Mammalian embryonic stem (ES) cells can either self-renew or generate progenitor cells that have a more restricted developmental potential. This provides an important model system to ask how pluripotency, cell commitment and differentiation are regulated at the level of chromatin-based changes that distinguish stem cells from their differentiated progeny. Here we show that the differentiation of ES cells to neural progenitors results in dynamic changes in the epigenetic status of multiple genes that encode transcription factors critical for early embryonic development or lineage specification. In particular, we demonstrate that DNA replication at a subset of neural-associated genes including *Pax3*, *Pax6*, *Irx3*, *Nkx2.9* and *Mash1* is advanced upon neural induction, consistent with increased locus accessibility. Conversely, many ES-associated genes including *Oct4, Nanog, Utf1, Foxd3, Cripto* and *Rex1* that replicate early in ES cells switch their replication timing to later in S-phase in response to differentiation. Detailed analysis of the *Rex1* locus reveals that delayed replication extends to a 2.8 Mb region surrounding the gene and is associated with substantial reductions in the level of histone H3K9 and H4 acetylation at the promoter. These results show that loss of pluripotency (and lineage choice) is associated with extensive and predictable changes in the replication timing of key regulator genes.

INTRODUCTION

Although the precise relationship between gene transcription, chromatin structure and DNA replication timing is not known, late replication is generally associated with constitutive heterochromatin and some facultative heterochromatin, whereas early replication correlates with more accessible chromatin including actively transcribed regions.[1-3] Pertinent examples of the inter-relationship between chromatin structure and DNA replication are provided by recent studies of the *β-globin* locus in erythroid cells and of X-inactivation in differentiating female ES cells. In the case of *β-globin*, locus activation in erythroblasts correlates with a switch to early replication and increased DNaseI sensitivity and histone acetylation.[4-6] In female ES cells where silencing of one X chromosome is induced upon differentiation, a switch to late replication is a relatively early event in the inactivation process that is preceded by Xist RNA recruitment, generalized deacetylation of histones H3 and H4 and reciprocal changes in methylated H3K4 and H3K9 levels consistent with heterochromatin formation.[7] Collectively these data suggest that changes in DNA replication reflect variations in chromatin structure and condensation[8] and that 'shifts' in replication timing may be a useful means to identify important epigenetic events that underlie different developmental programs in mammals.

During development neural specification occurs early in embryogenesis through the concerted activity of inducing factors, transcriptional activators and repressors.[9] Once specified, progenitors differentiate to generate a range of distinct neuronal cell types—a process that requires the establishment of stable patterns of neural-specific gene expression as well as restriction of alternative cell fates. Although many of key regulators of neural induction have been identified and their molecular interactions defined,[9] relatively little is known about the epigenetic basis of neural commitment and lineage restriction. This is in part due to difficulties in obtaining sufficient numbers of ex vivo purified progenitor cells for conventional chromatin-based analysis. However, recent advances in the induction of mouse ES cells have allowed the efficient generation of ES-derived neural populations without significant attrition.[10-14]

In this study we focus on the replication kinetics of genes encoding transcription factors that are either important for early embryonic development, or have a role in the specification of neural, haematopoietic and muscle tissues. By comparing undifferentiated ES cells and ES-derived neural progenitors we show that neural commitment is accompanied by a shift to later replication of multiple genes that regulate ES cells or have a role in maintaining pluripotency, and advances in the replication of many neural-associated genes. These data provide compelling and novel evidence of chromatin-based changes that underpin neural cell fate determination.

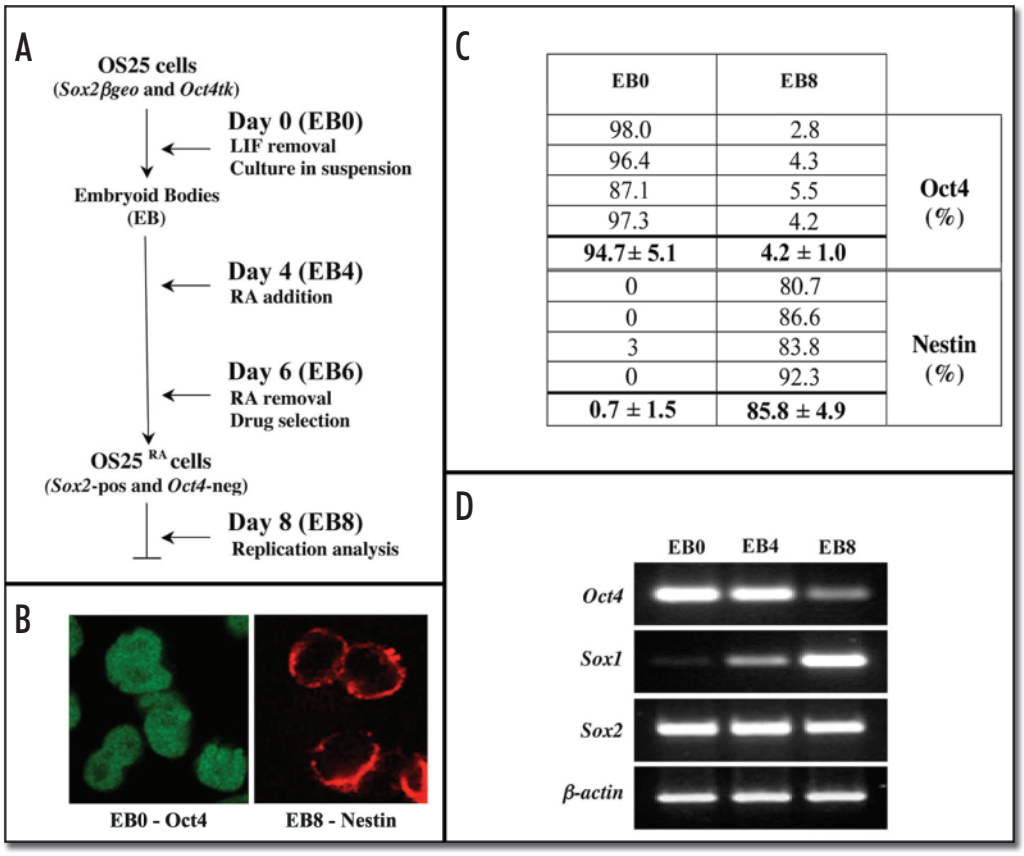

Figure 1. Retinoic acid-induced differentiation of OS25-ES cells into neural progenitors. OS25-ES (*Sox2βgeo/Oct4tk*) cells were cultured as embryoid bodies as outlined in (A). After four days, RA was added to stimulate the neuronal differentiation and two days later, cells were selected for the retention of *Sox2* expression by G418 and for loss of Oct4 expression by gancyclovir.[12] After eight days, RA-induced (EB8) and undifferentiated control cells (EB0) were analysed for the expression of Oct4 and nestin by immunofluorescence staining (B and C). Expression of *Oct4, Sox1, Sox2* or *β–actin* RNA was evaluated after 0, 4 and 8 days by RT-PCR (D).

MATERIALS AND METHODS

Cell Culture. CCE-ES cells were maintained in an undifferentiated state on irradiated SNL feeders in the presence of leukaemia inhibitory factor (LIF). The CGR8-derived OSG and E14Tg2a-derived OS25 ES cell lines were generated by sequential gene targeting of *βgeo* into the *Sox2* locus and *hygromycin-thymidine kinase* into the *Oct4* locus,[13] and maintained as previously described.[12] T lymphocytes were isolated from lymph nodes of 6–8 week old C57BL/6 mice, and primary embryonic fibroblasts (PEF) were prepared and cultured as previously described.[3]

ES Cell Differentiation. As outlined in Figure 1, exponentially growing OS25-ES cells were trypsinised on day 0 and 7 x 10^6 cells were plated on 10 cm bacterial dishes (Sterilin) without LIF to allow the formation of embryoid bodies (EBs). On day 4, retinoic acid (RA, 10^{-6}M) was added to the culture. On day 6, the media was replaced with a 50:50 mixture of Dulbecco's MEM-F12 medium containing N2 supplement and neurobasal medium containing B27 supplement, RA was removed and G418 (100 µg/ml) and gancyclovir (2.5 µM) drug selection was applied. After 2 more days, cells were dissociated with trypsin and subjected to RT-PCR analysis, immunocytochemistry, replication timing and chromatin immunoprecipitation analyses.

RT-PCR Analysis. OS25-ES cells were harvested by trypsinisation at days 0, 4 and 8 of differentiation and total RNA was extracted using RNeasy Protect Mini Kit (Qiagen) and RNase-Free DNase Set (Qiagen) to remove residual DNA. cDNAs were synthesised using Superscript™ First-Strand Synthesis system (Invitrogen) and PCR amplification of *Oct4, Sox1, Sox2* and *β-actin* was performed in 50 µl using 0.5 µM primers (*Oct4, Sox1, β-actin*) and 1 µM primers (*Sox2*) (primer sequences are available on request), 1.75U of HotStarTaq (Qiagen) and 60°C annealing temperature for 30 cycles (*β-actin* and *Sox2*), 28 cycles (*Oct4*) and 32 cycles (*Sox1*).

Immunocytochemistry. ES-derived cells were attached to Poly-D-Lysine-coated coverslips, fixed for 5 min in PBS with 4% paraformaldehyde at room temperature, washed in PBS and incubated for 30 min in 10% normal goat serum with 0.1%–0.4% Triton-X100. Slides were then incubated for 1 hour with either monoclonal anti-rat nestin antibody (Pharmingen) or monoclonal anti-mouse oct3/4 antibody (Santa Cruz), washed in PBS and incubated for 1 hour with appropriately diluted texas red-conjugated (Pharmingen) and Alexa 488-conjugated (Molecular Probes) goat anti-mouse antibodies, respectively. Coverslips were mounted in Vectashield (Vector) containing 1 µg/µl DAPI and examined using a confocal SP1 Leica microscope.

Replication Timing Analysis. BrdU labelling, fixation in 70% cold ethanol, cell cycle fractionation by flow cytometry and isolation of BrdU-labelled DNA by immunoprecipitation were carried out as previously described,[3] with the following modifications to BrdU-pulse labelling times: 30 min (ES cells and PEF), 45 min (activated peripheral T cells) and 90 min ($OS25^{RA}$). The abundance of newly replicated DNA was determined using 500–1000 cell equivalents of BrdU-labelled DNA by semi-quantitative PCR amplification and Southern blot hybridisation as previously described[3] or by real-time PCR amplification. Primer sequences used for this analysis are available on request.

Chromatin Immunoprecipitation (ChIP) Analysis. Approximately 10^7 OS25-ES cells were harvested with trypsin on day 0 and day 8 of differentiation and processed for ChIP analysis as described previously,[15] with minor modifications. 140 µg of chromatin was subjected to immunoprecipitation with 2 µl anti-Histone H3 antibody (input-control, Abcam 1791), 3 µl anti-acetyl-Histone H3K9 antibody (Upsate, 07-352), 5 µl anti-trimethyl-Histone H3K9 and anti-trimethyl-Histone H4K20 antibody (both kind gifts from Dr. Thomas Jenuwein), 5 µl anti-acetyl-Histone H4 antibody (Serotec, AHP418) and 2 µl of a rabbit anti-mouse-IgG antiserum (negative-control, Dako). After elution of immune complexes, DNA was resuspended in 75 µl Tris-EDTA solution. Quantification of recovered DNA was performed using real-time PCR amplification. Primer sequences are available on request.

Table 1 **CANDIDATE LOCI ANALYSED IN THIS STUDY**

Haematopoietic		Neuronal		Embryonic	
Scl	(4; C7)	Sox1	(8; A2)	*Oct4*	(17; B3)
Lmo2	(2; E2)	Sox2	(3; B)	*Nanog*	(6; F2)
Gata1	(X; A1.1)	Sox3	(X; A5)	*Utf1*	(7; F2)
Gata2	(6; D2)	Irx3	(8; C5)	*Rex1*	(8; B1.3)
Gata3	(2; A1)	Nkx2.2	(2; H1)	*Foxd3*	(4; C6)
T-bet	(11; D)	Nkx2.9	(12; C2)	*Esg1*	(X; A1.2)
Ebf	(11; B1.1)	Msx1	(5; B2)	*Cripto*	(9; F2)
Pax5	(4; B2)	Pax3	(1; -)	*Fgf4*	(7; F5)
Ikaros	(11; A2)	Pax6	(2; E3)	*Zfp57*	(17; B3)
NF-E2	(15; F3)	Pax7	(4; D3)		
C/EBPα	(7; B1)	Olig2	(16; C3.3)		
PU.1	(2; E1)	Neurod	(2; D)		
c-myb	(10; A3)	Mash1	(10; C2)		
		Math1	(6; C1)		
Muscle		Ngn1	(13; B2)		
Myog	(1; F)	Ngn2	(3; H1)		
MyoD	(7; B3)	Hes1	(16; B2)		
Myf5	(10; D1)	Hes5	(4; E2)		

43 Loci that are expressed in haematopoietic, muscle, neuronal or early embryonic and ES cells were selected to represent 17 chromosomes, shown with their band location according to www.ensembl.org.

immunoprecipitated DNA as a template in a 50 µl reaction volume. The relative abundance of amplified sequences was obtained from calibration plots generated with genomic DNA standards. Each measurement was performed in duplicate.

RESULTS

To investigate whether neural induction results in predictable changes to the replication timing of genes regulating lineage specification, we examined ES cells before and after neural induction by retinoic acid. We took advantage of the ES cell line OS25 (*Sox2βgeo/Oct4tk*), which allows for the efficient enrichment of ES-derived neural cells by combining selection for continued *Sox2βgeo* expression (by G418) and against *Oct4tk* expression (by Gancyclovir).[13] As described previously[12,13] and outlined in Figure 1A, actively dividing ES cells were cultured in suspension and in the absence of LIF to induce embryoid bodies (EBs). After 4 days (EB4), retinoic acid (RA) was added to promote neural development. At day 6 (EB6), cultures were treated with G418 and Gancyclovir. Two days later (EB8) cultures contained more than 85% of cells expressing the neuroepithelial protein nestin and fewer than 5% of cells expressed Oct4 as monitored by immunofluorescence and microscopy (Fig. 1B and C). Efficient neural induction was confirmed by RT-PCR, which showed the upregulation of *Sox1* mRNA, a specific marker of cycling neural progenitors[16] (Fig. 1D).

The replication timing of a panel of 43 candidate genes encoding transcriptional regulators of lineage specification (Table 1) was assessed using a previously established PCR-based approach.[2,3,17,18] Nonsynchronised cells were pulse-labelled with 5'-bromodeoxyuridine (BrdU), stained with propidium iodide (PI) and sorted according to DNA content by flow cytometry. Typical DNA profiles for undifferentiated ES (OS25) and RA-induced ES (OS25^{RA}) cells are shown in Figure 2A, where gates used to define cells in G_1, S and G_2/M phase are indicated. S-phase cells were subdivided into four populations with increasing DNA content (S1-S4). Newly synthesised BrdU-labelled DNA was isolated from each fraction by immunoprecipitation with anti-BrdU antibody, and the relative abundance of each of the candidate genes was compared between each cell cycle fraction by PCR amplification using locus-specific primers. As controls for this analysis known early-replicating (*α-globin*) and late-replicating loci (*β-globin* and X141, a region of constitutive heterochromatin on the X chromosome) were included.[3] As shown in Figure 2B, *α-globin* was abundant in S1 fractions isolated from ES (left) and ES-derived neural cells (right), consistent with replication of *α-globin* early in S-phase.[19] In contrast, *β-globin* and X141 showed peak abundance in S4 and G_2/M fractions respectively, consistent with the reported replication and chromatin properties of these loci.[1,3,20] To confirm equivalent precipitation of BrdU-labelled template DNA, cell cycle fractions were 'spiked' with a constant amount of BrdU-labelled *Drosophila* S2 cell DNA prior to immunoprecipitation. The detection of similar levels of *Drosophila Gbe* PCR products in each fraction confirmed uniform recovery of BrdU-labelled DNA (Fig. 2B, bottom panels). Replication timing of candidate loci was scored by evaluating the peak of

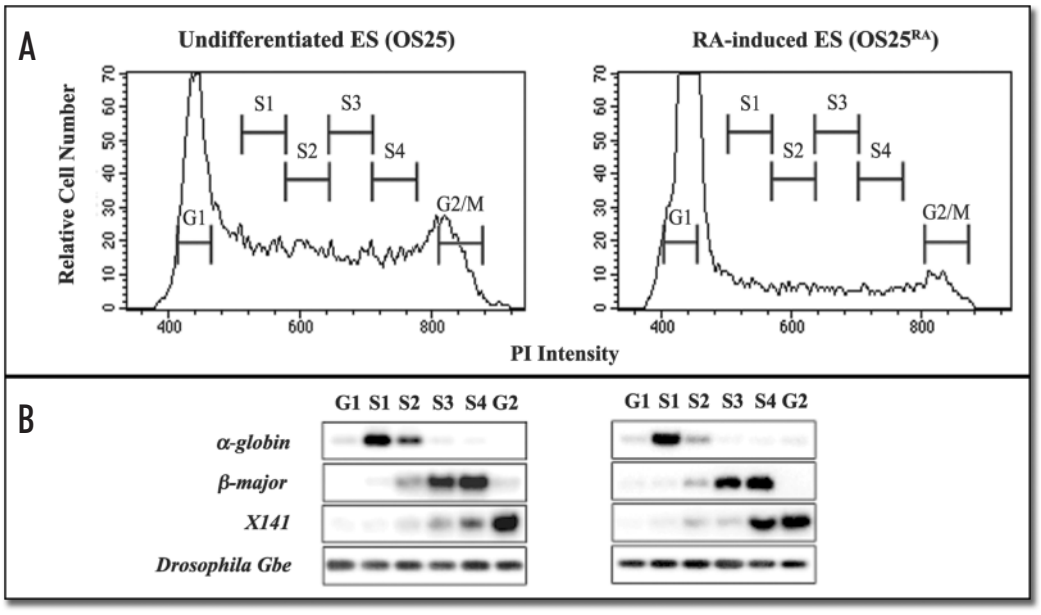

Figure 2. PCR-based analysis of replication timing. Undifferentiated ES (OS25) and RA-induced ES (OS25^{RA}) cells were pulse-labelled with BrdU, stained for DNA content with propidium iodide (PI) and sorted by flow cytometry into six cell cycle fractions (G_1, S1, S2, S3, S4 and G_2/M) according to DNA content (A). Replication timing was determined by analysing the abundance of locus-specific DNA within newly synthesised (BrdU-containing) strands isolated from each cell fraction following anti-BrdU immunoprecipitation. Southern blots of PCR products from each cell population (B). Early-replicating (*α-globin*) and late-replicating loci (*β-major* and *X141*) are indicated. The *D. melanogaster Gbe* gene provides a control for uniform recovery of BrdU-labelled DNA.

Real-Time PCR Analysis. Real-time PCR analysis was carried out on an Opticon™ DNA engine (MJ Research Inc.), running the following program: 94°C for 8 min, then 40 cycles of 94°C for 30 sec, 55°C (or 60°C) for 30 sec, 72°C for 1 min, followed by plate read. PCR reactions included 2x SYBR Green PCR Master Mix (Applied Biosystems), 300 nM primers and 2 µl of

DNA abundance in G_1/S1 (early, E), S2 (middle-early, ME), S2/S3 (middle, M), S3 (middle-late, ML) or S4/G_2M (late, L).

A summary of the analysis is shown in Figure 3A. In undifferentiated ES cells, the majority of genes studied (28/43) replicated early in S-phase. This included genes such as Oct4, *Nanog*, *Utf1*, *Rex1*, *Foxd3*, *Cripto*, *Fgf4* and *Zfp57* which are expressed by ES cells,[21-23] as well as genes that are not thought to be expressed at significant levels in ES cells, but are restricted to specific lineages such as *Ikaros* (haematopoietic), *Myog* (muscle) and *Math1* (neuronal). This finding is consistent with previous observations in lymphocytes and in fibroblasts, where many leukocyte-specific genes were found to replicate early in S-phase, irrespective of whether they were actively transcribed or not.[3] Upon neural induction, several loci did not change their replication timing, including transcriptional regulators of haematopoiesis (10/13) and the muscle regulatory factor *Myog*. *Sox2*, a gene that is expressed in both ES (OS25) and RA-induced ES (OS25^{RA}) cells remained early replicating while *Neurod*, which is expressed only in terminally differentiated (postmitotic) neurons, remained late replicating (Fig. 3B). Among the neural-associated genes studied, approximately 50% showed a clear shift to earlier replication in response to neural induction (green in Fig. 3A). The most dramatic differences were seen for *Mash1, Sox3, Pax3* (Fig. 3C) and *Pax6* where replication shifted from late (S3/S4 fractions) or middle (S3/S2) to early (S1) S-phase. Several other neural-associated genes including *Irx3, Nkx2.9, Msx1, Olig2* and *Ngn1* also displayed a marked advance in replication, in agreement with the idea that some tissue-specific genes replicate earlier upon transcriptional activation.[4,5,24]

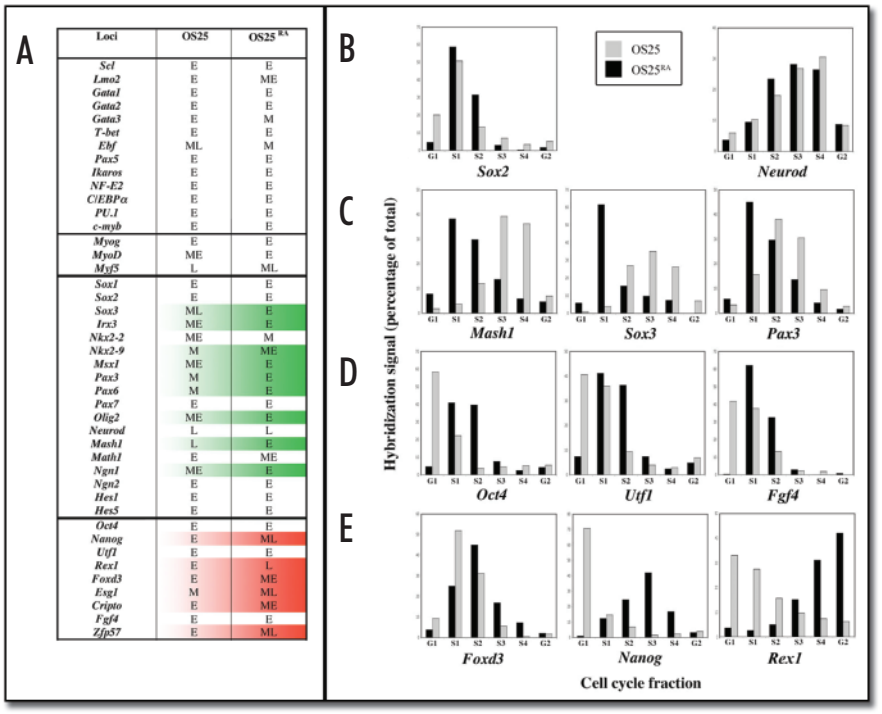

Figure 3. Changes in replication timing accompany neuronal differentiation of ES cells. Replication timing of the 43 candidate genes (see Table 1) analysed in OS25 and OS25^{RA} cell populations (A) where colour backgrounds in the neuronal and embryonic subsets indicate a marked delay (red) or advance (green) in the time of locus replication. Examples of loci that do not change their replication timing in (B), those that switch to early replication in (C) or those that show a slight temporal (D) or a marked (E) delay between OS25 (grey bars) and OS25^{RA} (black bars).

A striking feature of neural induction was a generalized delay in the replication of early-embryonic genes, including those involved in maintaining ES cell pluripotency (red in Fig. 3A). Three loci, *Oct4, Utf1* and *Fgf4* showed a subtle but reproducible delay in DNA replication upon neural induction. While still replicating within the first half of S-phase, these loci showed a consistent shift in their peak abundance from G_1/S1 to S1/S2 fractions in OS25 and OS25^{RA} cells, respectively (Fig. 3D). *Foxd3* and *Nanog* (see Fig. 3E), *Esg1, Cripto* and *Zfp57* all displayed a marked retardation

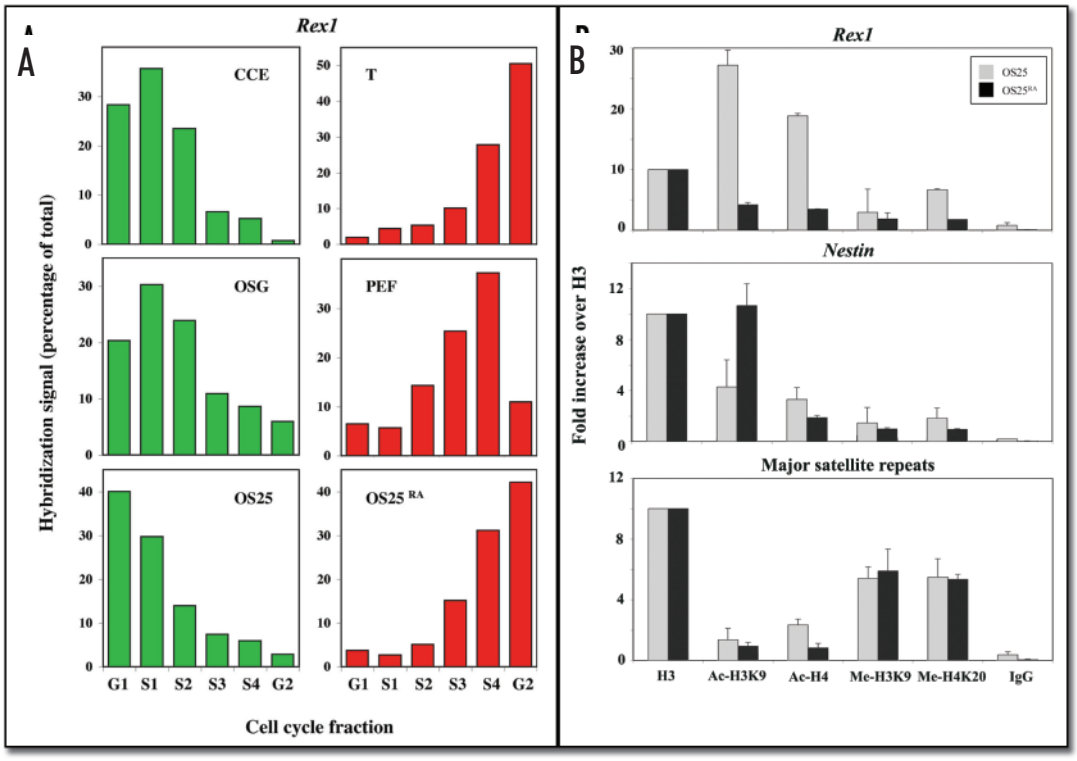

Figure 4. Late replication and histone deacetylation of the *Rex1* locus in differentated cell types. Replication timing of the *Rex1* locus in three undifferentated ES cell lines (CCE, OSG and OS25) (green), RA-induced ES cells (OS25^{RA}), T cells and primary embryonic fibroblasts (PEF) (red) (A). Chromatin immunoprecipitation analysis of the *Rex1* promoter (top), the *nestin* promoter (middle) and major satellite repeats (bottom) in undifferentiated (grey bars) and RA-induced ES cells (black bars). The values of two independent chromatin immunoprecipitation experiments using antibodies to acetylated H3K9, acetylated H4, tri-methylated H3K9 and tri-methylated H4K20 are shown relative to H3 (input control) (B).

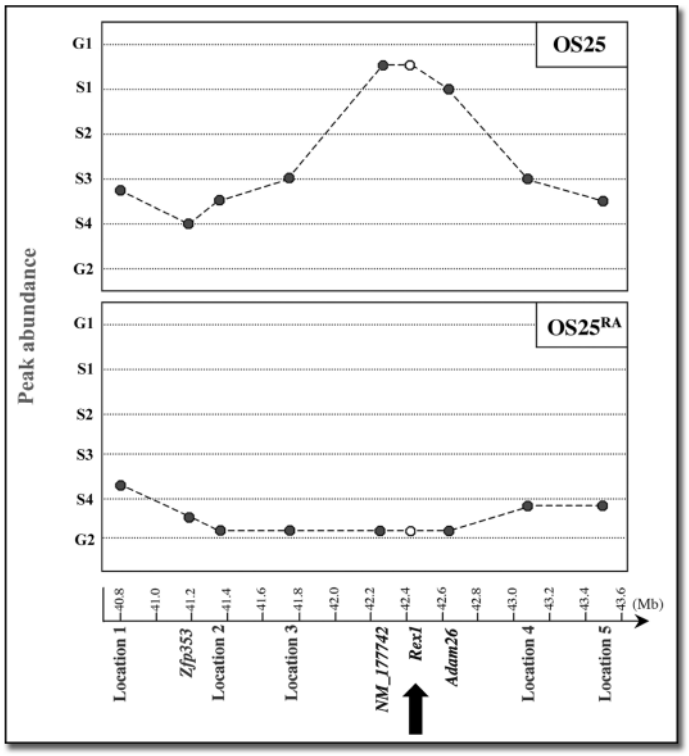

Figure 5. The replication profile of the *Rex1* locus in its genomic context. Replication timing analysis of the *Rex1* locus (arrow, open symbols) and 8 surrounding loci over a 2.8 Mb region (closed symbols) in undifferentiated OS25 (upper panel) and OS25^{RA} (lower panel) cells. A physical map of the four genes and the five genomic locations analysed is shown according to www.ensembl.org.

in replication. Replication of *Rex1*, shifted from early (G_1/S1) to very late (S4/G_2M) (Fig. 3E).

To determine whether early replication of the *Rex1* locus is a consistent feature of undifferentiated ES cells, we analysed two additional, independently isolated ES cell lines as well as differentiated fibroblasts and lymphocytes. As shown in Figure 4A, the *Rex1* locus replicated early in S-phase in CCE, OSG and undifferentiated OS25 ES cells. In differentiated ES cells (OS25^{RA}), primary embryonic fibroblasts (PEF) and mature T lymphocytes, replication of *Rex1* was consistently late. To define changes in the chromatin structure of the *Rex1* locus that accompany and may underlie this switch in replication timing, we performed a chromatin immunoprecipitation analysis of the *Rex1* promoter region in undifferentiated (OS25) versus neural induced (OS25^{RA}) cells. At the *Rex1* promoter acetylated H3K9 and acetylated H4 were 4 to 5 fold more abundant in undifferentiated ES cells (where *Rex1* was expressed and early replicating) than after neural induction (when *Rex1* was silent and late replicating) (Fig. 4B, upper panel). Conversely, acetylation of H3K9 at the *Nestin* promoter increased as a result of neural induction (Fig. 4B, middle panel). As a control for sequences that replicate late in undifferentiated ES cells and following neuronal induction, major satellite repeats were found to be enriched for methylated H3K9 and H4K20.

To explore changes in the replication timing of the *Rex1* locus in its genomic context, we determined the replication timing profile of eight loci spanning a ~2.8Mb region around *Rex1* before and after neural induction. In undifferentiated ES cells, early replication of *Rex1* extended to the two flanking loci *MN_177742* and *Adam26* (Fig. 5). The resulting early replicating domain of ~400kb was embedded within late replicating DNA. Silencing of *Rex1* in RA-induced ES cells resulted in the loss of this early-replicating domain and uniformly late replication of the entire 2.8 Mb region, including *Rex1*.

DISCUSSION

Conventional R- and G-banding patterns for individual mouse and human chromosomes[25,26] have suggested that large portions of the genome may be relatively stable in terms of when during S-phase DNA is replicated. However, studies examining DNA replication in amphibian and fly embryos have provided evidence that genome replication is modified as cell specification begins.[27,28] At early stages of embryogenesis cell division is rapid, S-phase is remarkably short and DNA synthesis is initiated more or less synchronously from regularly spaced origins to allow rapid chromosome duplication.[29] In *Xenopus*, S-phase lengthens at the midblastula stage as zygote transcription and lineage specification are initiated and DNA synthesis becomes asynchronous. At this time distinctions between early- and late-replicating regions of the genome become apparent (reviewed by refs. 30 and 31). In the present study we examined the replication timing profiles of approximately 50 genes in undifferentiated mouse ES cells and ES-derived neural progenitors and provide evidence that replication timing is subject to dynamic change. By comparing 43 genes that encode transcription factors involved in cell fate specification, we demonstrate that the replication timing of many of these genes differs between undifferentiated and differentiated ES cells and that replication switches as cells both acquire specialized function and lose their pluripotent status.

Approximately half of the neural-associated genes that we have analysed show an advance in their replication timing as ES cells differentiate and express a neural-specific gene program. Examples include *Irx3, Msx1* and *Nkx2.9* that encode basic helix-loop-helix transcription factors implicated in the sub-specification of neural progenitors[9] and *Pax3* and *Pax6* that are expressed in dividing progenitors within the embryonic neural tube.[32,33] However, not all neural-associated genes change their replication timing during neural differentiation. This likely reflects the context of individual gene within the genome, in particular whether they lie in close proximity to neighbouring genes. In addition, a recent genome-based analysis of replication kinetics of human chromosome 22q revealed that gene-dense and GC-rich regions tend to replicate in early S-phase.[34]

A key finding of our current study is that the replication timing of many early-embryonic genes is retarded when ES cells lose their pluripotent status. This includes genes encoding the transcription factors *Oct4* and *Nanog*, which are critical for maintaining pluripotency.[35-37] Perhaps the simplest interpretation of these data is that during ES cell differentiation progressive chromatin modifications render this subset of genes less accessible (as implied by a shift towards late replication) while some neural-associated genes become more accessible (suggested by earlier replication). Consistent with this idea we show that a switch in replication from early to late S-phase is accompanied by substantial histone deacetylation at the *Rex1* locus. A link between replication timing and histone acetylation has been implied by several recent studies. For example, histone deacetylase inhibitors have been shown to affect the replication timing of specific loci[38] and studies of the replication timing of transgenes integrated within late-replicating heterochromatin show a strong correlation between early replication and high local levels of histone acetylation.[3,39] In addition, experiments in the yeast *Saccharomyces cerevisiae* have shown that modifying histone acetylation[40] can directly alter origin firing. In the case of *Rex1*, we performed a bi-directional 'chromosome walk' to determine the extent of replication timing differences between undifferentiated ES and ES-derived neural cells. Within a 2.8 Mb domain, we show that differences

replication timing extend over 400kb around the *Rex1* gene. Whether neural induction promotes the extinction (or delayed firing) of an origin, located, for example, close to the *Rex1* locus is not currently known, but this explanation would fit well with previous findings in yeast and animals, as discussed previously.

In summary, we provide novel evidence that replication timing is dynamically altered in ES cells following neural induction. We show that replication of 9/9 ES-associated genes is delayed upon differentiation and loss of ES cell pluripotency. Collectively, our data also suggest that genome-wide analysis of replication timing provides a novel means to identifying 'addresses' within the genome where critical epigenetic changes associated with cell differentiation or lineage restriction are located.

Acknowledgements

The authors thank Austin Smith and Thomas Jenuwein for providing the OS25 and OSG-ES cell lines and antibodies against trimethyl-histone H3K9 and H4K20, respectively. Claudia Juno and Maria Dvorkina are acknowledged for assistance and advice.

We thank Martin Raff, Vasso Episkopou, Neil Brockdorff, Jacqueline Mermoud, Ana Pombo and the members of the Lymphocyte Development Group for helpful discussions, the FACS Laboratory at CRUK for help and advice, G. Reed, R. Newton and I. Devonish for photographic and secretarial assistance.

This work was supported by the Medical Research Council UK and through a MRC Collaborative Career Development Fellowship in Stem Cell Research funded by the UK Parkinson's disease Society to V.A.

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