Experimental Cell Research 339 (2015) 20–34

Contents lists available at ScienceDirect

Experimental Cell Research

journal homepage: www.elsevier.com/locate/yexcr

Research Article

The effect of DNA damage on the pattern of immune-detectable DNA methylation in mouse embryonic fibroblasts

Selcen Çelik*, Yan Li, Chris O'Neill

Human Reproduction Unit, Kolling Institute for Medical Research, Sydney Medical School, University of Sydney, Sydney 2065, Australia

ARTICLE INFO

Article history:
Received 26 March 2015
Received in revised form
31 July 2015
Accepted 27 August 2015
Available online 26 September 2015

Keywords:
Epigenetics
Cytosine methylation
methyl-binding protein-1
Heterochromatin
Genotoxic stress
DNA damage
DNA repair
trypsin
Antigen retrieval
İmmune-staining
Mouse
Fibroblast

ABSTRACT

The methylation of cytosine at CpG dinucleotides (5meC) is an important epigenetic mechanism that governs genome stability and gene expression. Important ontological and pathological transitions are associated with marked global changes in detectable levels of methylation. We have previously found two pools of immune-detectable 5meC exist within cells, a pool that can be detected after acid treatment of fixed cells to denature chromatin and another large but variable pool that requires a further tryptic digestion step for complete epitope retrieval. The trypsin-sensitive pool has been shown to be largely associated with the heterochromatic fraction (by a heterochromatin marker, HP1-β) of the genome, and the size of this pool varies with the growth disposition of cells. Since DNA damage imposes large changes on chromatin structure the present study analyzed how such changes influences the faithful immunological detection of 5meC within mouse embryonic fibroblasts. DNA damage was induced by either UV-irradiation or doxorubicin treatment, each of which resulted in increased levels of immune-detectable 5meC at 24–48 h after treatment. There was a marked trypsin-sensitive pool of 5meC in these cells which was significantly increased after DNA damage. The increased levels of 5meC staining predominantly co-located with heterochromatic foci within nuclei, as assessed by HP1-β staining. The relative amount of masked 5meC after DNA damage was positively associated with increased levels of HP1-β. The methyl binding protein, MBD1, was a less reliable measure of changes in 5meC, with a significant fraction of 5meC not being marked by MBD1. The cyto-epigenetic approaches used here reveal dynamism in the levels and localization of immune-detectable 5meC within the nuclei of fibroblasts in response to DNA damage.

1. Introduction

Methylation of cytosine at CpG dinucleotides (5meC) of the genome has important roles in the maintenance of genome stability as well as influencing the patterns of gene expression. Methylation can also influence chromatin structure within the nucleus as DNA in heterochromatic regions is commonly hypermethylated [1–4]. Since major changes in chromatin conformation and structure are known to occur during ontogeny [5–7], throughout the cell-cycle [6,8,9] and in response to DNA damage [6,10], it is of interest to understand whether 5meC levels are associated with these changes.

The use of immunolocalization with specific antibodies provides the significant advantage of allowing the detection of changes in the localization of 5meC within the architecture of the nucleus of individual cells [11,12]. It has the further very important advantage of being able to distinguish between the known range of covalent modifications to cytosine (5meC, 5hmC-5′-hydoxymethylcytosine; 5fC, 5′-formylcytosine; and 5caC, 5′-carboxycytosine) in the same cell, an analysis not possible with modern forms of chemical measurement. The power of immunolocalization can only be fully realized, however, if the conditions used faithfully allow detection of the antigens present. This requires that antibody binding is performed under equilibrium binding conditions and that sample preparation allows the all of the epitope to be exposed to the aqueous phase so that antibody binding can be achieved. These pre-conditions are particularly challenging for antigens within chromatin because of the complex and variable conformation and structure of chromatin [2,3,7,13].

DNA is by its nature highly coiled and forms many higher order structures. This complexity is compounded by the many histone and non-histone proteins that bind to DNA and the range of

Abbreviations: 5meC, 5-methyl cytosine; MBD1, methyl-binding protein-1; HP1-β, heterochromatin protein 1-β; MEFs, mouse embryonic fibroblasts

* Corresponding author. Current address: Department of Molecular Biology and Genetics, Faculty of Science, Karadeniz Technical University, Trabzon 61080, Turkey.
E-mail address: selcencelik83@yahoo.com (S. Çelik).

http://dx.doi.org/10.1016/j.yexcr.2015.08.017
0014-4827/

covalent and other modifications of chromatin components that can occur. Conventionally, the detection of 5meC has relied upon denaturation of chromatin by brief acid treatment to cause solvent exposure of 5meC. We have recently shown, however, that this form of epitope retrieval leaves a large amount of 5meC masked from immunodetection [14–16]. This was most evident in the newly fertilized mouse embryo where the use of conventional immuno-staining methods showed a progressive loss of 5meC staining resulting in an appearance of almost complete demethylation by the time of the first cell division [17–19]. Further analysis showed that this loss of 5meC staining was not primarily due to a loss of 5meC but was due to a progressive trypsin-sensitive masking of the antigen that accompanied changes to chromatin conformation and structure during the zygotic cell-cycle. Conventional immune-localization of 5meC has typically involved a brief exposure of fixed cells to acid treatment and it has been generally reasoned that this provides sufficient denaturation of chromatin to expose the 5meC. Yet a brief period of tryptic digestion of the fixed embryos allowed much more 5meC antigen to be retrieved. This methodology revealed that there were only relatively minor changes in the levels of 5meC in the early embryo, and did not support claims of active global demethylation in the zygote. Analysis of mouse embryonic fibroblasts (MEFs) showed that these cells also had a degree of trypsin-sensitive masking of 5meC [14]. While the near complete masking of 5meC seen in the zygote was not evident in MEFs, there was still a large trypsin-sensitive pool of 5meC. This pool was predominantly associated with the heterochromatic fraction of the genome. Most notably, it was found that 5meC associated with heterochromatin varied with the growth disposition of cells [14]. For instance, proliferative cells displayed a relatively greater accumulation of 5meC within the trypsin-sensitive pool than quiescent cells, and this was in large part associated with changes in the heterochromatin fraction between these two growth states [14].

DNA damage and the resulting repair processes are associated with marked changes in the extent of chromatin compaction and heterochromatin expansion, and this is considered to allow repair factors to access damaged sites of DNA [6,10]. It is also noteworthy that molecular models for the catalytic demethylation of cytosine implicate the involvement of DNA repair processes, including the base excision repair pathway [20–26]. Given the changes in chromatin organization caused by DNA damage and repair processes we investigated if various forms of genotoxic stress influenced the levels of solvent exposure and trypsin-sensitive masking of 5meC in MEFs. We chose two treatments to induce DNA damage; UV irradiation can predominantly induce single strand DNA breaks (SSBs) [27,28], while doxorubicin causes double strand DNA breaks (DSBs) [29,30]. The study finds that both forms of DNA damage caused changed levels, localization and trypsin-sensitive masking of immune-detectable 5meC in MEFs. This was associated with the marked changes within the heterochromatin fraction of the genome. The findings show that 5meC within the fibroblast nucleus displayed significant dynamism that can be detected in a cost-effective and efficient manner by careful attention to the conditions required for full antigen retrieval during immunostaining.

2. Material and methods

2.1. Cell culture

Mouse embryonic fibroblasts (MEFs) from Day 13.5 embryos were collected using standard methods and then cultured in Dulbecco's modified eagle medium (DMEM) (Thermo Fisher Scientific Inc., Utah, USA; Cat. no. SH30243.FS). Media were supplemented with 10% (v/v) fetal bovine serum (FBS) (Invitrogen Life Technologies, Carlsbad, USA; Cat. no. 10099-141), 1% (v/v) 1 × MEM-Non-Essential Amino Acids solution (Invitrogen, Cat. no. 11140-050), 50 U/ml penicillin (Sigma-Aldrich Co.; St. Louis, MO, USA; Cat. no P3032) and 50 μg/ml streptomycin (Sigma, Cat. no. S6501) at 37 °C with 5% CO_2 in air. Cells were grown as proliferative (1 day-culture) or quiescent (24 h serum-starved confluent culture) cultures.

2.2. Genotoxic stress

Quiescent MEFs were exposed to UV-irradiation (253.7 nm at a dose of 1.95 J/cm^2) for (i) 15 s followed by post-incubation either for 1 h or (ii) 12 min followed by culture for 48 h. Cells were in HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) buffered media (pH 7.4) (Invitrogen, Cat. no. 12430) at 37 °C, during exposure. HEPES-buffered media included bovine serum albumin (BSA) (3 mg/ml). Control cells were untreated at 37 °C with 5% CO_2 in air. Proliferative MEFs were treated with doxorubicin (Sigma, Cat. no. D1515) at doses of 0 or 50 nM in complete media for 24 h.

2.3. DNA damage – immunofluorescence for γ-H2A.X (phosphoS139) staining

After treatment, MEFs were washed with 1 × phosphate-buffered saline (PBS) and fixed with 2% (w/v) paraformaldehyde (PFA) (Sigma, Cat. no. P6148) for 30 min at room temperature (RT). Cells were permeabilized with 2% (w/v) PFA containing 0.3% (v/v) Tween-20 (Sigma, Cat. no. P7949) and 0.2% (v/v) Triton-X (Bio-Rad Laboratories Inc., CA, USA, Cat. no. 161-0407) for 30 min at RT. Cells were washed in 1 × PBS (w/v) (Sigma, Cat. no. D5773) for 30 min and blocked in 30% (v/v) goat serum (Sigma, Cat. no. G9023) in 2 mg/ml BSA in PBS with 0.05% Triton-X (w/v) (BSA, from Sigma, Cat. no. A1470) for 3 h at RT. Cells were washed in 1 × PBS for 30 min at RT. Cells were incubated in primary antibodies (1:250): rabbit polyclonal anti-γ-H2A.X (phosphoS139) (Abcam, UK; Cat. no. ab2893) to detect DNA breaks; or a non-immune rabbit IgG (Sigma, Cat. no. I5006) in 2 mg/ml BSA in PBS with 0.05% Triton-X (w/v) at 4 °C overnight. Cells were washed in 1 × PBS for 30 min, and then were incubated with fluorescein isothiocyanate (FITC) (1:200) (Sigma, Cat no. F1262) in 2 mg/ml BSA in PBS with 0.05% Triton-X (w/v) for 1 h at RT in the dark. Cells were washed in 1 × PBS for 30 min, and then mounted with PBS.

2.4. Immunofluorescence for DNA methylation (double staining for 5meC and MBD1)

After treatment, MEFs were washed with 1 × PBS (w/v) and fixed with 4% (w/v) PFA for 30 min at RT. Cells were then washed with 1 × PBT (w/v) (PBT, phosphate-buffered saline with Tween-20) for 30 min. Cells were permeabilized with 1 × PBS including 0.5% (v/v) Triton-X and 0.5% (v/v) Tween-20 for 40 min at RT. Cells were treated with 4 N HCl containing 0.1% (v/v) Triton-X for 10 min at RT. Acid was removed by extensive washing with 1 × PBT. Cells were treated with trypsin (0.25%) either for 0 or 1 min at 37 °C. Trypsin was inactivated by washing with an equal volume of pre-warmed media including 90% (v/v) DMEM, 3 mg/ml BSA and 10% (v/v) sheep serum, for 1 min at 37 °C and then washed with 1 × PBT (w/v) followed by blocking in 10% (v/v) goat and 30% (v/v) sheep serum in 1 × PBT at 4 °C overnight. Cells were incubated in primary antibodies: rabbit anti-MBD1 (1:50) or non-immune rabbit IgG (1:50) in 2 mg/ml BSA in 1 × PBT overnight at 4 °C. Cells were washed with 1 × PBT for 10 min at RT followed by incubation with primary antibodies: mouse anti-5meC (1:60) or non-immune mouse IgG (1:60) in 2 mg/ml BSA in 1 × PBT for 1 h at RT. Cells were washed with 1 × PBT (w/v) for 30 min. Cells then were