DNA Replication Fading As Proliferating Cells Advance in Their Commitment to Terminal Differentiation

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Terminal differentiation is the process by which cycling cells stop proliferating to start new specific functions. It involves dramatic changes in chromatin organization as well as gene expression. In the present report we used cell flow cytometry and genome wide DNA combing to investigate DNA replication during murine erythroleukemia-induced terminal cell differentiation. The results obtained indicated that the rate of replication fork movement slows down and the inter-origin distance becomes shorter during the precommitment and commitment periods before cells stop proliferating and accumulate in G1. We propose this is a general feature caused by the progressive heterochromatinization that characterizes terminal cell differentiation.

Throughout the last 2–3 decades significant progress was made in the understanding of how proliferating cells control and regulate initiation and progression of DNA synthesis⁴⁻⁷. In contrast, the fading of DNA replication as cells stop proliferating and differentiate, received little or no attention at all. Progressive differentiation of somatic stem cells differs from the terminal differentiation of those cells that, although might be reprogrammed in some cases⁵, are usually not committed to proliferate thereafter. This is one of the most important choices each single cell makes at some point⁶. It is a complex decision that involves dramatic changes in gene expression and chromatin organization²⁻⁴,⁹.

Murine erythroleukemia (MEL) cell lines derive from proerythroblasts transformed with the Friend complex¹⁰. As in the case of untransformed cells, MEL cells proliferate indefinitely in the absence of erythropoietin. MEL cells may overcome the blockage, however, and reinitiate differentiation when exposed to a number of different chemical agents, such as hexamethylene-bis-acetamide - HMBA. A precommitment period of 12–24 hours, however, is required before cells become irreversibly committed to terminal differentiation¹⁰,¹¹. In the presence of the inducer MEL cells continue to cycle 4–5 times before proliferation stops and cells accumulate in G1¹⁰. These features make MEL cells an invaluable model to study reprogramming of tumour cells to a non-malignant phenotype and to analyse the mode of action of different chemotherapeutic compounds. Some observations indicate that MEL phenotypic differentiation and terminal cell division, however, are not necessarily coupled¹². Down regulation of genes characteristic of proliferating cells, including several oncogenes such as myc, myb and PU.1, goes along with cell cycle arrest¹³,¹⁴. Concomitantly, expression of a number of differentiated cell-gene markers leads to reactivation of the erythroid differentiation program¹⁵⁻¹⁸.

Here we used cell flow cytometry and genome wide DNA combing to examine for the first time DNA replication during the precommitment and early commitment periods of MEL cells before they stop proliferation and differentiate in the presence of HMBA. The results obtained indicated that replication forks progressively slow down as cells advance in their commitment to differentiate. Concomitantly, the inter-origin distance becomes shorter, indicating that replication origins that were dormant in actively proliferating cells became activated as cells approached terminal differentiation. We confirmed that cells continue cycling for 4–5 rounds in the presence of HMBA, which induced no DNA damage, before proliferation stopped and cells accumulated in G1. In addition, we confirmed that HP1α, a marker for heterochromatinization¹⁹, increases as cells differentiate. As different loci are known to behave disparately during terminal cell differentiation²⁻¹⁰,²¹, these observations strongly suggest that heterochromatinization, which affects most but not all the genome, modulates origin choice and inter-origin spacing during terminal cell differentiation.
Results

To confirm that proliferating MEL cells differentiate in the presence of HMBA, samples were taken from three different cultures every 24 hours and cell differentiation was monitored by the benzidine staining reaction. Benzidine reacts with the heme groups of hemoglobin leading to a light blue colour. The number of stained cells remained below 1% in logarithmically growing MEL cells as well as up to 48 hours after the addition of HMBA and increased progressively to over 90% at 120 hours (Supplementary Figure 1). As cells become irreversibly committed to terminal differentiation 48 hours after exposure to the inducer, we decided to examine DNA replication in cells that were exposed to HMBA for 0, 24 and 48 hours. First, a 20 minutes bromodeoxyuridine (BrdU)-labelling pulse and cell flow cytometry was used to determine the distribution of cells along the cell cycle. Figure 1 shows that the number of replicating cells, those cells that incorporated BrdU, progressively dropped from 65.13% at 0 hr to 42.73% at 24 hr and 34.48% at 48 hr in the presence of HMBA. The number of cells in G2/M also dropped from 22.23% to 17.28% and 15.09%, respectively. On the other hand, the number of cells in G1 progressively increased from 10.64% to 36.41% and 48.33%, respectively. These observations confirmed that although cells continue to cycle after addition of the inducer, they progressively stopped proliferating and accumulated in G1.

To determine the rate of DNA replication fork progression and the inter-origin distance genome wide, we used DNA combing and immunocytochemistry. This technique has been successfully used to measure both parameters for several cell types in untreated as well as after cells were exposed to different treatments. MEL cells were exposed to two consecutive 20 min pulses with Idodeoxyuridine (IdU) and Chlorodeoxyuridine (CldU) respectively, after they were treated with HMBA for 0, 24 and 48 hours. Selected molecules from this experiment are shown in Figure 2a. Figures 2b and c show the track length of the second (CldU) pulse for molecules that were labelled with both (CldU and IdU) pulses. The most abundant CldU track (corresponding to 25.51%, 62 out of 243 molecules scored) was 30–40 kb long for MEL 0 hours. It dropped to 20–30 kb long (corresponding to 21.61%, 59 out of 273 molecules scored) for MEL 24 hours and to 10–20 kb long (corresponding to 38.59%, 110 out of 285 molecules scored) for MEL 48 hours. This data clearly showed that the number of molecules with longer track lengths progressively diminished with time, indicating that replication forks slowed down as cells progressed throughout the precommitment and commitment stages of terminal differentiation. Inter-origin distance was measured where CldU tracks were found to flank IdU tracks in two neighbour replications on the same molecule. Selected molecules corresponding to cells that were labelled 0, 24 and 48 hours after the addition of HMBA are shown in Figure 3a. Figures 3b and c show the inter-origin distance measured in each case. The most abundant inter-origin distance class (corresponding to 18.24%, 29 out of 159 molecules scored) was 80–100 kb long for MEL 0 hour. It dropped to 60–80 kb long (corresponding to 18.01%, 29 out of 161 molecules scored) for MEL 24 hours and to 40–60 kb long (corresponding to 31.58%, 78 out of 247 molecules scored) for MEL 48 hours. The total number of initiation events per megabase of DNA was 1.39 for MEL 0 hour, 1.40 for MEL 24 hour and 2.10 for MEL 48 hour. It is evident that the inter-origin distance turned progressively shorter as cells advanced throughout the precommitment and commitment stages of terminal differentiation.

We wondered if the response observed was caused by the lack of proliferation by itself and not necessarily associated to differentiation. To test the latter, we first used flow cytometry to check the progression of MEL cells along the cell cycle when they were forced to stop proliferating by serum deprivation. The results obtained are shown in Figure 1d. Contrary to what happened in the presence of 10% FCS and HMBA (Figure 1c), serum deprivation caused no significant redistribution of cells along the cell cycle compartments. As expected, the total number of cells remained unchanged indicating that cell proliferation has ceased indeed. Despite the abridged incorporation of BrdU, it was sufficient to allow the quantitation of replication fork movement and inter-origin distance after 24 of serum deprivation. After logarithmically growing MEL cells were shifted to a culture medium without FCS for 24 hours they were exposed to two consecutive 20 min pulses with Idodeoxyuridine (IdU) and Chlorodeoxyuridine (CldU) respectively. The rate of DNA replication fork progression and the inter-origin distance genome wide, were determined by DNA combing and immunocytochemistry as described before. The results obtained for the rate of replication fork progression are shown in supplementary figure 3A. Compared to the data obtained when the cells were grown for 24 hours in the presence of 10% FCS, the most abundant CldU track remained 20–30 kb long, although its abundance increased from 22 to 33%. The mean track length dropped, though, from 54.03 to 28.89 kb. This is explained by the absence of longer tracks in the sample corresponding to 0% FCS. Surprisingly, though, the inter-origin distance remained almost unchanged (Supplementary figure 3B). The most abundant inter-origin distance class shifted from 60–80 kb long for cells grown in the presence of 10% FCS to 100–120 kb long in the absence of FCS. The mean inter-origin distance, though, remained almost unchanged (84.87 kb for cells grown in the presence of 10% FCS to 71.77 kb for cells grown without FCS). Altogether, this data showed that in the absence of FCS the number of molecules with longer track lengths progressively diminished with time, indicating that replication forks slow down. Contrary to what happens as cells differentiate in the presence of HMBA, though, the inter-origin distance showed no significant changes for cells grown without FCS indicating that silent replication origins were not activated when cells stop proliferating due to senescence by itself.

It is well known that nucleotide pools vary along the cell cycle expanding during the S-phase and contracting significantly in G1. Moreover, the effective concentration of deoxyribonucleoside 5’-triphosphates (dNTPs) at sites of DNA replication in vivo are higher than the concentrations of dNTPs averaged over the entire cell volume. For this reason, in order to evaluate dNTP levels in asynchronously growing cells nowadays many biochemists prefer to determine the transcription level of ribonucleotide reductase (RNR), the enzyme that catalyses a rate-limiting step in the biosynthesis of all four dNTPs. Here we used real-time PCR to determine the transcription level for the subunit 1 of RNR during HMBA-induced MEL cell differentiation (0, 24 and 48 hours). As a control we used proliferating cells exposed to 2 mM hydroxyurea (HU) for 2 and 18 hours. The results obtained indicated that the levels of RNR-S1 dropped in the presence of HMBA but remained constant after 24 and 48 hours in the presence of the drug (data not shown). On the contrary, RNR-S1 also dropped abruptly after just 2 hours in the presence of HU, but recovered and achieved higher levels after 18 hours. This was probably due to the induction of DNA damage by HU. DNA damage is known to activate RNR to increase the levels of dNTPs. To check whether or not HMBA also caused DNA damage we used immunocytochemistry to determine the levels of γH2AX in cells treated with HMBA for 0, 24 and 48 hours. For comparison we used MEL cells exposed to 2 mM HU for 24 and 48 hours as well. The results obtained are shown in Supplementary Figure 2. Although γH2AX was detected in untreated proliferating cells (MEL 0 hr), the intensity of the signal remained similar after 24 and 48 hours in the presence of HMBA. On the contrary, the intensity of the signal increased significantly when the cells were cultured in the presence of HU. These observations confirmed that no DNA damage occurred during the precommitment and commitment periods of differentiating MEL cells in the presence of HMB.

Discussion

In prokaryotes and some unicellular eukaryotes, such as Saccharomyces cerevisiae, it is well established that replication origins are sequence specific, although its nature is less clear in higher eukaryotes.
Figure 1 | Distribution of MEL-DS19 cells throughout the cell cycle during HMBA-induced differentiation. MEL-DS19 cells were pulse-labelled with BrdU, fixed and double stained with propidium iodide (PI) and BrdU-FITC. (a) The upper panels show a bivariate analysis of total DNA content using propidium iodide against BrdU incorporation (BrdU-FITC). Unlabelled cells lie below 10^6 of the y-axis while BrdU-labelled cells lie above it. (b) Lower panels show the PI fluorescence distribution of unlabeled (left) or BrdU-labelled (right) nuclei of MEL untreated (0 h) or HMBA-treated for 24 or 48 h. Table (c) indicate the calculated percentage of cells in each phase of the cell cycle and the total number of cells per ml at 0, 24 and 48 hours, respectively. Finally, table d shows the calculated percentage of cells in each phase of the cell cycle and the total number of cells per ml after the cells were grown without FCS for 0, 24 and 48 hr.
Figure 2 | The rate of replication fork progression slows down as cells differentiate. MEL-DS19 cells untreated or treated with HMBA for 24 or 48 hr were pulse-labelled for 20 min with IdU (red) followed by another 20 min pulse with CldU (green). DNA molecules were stretched by DNA combing as described in Materials and Methods. (a) Representative images of DNA fibres arbitrarily assembled from different fields. Only fibres showing both red and green fluorescence followed by unlabelled DNA (visualized in blue) were measured to guarantee the selection of complete intervals. (b) Distribution of CldU track lengths clustered by frequency measured in 10 kb intervals for fibres of uninduced MEL (MEL 0) or cells treated with HMBA for 24 (MEL 24) or 48 hours (MEL 48). In the histogram, coloured bars point to MEL 0, 24 and 48 from left to right, respectively. P value < 0.0001 in the Mann Whitney test. The table (c) indicates the calculated percentage of molecules in each interval.
Figure 3 | The inter-origin distance turns shorter as cells advance to terminal differentiation. MEL-DS19 cells untreated or treated with HMBA for 24 or 48 hr were pulse-labelled for 20 min with IdU (red) followed by another 20 min pulse with CldU (green). DNA molecules were stretched by DNA combing as described in Materials and Methods. (a) Selected DNA molecules isolated from MEL DS19 untreated cells (MEL 0 hr) or treated with HMBA for 24 (MEL 24 hr) or 48 hr (MEL 48 hr) containing replication initiation sites. Detection of sequential labelled tracks with IdU (red) followed by CldU (green) and unlabelled (blue) plus subsequent cell harvest guaranteed that the center-to-center distance between adjacent IdU tracks corresponded to neighbor inter-origin distances. (b) Distribution of inter-origin distances clustered by frequency measures in intervals of 20 kb for fibres of uninduced MEL (MEL 0) or cells treated with HMBA for 24 (MEL 24) or 48 hours (MEL 48). In the histogram, coloured bars point to MEL 0, 24 and 48 from left to right, respectively (see text for details). P value < 0.0001 in the Mann Whitney test. The table (c) indicates the calculated percentage of cases in each interval.
In summary, here we used genome-wide analysis of DNA replication to show that in general the rate of replication fork movement slows down and the inter-origin distance becomes shorter during the precommitment and commitment periods of MEL terminal differentiation before cells stop proliferating and accumulate in G1. We propose this is a general feature that occurs as a consequence of the progressive heterochromatization that takes place during terminal cell differentiation.

**Methods**

**Cell culture.** MEL DS-19 cells were maintained in Dulbecco’s modified Eagle’s medium DMEM, (Gibco) supplemented with 10% fetal bovine serum, PBS (1 mg/ml of penicillin and streptomycin) and 100 units/ml of penicillin and streptomycin. Cell synchronization was induced by exposing logarithmically growing cultures to 5 mM HMBA. Hemoglobinized cells were monitored by determining the proportion of benzidine-staining cells (B+) in the culture. Briefly, aliquots (0.1 ml) of culture containing from 105 to 106 cells were mixed with 0.1 ml of a 77.7 mM benzidine solution and 15 ml of 30% H2O2. After 20 minutes of incubation at room temperature 500–700 cells per sample were counted with a hemocytometer. To induce quiescence, cells were washed with PBS to completely eliminate fetal bovine serum and maintained in Dulbecco’s modified Eagle’s medium, DMEM (Gibco), supplemented with 100 units/ml of penicillin and streptomycin (Gibco) for 24 and 48 hours.

**BrdU labelling and flow cytometry.** MEL DS-19 cultures, 3·105 cells/ml growing in the absence (0 hr) or in the presence of 5 mM HMBA (24 and 48 hr) were pulse-labelled with 20 μM 5-bromo-2’-deoxyuridine (BrdU, Sigma Aldrich) for 20 minutes at 37°C, fixed with 70% ethanol and kept overnight at 4°C. Cells were permeabilized for 30 minutes at room temperature with a solution containing 200 μg of pepstatin (Sigma Aldrich) in 2 M HCl. After permeabilization, cells were incubated 1 hour at room temperature. The pellet was resuspended in 0.3 ml of PBS supplemented with 0.5 % Tween 20 and 0.5% BSA, containing 15 μl of anti-BrdU-FITC-Ab (Becton Dickinson) for 1 hour at room temperature. Samples were resuspended in PBS, stained with 20 μg/ml of propidium iodide and analyzed by flow cytometry (Coulter, XL, cytometer). Data were analyzed with Flowjo 8.2 software.

**DNA Combing.** Exponential growing MEL DS-19 cultures at 5·105 cells/ml were pulse labelled for 20 minutes with 40 μM IdU (Sigma), followed by a second 20 min pulse with 400 μM CldU (Sigma). After harvest, the cells were embedded in low melting point agarose (BioRad) plugs, at a density of 4·105 cells per plug. Plugs were digested for 48 hours at 50°C in ESP buffer (10 mM Tris-HCl pH 7.5, 50 mM EDTA pH 8.0, 1% Sarcosyl) containing proteinase K (2 mg/ml), washed with 0.5 M EDTA pH 8.0 at 50°C and kept at 4°C. For DNA extraction, plugs were washed 10 minutes with TE (10 mM Tris-HCl pH 8.0/ 1 mM EDTA pH8.0) and stained with 1.5 μg YoYo (Invitrogen) in 100 μl of TE for 1 hour in the dark on a rocking platform at 30°C. Plugs were melted in 50 mM MES pH 5.7 at 65°C for 1 hour and the agarose was digested overnight with β-agarase (BioLabs), 3 units per plug. Combing of DNA fibres was performed at Philippe Pasero’s lab as previously described (Pasero et al, 2002). Briefly, sidelined coverslips (Skeeb’s lab) were incubated in DNA solutions for 15 minutes at room temperature and removed from the reservoir at a constant speed of 300 μm/s. To fix the fibres the coverslips were baked for 2 hours at 65°C.

**Immunofluorescent Detection.** Slides were dehydrated for 5 minutes in successive ethanol baths of 70%, 90% and 100%, denatured in 1 M NaOH for 25 minutes, neutralized with PBS and blocked in 1x PBS, 1% BSA and 0.1% TritonX100 for 60 minutes. Hybridization was carried out in a humid chamber at 37°C. 1μl of IgD and anti-IgD signals were developed by sequential incubations with antibodies separated by PBS washings, as follows: primary antibody mix, 1/20 Mous Anti-BrdU clone B44, (Becton Dickinson) (anti-IgD) and Rat Anti-BrdU clone BU1/75, (ABCys SA) (anti-CldU) for 45 minutes, secondary antibody mix, 1/50 Goat anti-Mouse Alexa 546 (Molecular Probes) and Goat anti-Rat Alexa 488 (Molecular Probes) for 30 minutes. DNA detection was developed by 1/100 Mouse anti ssDNA (Chemicon) for 30 minutes and Goat anti- Mouse IgG2a Alexa 647 (Molecular Probes) for 30 minutes.

**Image acquisition and analysis.** Image acquisition was performed with a Leica DM6000B microscope equipped with a CoolSNAP HQ CCD camera and controlled with MetaMorph (Roper Scientific). On images acquired with this CCD camera and a 40x objective, 1 pixel = 340 μm. IgD and CldU tracks were measure manually with Photoshop (Adobe CS3) and data were transferred to an Excel Spreadsheet (Microsoft). Statistical analysis of CldU track length and inter-origin distance was performed with Prism 4.0 (GraphPad).

**Indirect immunofluorescent staining.** MEL DS-19 cells were fixed with 70% cold ethanol overnight at 4°C. Cells were permeabilised with 0.1% Triton X-100 in PBS for 30 minutes, blocked in 10% normal goat serum in PBS at room temperature for 1 hour. Primary and secondary antibody incubations were carried out in blocking buffer at 1 hour each one and washed with PBS before using PBS. The primary antibodies used were: anti-H2A (clone 15.19s2, #05–689, Upstate, USA) at a dilution of 1: 200 and anti-phospho-Histone H2A.X Ser 139, (clone JBW30, 0.5-656 Millipore) at a dilution of 1: 500. In both cases the secondary antibody...
used was anti-mouse Alexa 488 (Molecular Probes) at a dilution of 1:400 and 1:1000, respectively. Cells were counterstained with DAPI (4’,6-diamidino-2-phenylindole, 0.2 μg/ml, Sigma). Cells were spinne to slides and mounted with Pro Long Gold (Invitrogen). Images of immunostained cell nuclei were acquired with a Leica TCS SP5 Confocal Laser microscope equipped with a 63x immersion oil objective. Pictures were processed using Adobe Photoshop 4.0 software.

**HP1α flow cytometry.** MEL DS-19 cells were fixed with 70% cold ethanol overnight at 4°C. Cells were permeabilised with 0.1% Triton X-100 in PBS for 30 minutes and incubated with anti-HP1α at a dilution of 1:500 for 1 hour at room temperature. After three washes with PBS cells were incubated with the secondary antibody: anti-mouse Alexa 488 (Molecular Probes) at a dilution of 1:1000 for 1 hour at room temperature. Cells were washed with PBS twice and incubated with 20 μg/ml of propidium iodide and 100 μg/ml of RNAsa for 30 minutes at room temperature. Samples were analysed by flow cytometry (Coulter, XL, cytometer) and data was analyzed with FlowJo 8.7 software.

**Ribonucleotide reductase expression analysis.** Total RNA was extracted from cells using Trizol reagent. First-strand cDNA was synthesized from 5.0 μg of total RNA using the Superscript II (Invitrogen) in a final volume of 20 μl with 0.5 μg of Oligo dT (Invitrogen), 20 units of SUPERase In RNase Inhibitor (Ambion) and 200 units of Superscript II reverse transcriptase. The reaction mixture was incubated at 42°C for 50 min. Quantitative real time PCR was carried out in iQ5 system (Bio-Rad). The reaction mixture of 20 μl consisted of 1X iQ SYBR Green Supermix (Bio-Rad), 1μl cDNA and 0.2 μM of each primer. The PCR protocol was: 95°C for 5 min, followed by 50 cycles of 95°C for 30 s and 60°C for 30 s. The following primers were used: RNRM1, 5’-CTGGGCGAGGTCAGCAGTCT-3′ (forward) and 5’-GGACCTGCTTCTGAACT-3′ (reverse). RNRM2, 5’-TTTCTTTGCAAGCGATGTAG-3′.
(forward) and 5'-CGGGCCCTGCTATAAATACGGAGC-3' (reverse), GAPDH, 5'-GGG-TTCCTATATAGCCGTCGT-3' (forward) and 5'-CCATTGGTTCGAGGAG-CAAG-3' (reverse). Beta-Actin, 5'-CTAAGGCAACCTGGTAAAAG-3' (forward) and 5'-ACCGAGGCATAAGCGGACA-3' (reverse). Relative gene expression quantification method was used to calculate the fold change of mRNA expression according to the comparative C\(\text{t}\) method using GAPDH and \(\beta\)-actin as endogenous controls. The expression was determined to be 1 for the control sample, and the fold change of the target gene was calculated using the \(\Delta\Delta\text{Ct}\) method, where \(\Delta\text{Ct}\) values of the control and sample were determined by subtracting the \(\text{Ct}\) value of the target gene, RNR, from the value of the housekeeping gene: GAPDH and \(\beta\)-actin.

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Author contribution Statement
Mª Estefania Monturro performed all the experimental work. Olivier Ganier and Marcel Mechali designed, participated and advised all the experiments involving DNA combing. Pablo Hernandez participated in the analysis of cell flow cytometry data. Jorge B. Schwartzman and Dora B. Krimer designed the experiments, analysed the results and wrote the manuscript.