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Remodeling of ribosomal genes in somatic cells by *Xenopus* egg extract

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ABSTRACT

Extracts from *Xenopus* eggs can reprogram gene expression in somatic nuclei, however little is known about the earliest processes associated with the switch in the transcriptional program. We show here that an early reprogramming event is the remodeling of ribosomal chromatin and gene expression. This occurs within hours of extract treatment and is distinct from a stress response. Egg extract elicits remodeling of the nuclear envelope, chromatin and nucleolus. Nucleolar remodeling involves a rapid and stable decrease in ribosomal gene transcription, and promoter targeting of the nucleolar remodeling complex component SNF2H without affecting occupancy of the transcription factor UBF and the stress silencers SUV39H1 and SIRT1. During this process, nucleolar localization of UBF and SIRT1 is not altered. On contrary, azacytidine pre-treatment has an adverse effect on rDNA remodeling induced by extract and elicits a stress-type nuclear response. Thus, an early event of *Xenopus* egg extract-mediated nuclear reprogramming is the remodeling of ribosomal genes involving nucleolar remodeling complex. Condition-specific and rapid silencing of ribosomal genes may serve as a sensitive marker for evaluation of various reprogramming methods.

1. Introduction

Reprogramming somatic cells to pluripotency entails a stable switch in the transcriptional program. Use of *Xenopus laevis* egg extracts has notably been of interest due to ample availability of reprogramming material (eggs) and ease of manipulation of the system to investigate mechanisms of reprogramming [1,13,18]. A more widespread and practical use of extracts for reprogramming has, however, been hampered by phenotypic and functional instability of extract-treated cells in long-term culture [13,26,27], probably because of inefficient epigenomic rebuilding of the genome [3,8,42]. Moreover, the mechanisms of nuclear reprogramming remain largely unidentified although chromatin remodeling factors and associated changes in DNA methylation and histone modifications have been implicated, e.g. treatment of cells with the DNA methyltransferase (DNMT) inhibitor 5-aza-2′deoxycytidine (AZA) [25,43] suggesting that changes towards a more "open" chromatin state may favorably prime the genome.

Changes in chromatin states may, however, under certain circumstances reflect stress-related mechanisms rather than true reprogramming [17,44]. Ribosomal RNA (rRNA) synthesis is tightly regulated in response to metabolic and environmental changes [10,29]. Interestingly, rRNA genes are epigenetically remodeled within a few hours after induction of differentiation [33,36] or stress [29]. These two events involve two distinct epigenetic mechanisms, each with their own set of remodeling factors.

Inactivation of rRNA genes during genomic reprogramming is mediated by the nucleolar remodeling complex (NoRC), which triggers heterochromatin formation and transcriptional silencing [22]. NoRC is a member of the ATP-dependent chromatin remodelers. Notably, nucleolar protein TIP5 in combination with the ATPase SNF2H [40] recruits DNMTs, histone deacetylases (HDACs) and histone methyltransferases (HMTs) to rDNA promoters [41], causing their silencing.

Ribosomal DNA transcription is also regulated by ATP through a mechanism by which rDNA silencing significantly decreases energy expenditure and thus protects cells from energy deprivation-induced apoptosis accompanying stress [15,37]. This mechanism operates via energy-dependent nucleolar silencing complex (eNoSC), which balances rDNA transcription vs. silencing through metabolic feedback loops [29]. Two components of the eNoSC are the NAD-dependent deacetylase SIRT1 and histone methyltransferase SUV39H1 [4,29,30]. eNOSC activation may, thus, constitute a marker of recruitment of protective mechanisms to rDNA loci in a reprogramming context.

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In this study, we examined early ribosomal chromatin-linked events induced in somatic cell nuclei by *Xenopus* egg extract. We show for the first time that extract exposure induces reprogramming-related silencing of ribosomal genes, and initiates large-scale chromatin reorganization suggestive for cellular dedifferentiation. Moreover, as shown on stress-response detected in cells pre-treated with AZA, specific silencing of ribosomal genes is a sensitive marker for evaluation of reprogramming methods.

2. Material and methods

2.1. Cell culture

Human embryonic kidney-derived epithelial 293T cells were cultured in RPMI 1640 (Sigma) containing 10% FCS, 2 mM L-glutamine, 1 mM sodium pyruvate and non-essential amino acids (complete RPMI). Treated and control cells were cultured in parallel and experiments were performed in three biological replicates.

2.2. Xenopus egg extract preparation

Xenopus egg extracts were prepared as described previously [14] with minor changes. Briefly, freshly laid eggs were washed in 1× Mare's Modified Ringers buffer (MMR). Eggs were dejellied in 20 mM Tris–HCl/100 mM NaCl/1 mM dithiothreitol for 4 min and washed in 0.25× MMR. Eggs were rinsed in extraction buffer (5 mM $MgCl_2$, 50 mM KCl, 2 mM 2-mercaptoethanol, protease inhibitor cocktail, 5 mM EGTA, 10 μg/ml cytochalasin B and 50 mM HEPES, pH 7.4) and transferred to centrifuge tubes. Excess buffer was removed and eggs were crushed at 15,000*g* for 30 min at 4 °C. The cytoplasmic layer was collected and re-centrifuged at 15,000*g* for 20 min at 4 °C. The cleared cytoplasm was mixed with 5% glycerol, snap-frozen in aliquots in liquid nitrogen and stored at −80 °C until use.

2.3. Egg extract treatment

Cells with or without 48 h incubation with 5-azacytidine (Sigma, A2385) were washed twice in cold PBS and twice in cold Ca^{2+}- and Mg^{2+}-free Hanks' balanced salt solution (HBSS). HBSS (1.5 ml) was added and samples heated to 37 °C for 2–3 min prior a 30-min permeabilization with 500 μl Streptolysin-O solution (Sigma; 1000 μg/ml). Permeabilization was assessed by monitoring uptake of a 70,000 M_r Texas Red-conjugated dextran (50 μg/ml) in a separate sample 24 h after resealing plasma membranes. Permeabilization efficiency under these conditions was 80% (not shown). After permeabilization, cells were incubated for 1 h at 37 °C with egg extract diluted 1:1 in MilliQ water and containing an ATP-regenerating system. To reseal plasma membranes, cells were cultured in complete RPMI containing 2 mM $CaCl_2$. After 2 h, floating cells were removed and plated cells were cultured in complete RPMI. As negative controls, permeabilized cells were resealed without extract exposure. These mock-treated cells did not exhibit any difference from untreated cells for any of the markers examined (data not show).

2.4. Reverse transcription (RT)-PCR

Total RNA was isolated with the RNeasy Mini Kit (Qiagen), DNAase treated and reverse transcribed using random hexamers. Quantitative (q)PCR was performed with the iCycler MyiQ real time PCR detection system (BioRad) and SYBR Green using primers specific for the 5′external transcribed spacer of pre-rRNA (5′-GAACGGTGGTGTGTCGTTC-3′ and 5′-GCGTCTCGTCTCGTCTCACT-3′) and beta actin mRNA (5′-ATCGTCCACCGCAAATGCTTCTA-3′ and 5′-AGCCATGCCAATCTCATCTTGTT-3′). Primers pairs gave no signal in PCRs lacking template (not shown). Relative expression was determined by the $\Delta\Delta C_T$ method.

2.5. Immunocytochemistry

Cells were fixed with 4% paraformaldehyde for 10 min, or in ice-cold acetone:methanol (1:1) for 5 min (lamin B staining), permeabilized in 0.1% Triton X-100 for 10 min, blocked with 2% bovine serum albumin for 15 min and incubated for 30 min with primary antibodies. Antibodies were mouse anti-nucleophosmin (B23; Invitrogen 32-5200), mouse anti-UBF (Abnova, H00007343-Q01), goat anti-lamin B (Santa Cruz, sc-6217), and mouse anti-SIRT1 (Abnova, H00023411-M01). Secondary antibodies were Alexa fluor 488 anti-mouse (B23, SIRT1), Alexa fluor 594 anti-mouse (UBF) and Alexa fluor 594 anti-goat (lamin B) (all from Jackson Laboratories). DNA was stained with Hoechst 33258. For negative control immunostaining was performed by omitting the primary antibodies and resulted in the lack of labeling (not shown).

B-type lamin staining classification: cells displaying intact nuclear envelope (intact); cells with partial/incomplete staining (partial); and cells lacking the staining (absence). Double staining for UBF and B23 classification: Type 1 cells with distinct nucleolar localization of UBF into foci and B23 into sharp margin shells; Type 2 cells with diffuse nucleoplasmic labeling of UBF and distinct B23 labeling; and Type 3 cells with diffuse labeling of UBF and B23. In each experimental group, minimum 200 cells were analyzed in three biological replicates.

2.6. Chromatin immunoprecipitation (ChIP)

ChIP was performed as described [5]. Antibodies used were UBF (Abnova, H00007343-Q01), SNF2H (Abcam, ab3749), SUV39H1 (Abnova, MAB1156) and SIRT1 (Abnova, H00023411-M01). ChIP DNA was analyzed by qPCR using primers specific for the rDNA promoter region −49 to +32 (5′-GGTATATCTTTCGCTCCGAG-3′ and 5′-GACGACAGGTCGCCAGAGGA-3′) and related to input chromatin eluted without previous ChIP. No antibody control was performed and resulted in marginal chromatin pull down. Three biological replicates were analyzed each by 2 ChIPs, statistically analyzed by *t*-test ($p < 0.05$).

3. Results

3.1. Remodeling of the nuclear envelope, chromatin, and rRNA transcription machinery by egg extract

Xenopus egg extracts have been shown to induce remodeling of chromatin and reprogram gene expression in somatic cells [1,13]. We first examined changes in nuclear morphology. Destabilization of the nuclear envelope enables interaction of somatic chromatin with egg-specific remodeling factors [18]. B-type lamins, major structural components of the nuclear envelope [20], were gradually disassembled from the nuclear periphery such that nearly all nuclei lacked a detectable lamina by 1 h in extract (Fig. 1A). Moreover, DNA staining showed evidence of global chromatin remodeling as seen by profound heterochromatin areas (Fig. 1E; Condensed). Likewise, the rRNA transcription factor UBF de-localized from nucleoli (Fig. 1C and D; Type 2), followed by delocalization of rRNA processing protein nucleophosmin (B23) (Fig. 1C and D; Type 3). Importantly, both chromatin and nucleolar alterations were reversible, as nuclei regained normal morphology within 1 h after extract treatment (Fig. 1A and C).

In addition, extract treatment elicited dramatic changes in cell phenotype, judged by the formation of cell aggregates on day 1