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Ribosome biogenesis is dynamically regulated during osteoblast differentiation

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ABSTRACT

Changes in ribosome biogenesis are tightly linked to cell growth, proliferation, and differentiation. The rate of ribosome biogenesis is established by RNA Pol I-mediated transcription of ribosomal RNA (rRNA). Thus, rRNA gene transcription is a key determinant of cell behavior. Here, we show that ribosome biogenesis is dynamically regulated during osteoblast differentiation. Upon osteoinduction, osteoprogenitor cells transiently silence a subset of rRNA genes through a reversible mechanism that is initiated through biphasic nucleolar depletion of UBF1 and then RNA Pol I. Nucleolar depletion of UBF1 is coincident with an increase in the number of silent but transcriptionally permissible rRNA genes. This increase in the number of silent rRNA genes reduces levels of ribosome biogenesis and subsequently, protein synthesis. Together these findings demonstrate that fluctuations in rRNA gene transcription are determined by nucleolar occupancy of UBF1 and closely coordinated with the early events necessary for acquisition of the osteoblast cell fate.

1. Introduction

Ribosome biogenesis is a fundamental cellular process that is necessary for all protein production. As requirements for protein production differ depending on the cellular state, the rate of ribosome biogenesis fluctuates over a cell's ontogeny. The rate of ribosome biogenesis is largely regulated at the level of ribosomal RNA (rRNA) gene transcription, which is carried out by RNA Polymerase I (RNA Pol I) (Learned et al., 1986; Bell et al., 1988; Moss et al., 2007). Differences in the level of rRNA gene transcription correlate with the state of cellular differentiation: progenitor cells synthesize higher levels of rRNA compared to their terminally differentiated counterparts (Bowman, 1987; Zahradka et al., 1991; Ali et al., 2008). Strong evidence suggests that this relationship is functionally important to cell fate decisions. Signals for differentiation trigger transcriptional repression of rRNA genes in stem and progenitor cells (Larson et al., 1993; Poortinga et al., 2004). Furthermore, evidence suggests that rRNA levels play an active role in phenotypic determination. Up-regulation of rRNA gene transcription delays differentiation, whereas its down-regulation triggers differentiation (Hayashi et al., 2014; Watanabe-Susaki et al., 2014; Zhang et al., 2014).

The lineage-specific mechanisms that regulate rRNA gene transcription during differentiation are best understood in bone. During bone formation Runx2, the master transcriptional regulator of the osteoblast phenotype, directly represses rRNA gene transcription (Ali et al., 2008). RUNX2 and its coregulator TLE bind the rRNA gene promoter, where they interact with the RNA Pol I transcription factor UBF1, and promote removal of active histone marks through recruitment of HDAC1 (Young et al., 2007; Ali et al., 2010; Ali et al., 2012). These repressive actions for RUNX2 are opposed by an unorthodox activity of fibroblast growth factor receptor 2 (FGFR2) in the nucleolus. FGFR2 regulates the binary choice of osteoprogenitor cells to either self-renew or terminally differentiate (Ornitz and Marie, 2002). We previously demonstrated that nucleolar FGFR2 holds osteoprogenitor cells in a self-renewing state that resists differentiation by interacting at the rRNA gene promoter with UBF1 and FGF2 at the rRNA gene promoter to limit occupancy of RUNX2 (Neben et al., 2014).

That regulators of osteoblast differentiation, like RUNX2 and FGFR2, influence the level of rRNA gene transcription suggests a strong mechanistic link between ribosome biogenesis and osteogenesis. Further support for ribosome biogenesis playing a role in bone is seen in the skeletal deficiencies of congenital ribosomopathies (Trainor and Merrill, 2014). Given the universal importance of ribosomes in all cell types, it is striking that disruptions in ribosome biogenesis lead to specific skeletal abnormalities. Here we attempt to better understand why bone is highly sensitive to changes in ribosome biogenesis using MC3T3-E1 mouse calvarial osteoprogenitors, which upon osteoinduction transition

Abbreviation: rRNA, ribosomal RNA; RNA Pol I, RNA Polymerase I; TSS, transcription start site; NOMe, nucleosome occupancy and methylome assay; OPP, O-propargyl-puromycin.

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through a development-like sequence from proliferative preosteoblasts to mature osteoblasts (Quarles et al., 1992; Wang et al., 1999). We find that rRNA gene transcription is dynamically regulated during osteoblast differentiation. During early stages of cell fate determination, rRNA gene transcription is transiently decreased. This decrease is temporally correlated with biphasic nucleolar depletion first of UBF1 and then RNA Pol I. Concomitant with nucleolar depletion of UBF1, we find an increase in the number of inactive rRNA genes without a corresponding increase in endogenous CpG methylation. Finally, we show that changes in rRNA gene transcription during osteoblast differentiation correlate with levels in ribosome production that affect the cell's capacity for protein synthesis.

2. Materials and methods

2.1. Cells and osteoinduction

MC3T3-E1 preosteoblasts were grown in MEMα containing 10% FBS and 1× PSG. Cells were seeded at 6×10^3 cells/well in 96-well plates, 7.5×10^4 cells/well in 12-well plates, 2×10^4 cells/well in 4-well chamber slides, or 3×10^6 cells in 100 mm dishes. After 24 h, cells were collected or induced to differentiate with 50 mg/ml ascorbic acid and 10 mM β-glycerophosphate. After 2, 3, or 6 days of growth, cells were processed as specified below.

2.2. Quantitative PCR (qPCR)

RNA was isolated from cells using RNeasy Mini Kit (Qiagen). cDNA was synthesized by reverse transcription (Qiagen), and qPCR was performed using PerfeCTa SYBR Green FastMix (Quanta Biosciences) with the following primer sets: *45S pre-rRNA* (Forward 5′-GCTTGTTTC TCCCGATTGC-3′, Reverse 5′-CGCGAACCACTGAGAAAAGT-3′), *Runx2* (Forward 5′-CCGCACGACAACCGCACCAT-3′ and Reverse 5′-CGCTCCG GCCCACAAATCTC-3′), *Collagen I* (Forward 5′-TCTGTATCTGCCACAA TGGC-3′ and Reverse 5′-AGCTTCCCCATCATCTCCA-3′), and *Osteocalcin* (Forward 5′-GGACCATCTTTCTGCTCACT-3′ and Reverse 5′-CGGAGT CTGTTCACTACCTTAT-3′).

2.3. Immunofluorescence

Cells were seeded on chamber slides (Lab-Tek), fixed in 4% paraformaldehyde, permeabilized, blocked in 10% goat serum, and incubated with mouse anti-UBF1 (Santa Cruz, 1:400) and rabbit anti-POLR1D (Abcam, 1:500) antibodies overnight at 4 °C. Primary antibodies were detected using Alexa Fluor conjugated secondary antibodies (goat anti-mouse 488 and goat anti-rabbit 568, Invitrogen) diluted 1:400 in 1% donkey serum/PBS at room temperature for 1 h. Coverslips were mounted on slides using Vectashield with DAPI (Vector Laboratories) and imaged on Leica TCS SP5/8 confocal system using a 63× objective and 2.0× digital zoom.

2.4. Chromatin immunoprecipitation (ChIP)

ChIP was performed as previously described with slight modifications (Neben et al., 2014). Briefly, cells were cross-linked, and isolated nuclei were sonicated to generate DNA fragments. Chromatin was diluted, and 10% of the supernatant was kept for input. The remaining supernatant was pre-cleared and incubated overnight with 5 μg of antibody (anti-UBF1, Santa Cruz) followed by a 4-hour incubation with Protein G Dynabeads. Protein G bead complexes were washed, and protein-associated chromatin was eluted and cross-linking was reversed. DNA was then purified, precipitated, and quantified by qPCR (rRNA gene UCE Forward 5′-GCGGTTTTCTTTCATTGACC-3′ and Reverse 5′-GTATGA CTTCCAGGCGTCGT-3′; 5′-ETS Forward 5′-GCTTGTTTCTCCCGATTGC-3′ and Reverse 5′-ACTTTTCTCAGTGGTTCGCG-3′; and 28S Forward 5′-CGCCGGTGAAATACCACTAC-3′ and Reverse 5′-GTGTCCTAAGGCGAG CTCAG-3′). ChIP enrichment was determined as percentage of input.

2.5. Nucleosome occupancy and methylome (NOMe) assay

NOMe assays were conducted as previously described (Kelly et al., 2012). Briefly, cells were trypsinized and centrifuged for 5 min at 250*g*, then washed in ice-cold PBS and resuspended in 1 ml ice-cold lysis buffer (10 mM Tris, pH 7.4, 10 mM NaCl, 3 mM $MgCl_2$, 0.1 mM EDTA and 0.5% NP-40) and incubated on ice for 5 min. Nuclei were recovered by centrifugation for 5 min at 750*g* and washed in ice-cold wash buffer (10 mM Tris-HCl pH 7.4, 10 mM NaCl, 3 mM $MgCl_2$ and 0.1 mM EDTA). Washed nuclei were resuspended in 1× GpC buffer and incubated at 37 °C in a reaction mixture of 1 M sucrose, 10× GpC buffer, 32 mM SAM, and 4 U/μl M.CviPI. Reactions were quenched with an equal volume of stop buffer (20 nM Tris-HCl pH 7.9, 600 mM NaCl, 1% SDS, 10 mM EDTA, 400 μg/ml Proteinase K) and incubated overnight. DNA was purified by phenol-chloroform extraction and ethanol precipitation. Bisulfite conversion was performed using the EpiTect Bisulfite Kit (Qiagen), and amplified using the following primers targeted to the rRNA gene promoter region: Forward 5′-GGATTT TATTTTTTTTTTAATTTTTTTTTT-3′ and Reverse 5′-ATATCCTTTAATAT TAATAAAAAAAAAAACA-3′. PCR amplicons were subsequently cloned using the TOPO TA Kit (Invitrogen) and individual clones were then Sanger-sequenced.

2.6. Ribosome profiling

MC3T3-E1 cells were treated with 200 μg/ml cyclohexamide for 20 min, washed, and scraped in PBS supplemented with 100 μg/ml cyclohexamide. Cells were counted, and the pellets were resuspended in ice-cold lysis buffer (10 mM Tris-HCl pH 7.4, 5 mM $MgCl_2$, 100 mM KCl, 1% Triton X-100, 2 mM DTT, 20 U/ml Promega RNasin, 100 μg/ml cycloheximide, EDTA-free protease inhibitor) and incubated on ice for 10 min. Lysates were cleared at 10,000*g* at 4 °C for 10 min. Supernatants were loaded onto 10–50% continuous sucrose gradients (20 mM HEPES-KOH pH 7.4, 5 mM $MgCl_2$, 100 mM KCl, 2 mM DTT, 20 U/ml Promega RNasin, 100 μg/ml cycloheximide) and centrifuged at 40,000 rpm for 2 h in a SW-41 Ti rotor (Beckman Coulter). Samples were then inserted into a tube piercer connected to a syringe pump (Brandel) and fractionated with an ISCO gradient fractionator/UA-6 detector system (Teledyne). Polysome profiles were recorded using WinDaq data acquisition software (DATAQ INSTRUMENTS). The area under the 40S, 60S, and 80S curves were approximated using Riemann sums. Polysome-to-monosome ratios were quantitated by calculating the area under the curve corresponding to the polyribosome peaks (more than two ribosomes) divided by the area under the curve for the monosome (80S) peak.

2.7. Protein synthesis assay

Cells were processed using Click-iT® Plus O-propargyl-puromycin (OPP) Alexa Fluor® 488 Protein Synthesis Assay Kit (Life Technologies) according to the manufacturer's instructions with slight modifications. Briefly, cells were incubated with 20 μM Click-iT® OPP for 30 min, fixed with 3.7% formaldehyde, and permeabilized using 0.5% Triton X-100. OPP incorporation was detected by chemoselective ligation using Alexa Fluor® picolyl azide dye diluted in Click-iT® Plus OPP reaction cocktail at room temperature for 30 min, protected from light. Cells were resuspended in PBS and analyzed by flow cytometry using 488 nm excitation with a green emission filter (Beckman Coulter).

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