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Research Article

General and specific replication profiles are detected in normal human cells by genome-wide and single-locus molecular combing

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ABSTRACT

Mammalian genomes are replicated under a flexible program, with random use of origins and variable fork rates, and many details of the process must be still unraveled. Molecular combing provides a set of direct data regarding the replication profile of eukaryotic cells: fork rates; organization of the replication clusters; proportion of unidirectional forks; and fork dynamics. In this study the replication profiles of different primary and immortalized non-cancer human cells (lymphocytes, lymphoblastoid cells, fibroblasts) were evaluated at the whole-genome level or within reference genomic regions harboring coding genes. It emerged that these different cell types are characterized by specific replication profiles. In primary fibroblasts, a remarkable fraction of the mammalian genome was found to be replicated by unidirectional forks, and interestingly, the proportion of unidirectional forks further increased in the replicating genome along the population divisions. A second difference concerned in the proportion of paused replication forks, again more frequent in primary fibroblasts than in PBL/lymphoblastoid cells. We concluded that these patterns, whose relevance could escape when genomic methods are applied, represent normal replication features. In single-locus analyses, unidirectional and paused replication forks were highly represented in all genomic regions considered with respect to the average estimates referring to the whole-genome. In addition, fork rates were significantly lower than whole-genome estimates. Instead, when considering the specificities of each genomic region investigated (early to late replication, normal or fragile site) no further differentiating features of replication profiles were detected. These data, representing the integration of genome-wide and single-locus analyses, highlight a large heterogeneity of replication profiles among cell types and within the genome, which should be considered for the correct use of replication datasets.

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Introduction

DNA replication is a fundamental process, governed by a complexity of molecular and cellular events which has been the subject of investigations for decades [22–24].

Different genomic methods developed in recent years contributed to delineate the key features of the replication process in high eukaryotes [11]. A major effort has been put in defining the molecular characteristics and regulation of replication origins in metazoans: potential initiation sites are indeed about 10-fold more frequent than actually used per cell cycle [3,5,24]. For a long time, no consensus sequences were identified at metazoan replication origins, and epigenetic mechanisms were considered as major regulators of origin activation [22–24]; recently, G-quadruplexes were reported to be associated to replication origins, and to regulate activation and usage efficiency [3,5].

During S-phase, origins fire according to a deterministic program which defines the average time for activation (i.e., the replication timing); however, in agreement with the general notion that the frequency of potential origins is greater than the number of those actually activated per cell division, a same origin is not strictly committed to fire in all the cells. Indeed, origins can be either activated or passively replicated by adjacent forks, in a stochastic pattern leading to cell-to-cell plasticity [2]. Replication timing has been deeply investigated with special emphasis for its possible modulation along development and differentiation [24], and robust evidence was recently collected that the timing may be cell-type specific and reprogrammable [3,13,12]. Finally, in mammalian cells a tight coordination between replication and transcription has been demonstrated [4,21]; this regulation may be crucial for genome stability [14].

Molecular combing may be applied to describe the replication profiles of mammalian cells [11,15]. This single-molecule approach provides estimates of fork rates, fork dynamics, inter-origin distances at the whole-genome or single-locus level [11,15]. With respect to novel genomic approaches that focus the attention on the position and the activity of initiation sites [11], molecular combing returns additional direct information regarding the replication profile of mammalian cells: size and organization of the replication clusters, proportion of unidirectional forks, pause or arrest of the replication forks, fork rate variations along the bidirectional fork progression. Several contributions regarding DNA replication in eukaryotes were obtained by molecular combing [15]. Furthermore, new insights concerning the molecular basis for instability of common fragile sites derive from the recent characterization of the replication patterns of *FRA6E* [29], *FRA3B* [20] and *FRA16C* [27]. However, in the light of the complex organization of the mammalian genome, the flexibility of the replication process, and the possibility of its epigenetic regulation, a detailed picture of the mammalian replication program deserves further in-depth studies. In particular, single molecule analyses carried out with different normal cell types would provide valuable information to define the regular features of the process with respect to the events occurring under perturbed conditions.

Here we applied molecular combing to evaluate and compare the replication parameters of different primary and immortalized non-cancer human cells; in parallel to a genome-wide analysis, the replication profiles of reference genomic regions harboring coding genes were considered.

Materials and methods

Cells

Primary human peripheral lymphocytes (PBL) from healthy donors were obtained by standard procedures [29]; after PHA-stimulation, PBL were grown in RPMI 1640 medium supplemented with antibiotics and 10% fetal bovine serum (FBS) for 48–72 h before harvesting.

Immortalized B lymphoblastoid cells (TK6 and H691) were cultured in RPMI 1640 medium supplemented with antibiotics and 10% fetal bovine serum (FBS). Duplication time is 24–30 h for both the cell lines.

IMR-90 cells (a diploid fibroblast strain derived from fetal lung) were cultured in DMEM medium supplemented with antibiotics and 10% FBS. Fibroblasts were harvested at different population doublings (PD) in the interval 21–37, from independent cultures started separately from low PD frozen samples. For IMR-90 cells, duplication time is 28–30 h up to 30 PD; at PD 34–36 the proliferation is slowered, and duplication time is 45 h.

Cell lines were grown at the standard conditions (37 °C, 5% CO_2). All culture reagents were from Gibco, Invitrogen.

Assays of cell proliferation

To evaluate cell cycle distribution by flow cytometry, actively proliferating cells were harvested and fixed in 70% ethanol; cells were stained with propidium iodide (50 ng/ml, Sigma-Aldrich) immediately before analysis (Becton Dickinson FACSCanto II Flow cytometer). The proportion of S-phase cells has also been detected with an immunofluorescent approach which allowed to evaluate the early, mid and late stage distribution. Exponentially growing populations (PBL cell suspensions, or IMR-90 cells growing on 22 × 22 coverslips) were labeled for 30 min with 10 μM 5-Bromo-2'-deoxyuridine (BrdU, Sigma-Aldrich) before harvest. Immunodetection was performed as described in details in [29]. Per each cell sample, at least 120 BrdU-positive nuclei were scored; the total number of nuclei present in the same microscope fields was recorded in parallel.

Molecular combing

Immediately before harvesting, exponentially growing cells were exposed by two sequential pulses (30 min each) to 100 μM 5-Iodo-2'-deoxyuridine (IdU, Sigma Aldrich), and to 100 μM 5-Chloro-2'-deoxyuridine (CldU, Sigma Aldrich) (Supplementary Fig. 1). Cells were immobilized in agarose plugs (100–200,000 cells/plug); high molecular weight DNA was released from 1–2 plugs in 0.1 M MES, pH 6.5, after digestion with β-agarase (3 U, BioLabs). DNA combing on silanised surfaces was done according to a standard procedure. More details can be obtained in [29].

Genome-wide and single locus analyses on combed DNA

Replication tracks in combed DNA were detected by immunofluorescence. For genome wide analyses, DNA preparations were denatured in 1 M NaCl, 50 mM NaOH (15 min); slides were incubated with a mix of two primary anti-BrdU antibodies, cross-reacting respectively with IdU (Becton Dickinson, developed