Published in final edited form as:

Gene. 2017 May 15; 612: 29–35. doi:10.1016/j.gene.2016.11.010.

Ribosome biogenesis is dynamically regulated during osteoblast differentiation

Cynthia L. Neben^{1,2}, Fides D. Lay², Xiaojing Mao^{1,2}, Creighton T. Tuzon^{1,2}, and Amy E. Merrill^{1,2,*}

¹Center for Craniofacial Molecular Biology, Ostrow School of Dentistry, University of Southern California, Los Angeles, CA, 90033

²Department of Biochemistry and Molecular Biology, Keck School of Medicine, University of Southern California, Los Angeles, CA, 90033

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.

Keywords

osteoblast; rRNA; ribosome biogenesis; RNA Pol I; UBF1; osteoprogenitor; bone

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.,

*Correspondence: amerrill@usc.edu.

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 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 MEM α containing 10% FBS and 1X 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 100mm 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'-GCTTGTTTCTCCCGATTGC-3', Reverse 5'-CGCGAACCACTGAGAAAAGT-3'), *Runx2* (Forward 5'-CCGCACGACAACCGCACCAT-3' and Reverse 5'-CGCTCCGGCCCAAAATCTC-3'), *Collagen I* (Forward 5'-TCTGTATCTGCCACAATGGC-3' and Reverse 5'-AGCTTCCCCATCATCTCCA-3'), and *Osteocalcin* (Forward 5'-GGACCATCTTTCTGCTCACT-3' and Reverse 5'-CGGAGTCTGTTCACCTTAT-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 hour. Coverslips were mounted on slides using Vectashield with DAPI (Vector Laboratories) and imaged on Leica TCS SP5/8 confocal system using a 63X objective and 2.0X 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 μ g 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'-GTATGACTTCCAGGCGTCGT-3'; 5'-ETS Forward 5'-GCTTGTTTCTCCCGATTGC-3' and Reverse 5'-ACTTTTCTCAGTGGTTTCGCG-3'; and 28S Forward 5'-CGCCGGTGAAATACCACTAC-3' and Reverse 5'-GTGTCCTAAGGCGAGCTCAG-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 MgCl₂, 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 MgCl₂ and 0.1 mM EDTA). Washed nuclei were resuspended in 1x GpC buffer and incubated at 37°C in a reaction mixture of 1 M sucrose, 10x GpC buffer, 32 mM SAM, and 4 U/ul M.CviPI. Reactions were quenched with an equal volume of stop buffer (20 mM 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'-GGATTTTATTTTTTTTTTAATTTTTTTTTT-3' and Reverse 5'-ATATCCTTTAATATTAATAAAAAAAAAACA-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 MgCl₂, 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 minutes. Lysates were cleared at 10,000 *g* at 4°C for 10 minutes. Supernatants were loaded onto 10–50% continuous sucrose gradients (20 mM HEPES-KOH pH 7.4, 5 mM MgCl₂, 100 mM KCl, 2 mM DTT, 20 U/mL Promega RNasin, 100 µg/mL cycloheximide) and centrifuged at 40,000 rpm for 2 hours 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 minutes, 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 minutes, protected from light. Cells were resuspended in PBS and analyzed by flow cytometry using 488 nm excitation with a green emission filter (Beckman Coulter).

2.8 Statistical analysis

The Student's *t*-test was used to test for significance in each set of values, assuming equal variance. Mean values plus or minus standard deviation are plotted (SD). **P*<0.05 and ***P*<0.01.

3. Results

3.1 Transcription of rRNA genes fluctuates during osteoblast differentiation

During differentiation, mesenchymal progenitor cells undergo a phase of active proliferation, followed by increased gene expression of lineage-specific factors and withdrawal from the cell cycle (Sunters et al., 1998). This process is tightly coupled with transcription of the rRNA genes (Ali et al., 2008). To assess the extent to which rRNA gene transcription fluctuates during differentiation of MC3T3-E1 mouse calvarial osteoprogenitor cells, we assayed levels of the 45S precursor rRNA at 0, 3, and 6 days of osteoinduction. We found that rRNA synthesis was 27% lower at day 3 compared to day 0 (Fig. 1A). A decrease in 45S precursor rRNA at day 3 coincided with increased expression of osteoblast-specific genes in a clear temporal sequence (Fig. 1B). Levels of *Runx2*, one of the earliest transcription factors required for determination of osteoblast lineage, increased nearly 5-fold between days 0 and 3. These immature osteoblasts at day 3 also express high levels of *Collagen I* mRNA, which is subsequently processed and secreted (Fig. 1B). Upon collagen deposition and accumulation in the extracellular matrix, immature osteoblasts fully differentiate into mature osteoblasts and strongly upregulate the RUNX2 target gene *Osteocalcin* (Quarles et al., 1992). We found that levels of *Osteocalcin* were increased nearly 20-fold between days 0 and 3 (Fig. 1B). Interestingly, 45S precursor rRNA levels rose at day 6 to a level 1.4-fold higher than originally detected at day 0 (Fig. 1A). This increase is coincident with peak extracellular matrix production during MC3T3-E1 cell differentiation and likely represents an increased need for protein synthesis in maturing osteoblasts (Fig. 1A) (Quarles et al., 1992). Indeed, levels of *Collagen I* and *Osteocalcin* were increased nearly 6-fold and 70-fold, respectively, between days 0 and 6 (Fig. 1B). Together, these observations indicate that rRNA gene transcription fluctuates during osteoblast differentiation and that these fluctuations are concomitant with osteoblast determination and maturation.

3.2 Nucleolar localization of RNA Pol I and UBF1 are reduced following osteoinduction

The initial decrease in rRNA levels is associated with increased expression of the osteoblast determination factor RUNX2. This suggests that rRNA transcription is coordinated with osteoblast cell fate determination. To address the mechanisms through which rRNA transcription is reduced in early differentiating cells, we examined changes in the subcellular localization of RNA Pol I and its key transcription factor UBF1. UBF1, an HMG box architectural protein, associates with the promoter and transcribed regions of rRNA genes, where it promotes RNA Pol I recruitment, promoter escape, and elongation (Jantzen et al., 1990; Panov et al., 2006; Stefanovsky et al., 2006). Immunofluorescence detection of UBF1 and the RNA Pol I subunit POLR1D showed intense colocalization to mature nucleoli of preosteoblasts at day 0 (Fig. 2A). At day 2 of osteoinduction, preosteoblasts showed a decrease in the number and intensity of nucleolar UBF1 foci, while nucleolar localization of POLR1D remained robust (Fig. 2B). Thus, colocalization of UBF1 and POLR1D was greatly reduced at this time point. At day 3 of osteoinduction, early osteoblasts maintained reduced levels of UBF1 but now also decreased the number and intensity of nucleolar POLR1D foci (Fig. 2C). Furthermore, the nucleolar foci at day 3 were notably smaller than at day 0 (Fig 2A, C). Since nucleolar size and content is an indicator of transcriptional

activity at rRNA genes, this data suggests that decreased localization of UBF1 and RNA Pol I during osteoblast determination leads to a drop in the pool of active rRNA genes.

3.3 Osteoinduction decreases the number of active rRNA genes

Mammalian cells have approximately 200 copies of rRNA gene tandem repeats, of which only a fraction are transcribed at any given time (Sanij et al., 2008). UBF1 binding along the rRNA gene repeat is necessary to maintain active r-chromatin, and its loss is sufficient to induce a closed r-chromatin state (Sanij and Hannan, 2009). Chromatin immunoprecipitation (ChIP) for UBF1 detected decreased occupancy at the rRNA gene upstream core promoter (UCE) (2.8-fold) and in the 5' external transcribed spacer (5'-ETS) (2.6-fold) at day 3 of osteoinduction compared to day 0 (Fig. 2D). These results support our finding that UBF1 is depleted from the nucleoli of differentiating osteoblasts.

Depletion of UBF binding at rRNA genes leads to transcriptional silencing (Sanij et al., 2008; Hamdane et al., 2014). Therefore, we used the nucleosome occupancy and methylome assay (NOME) to examine two hallmarks of silent r-chromatin: CpG DNA methylation and promoter inaccessibility. NOME detects endogenous CpG methylation as well as nucleosome positioning information at an individual DNA molecule (Kelly et al., 2012). In NOME assay, a GpC methyltransferase is used to methylate GpC dinucleotides not protected by nucleosomes and/or DNA binding factors. Subsequently, methylated GpC dinucleotides along with any endogenous CpG methylation sites are detected by bisulfite conversion and sequencing to generate a footprint of chromatin accessibility. Our NOME analyses surveyed the sequence 400 bp upstream and 10 bp downstream of the transcription start site and included the CpG dinucleotides whose methylation has been shown to be critical for rRNA gene silencing (-143, -133, and +8) (Santoro and Grummt, 2001). Since rRNA gene silencing is correlated with the density of CpG methylation, clones were categorized based on this parameter (Ghoshal et al., 2004). Methylation of 30–60% of CpG sites was considered as partially methylated, while methylation of more than 60% of sites was scored as hypermethylated (Harris et al., 2010). Of the day 0 clones sampled, 11% were partially methylated, and none were hypermethylated (Fig. 3A). At day 2 of osteoinduction, 11% remained partially methylated and 5.5% hypermethylated (Fig. 3B). By day 3, partially methylated clones were decreased to 6.3%, while hypermethylated clones rose slightly to 6.3 % (Fig. 3C). This suggests that the number of CpG methylated rRNA genes slightly increases during the early stages of osteoblast determination.

To determine the relationship between levels of CpG methylation and r-chromatin configuration, we used the GpC accessibility footprint data from the same NOME clones to predict the location of the promoter-bound nucleosome. In silent rRNA genes, the promoter-bound nucleosome covers nucleotides -132 to +22, preventing assembly of the RNA Pol I pre-initiation complex (Li et al., 2006; Xie et al., 2012). On the other hand, in active rRNA genes, the nucleosome resides over nucleotides -157 to -2, which leaves the transcriptional start site (TSS) accessible. In osteoprogenitor cells at day 0, the GpC accessibility footprint indicated that 61% of the clones had the promoter-bound nucleosome in the active “on” position (Fig. 3A). Interestingly, the clones with inaccessible TSS were not those with partial CpG methylation. This suggests that in osteoprogenitor cells inactive r-chromatin is

not dependent on CpG methylation. CpG methylation represents a more enduringly silenced state. Thus, our data indicate that inactive r-chromatin in osteoprogenitor cells remains permissible, allowing for more rapid reactivation. At day 2 of osteoinduction, only 39% of clones had an accessible start site (Fig. 3B). This decrease in TSS accessibility is coincident with nucleolar depletion of UBF1 but not RNA Pol I (Fig. 2B). Clones with an inaccessible TSS at day 2 included the unmethylated clones, one of the partially methylated clones, and the only hypermethylated clone. By day 3, the GpC footprint showed that the clones with TSS accessibility increased to 62%, a level seen in osteoprogenitor cells prior to osteoinduction (Fig. 3C). As with the earlier time points, the clones with inaccessible TSS included unmethylated clones, one of the partially methylated clones, and the only hypermethylated clone. Together these results show that upon osteoinduction, rRNA genes are initially silenced through a reversible mechanism that reduces promoter accessibility by changing nucleosome occupancy. As osteoblast differentiation progresses, CpG methylation around the TSS is slightly increased. While CpG methylation increases, it is only inversely correlated with TSS accessibility when over 60% the CpG sites around the promoter are methylated. Partially methylated molecules, on the other hand, have variable TSS accessibility that did not correlate with methylation of any particular CpG sites.

3.4 Translation potential is decreased following osteoinduction

Since rRNA transcription is the rate-limiting step in building ribosomes and fluctuates during osteoblast differentiation, we examined the effect of osteoinduction on ribosome biogenesis (Panov et al., 2001). Polysome profiling showed an 11% and 32% decrease, respectively, in synthesis of 40S and 60S ribosomal subunits as well as a 19% decrease in 80S ribosomes in MC3T3-E1 cells at day 3 compared to day 0 (Fig. 4A). The drastic reduction in the 60S subunit is especially important, as nuclear export of the large subunit is tightly coordinated with the production of ribosomal proteins, which are needed for assembly of active ribosome (Stefanovsky et al., 2006). Consistently, active ribosomes, or polysomes, were reduced 21% at day 3 compared to day 0. Further quantification of the areas under the 80S ribosome, or monosome, and polysome peaks showed that the polysome/monosome ratio is not altered between day 0 and day 3 (71% and 68%, respectively). This indicates that despite reduced ribosome biogenesis during osteoblast differentiation, relative rates for translation initiation and elongation go unchanged. Since the level of ribosome biogenesis impacts protein production, protein synthesis was measured by O-propargyl-puromycin (OPP) incorporation. Differentiating osteoblasts at day 3 had lower levels of protein synthesis compared to their undifferentiated counterparts at day 0 (Fig. 5B). Together these results suggest that a reduction in rRNA transcription at day 3 of osteoblast differentiation leads to a decrease in ribosome biogenesis and subsequently, global levels of protein synthesis.

4. Discussion

Here we demonstrate that rRNA gene transcription is dynamically regulated during osteoblast differentiation. Using MC3T3-E1 calvarial-derived osteoprogenitors, we show that a relatively high level of 45S precursor rRNA is transiently decreased early in osteoblast differentiation, when the osteoblast-specific gene expression program is first activated.

Furthermore, we showed that levels of rRNA gene transcription are later restored in mature osteoblasts. Transcription of rRNA genes is the rate-limiting step in building ribosomes, and correspondingly, we found that fluctuating rRNA levels during osteoblast differentiation are linked to changes in ribosome biogenesis and protein synthesis. Specifically, our results demonstrate that osteoprogenitor cells have relatively higher levels of rRNA build more ribosome and synthesize more protein than their differentiating counterparts. We expect that fluctuations in rRNA gene transcription have a primary role in cell-fate determination in osteoprogenitor cells, as levels of rRNA transcription are a critical switch between proliferation and differentiation in other cell types (Sanij et al., 2008; Hayashi et al., 2014; Zhang et al., 2014).

The structure of the nucleolus is tightly linked to its output (Hernandez-Verdun et al., 2010). We found that the transient decrease in rRNA gene transcription during osteoblast differentiation is temporally correlated with changes in nucleolar content and size. As osteoprogenitor cells proceed through differentiation, nucleolar content is first depleted of UBF1 and then, RNA Pol I. Upon nucleolar eviction of RNA Pol I, differentiating cells show an overall decrease in nucleolar size. Nucleolar structure can be controlled by the epigenetic state of r-chromatin (McKeown and Shaw, 2009; Bacalini et al., 2014). Correspondingly, we found that the pool rRNA genes that have an accessible TSS is reduced upon nucleolar depletion of UBF1.

Nucleolar depletion of UBF1 leads to rRNA gene silencing and is critical for terminal differentiation of other cell types (Larson et al., 1993; Poortinga et al., 2004; Datta et al., 2007; Sanij and Hannan, 2009). Gene inactivation induced by loss of UBF1 is methylation-independent, reversible, and stably propagated through cell division (Sanij et al., 2008). Our data suggest that the transient decrease in rRNA gene transcription during the initial stages of osteoblast differentiation is primarily mediated through UBF1 and not CpG methylation for three reasons. First, the percentage of rRNA genes with active r-chromatin as denoted by the configuration of the promoter-bound nucleosome was rapidly increased between day 2 and 3 of osteoinduction, indicating reversibility. Second, the percentage of rRNA genes that was both TSS inaccessible and hypermethylated only increased slightly. Third, partially methylated rRNA genes had variable TSS accessibility, even if methylated at the CpG sites (−143, −133, and +8) previously shown to be critical for the formation of silent r-chromatin (Santoro and Grummt, 2001). These findings also indicate the utility of clonal analyses in correlating CpG methylation and TSS accessibility.

The number of rRNA genes far exceeds the needs for normal cellular processes, with only about 50% being transcribed in a somatic cell (Grummt, 2007). Silencing of rRNA genes early in development is allelic: one parental copy of each rRNA gene locus becomes late-replicating and marked for inactivation by *de novo* DNA methylation (Schlesinger et al., 2009). However, the upstream regulators that determine which rRNA gene copies will be inactivated during development are not well understood. There is evidence to suggest that in osteoprogenitor cells, the molecular rationale for such decisions is provided by RUNX2 and FGFR2. The osteoblast-specific transcription factor RUNX2 represses rRNA gene transcription during the early stages of osteoblast differentiation (Young et al., 2007; Ali et al., 2008). FGFR2 opposes the repressive action of RUNX2 in osteoprogenitor cells by

directly promoting rRNA gene transcription with UBF1 (Neben et al., 2014). Thus, competitive activities of FGFR2 and RUNX2 at rRNA genes could therefore be a key determinant of not only the specific rRNA gene copies selected for repression, but also the timing of fluctuation in rRNA gene transcription during osteoblast differentiation.

We have shown here that variations in the level of rRNA gene transcription during osteoblast differentiation are concordant with ribosome abundance. However, the ends of fluctuating rRNA levels on ribosome biogenesis and protein production are likely more complicated. Alterations in rRNA gene transcription levels in stem cells are linked to highly specific changes in protein synthesis during cell fate determination and commitment (Zhang et al., 2014). This might be explained by ever-growing evidence that heterogeneity in ribosome composition, including differences in the primary sequence and posttranslational modification of rRNA, contribute to specificity in the translational activity of ribosomes (Kuo et al., 1996; Belin et al., 2009; Xue and Barna, 2012; Marcel et al., 2013). Thus, it remains a possibility that the transient decrease in rRNA gene transcription we observed in osteoblast differentiation is indicative of selective silencing at a distinct set of rRNA genes.

That ribosomes exert regulatory control over translation rather than indiscriminately translating all mRNAs is exemplified by the skeletal phenotypes of the multiple human birth defects with known ribosomal dysfunction. These disorders, termed ribosomopathies, arise from deficient ribosome biogenesis and are characterized by distinct overlapping skeletal defects such as hypoplastic craniofacial bones, short stature, and anomalies of the digits (Trainor and Merrill, 2014). The effects of increased rRNA in the developing skeleton are unclear. However, there are strong correlations between increased rRNA and abnormal skeletal development in Bent Bone Dysplasia Syndrome (BBDS), a disorder caused by *FGFR2* mutations that enhance nucleolar localization of FGFR2 (Merrill et al., 2012; Neben et al., 2014). Furthermore, given the role of RUNX2 in repressing rRNA transcription, it is likely that increased levels of rRNA could be found in Cleidocranial Dysplasia, a skeletal disorder caused by *RUNX2* haploinsufficiency (Young et al., 2007). By gaining a more complete understanding of how rRNA gene expression is regulated during osteoblast differentiation, we hope to gain a more complete understanding of why skeletal tissue is particularly sensitive to changes in ribosome biogenesis during development and thereby lend new insight into the pathogenesis and treatment of skeletal birth defects resulting from ribosome dysfunction.

Acknowledgments

This work was supported by the National Institutes of Health [R01DE025222 to A.E.M and 5T32HD060549-02 to C.L.N.] and March of Dimes [#6-FY15-233 to A.E.M.]. We thank Joanna Salva, Ryan Roberts, and Judd Rice for their helpful discussions.

References

Ali SA, Dobson JR, Lian JB, Stein JL, van Wijnen AJ, Zaidi SK, Stein GS. A RUNX2-HDAC1 co-repressor complex regulates rRNA gene expression by modulating UBF acetylation. *J Cell Sci.* 2012; 125:2732–9. [PubMed: 22393235]

- Ali SA, Zaidi SK, Dacwag CS, Salma N, Young DW, Shakoori AR, Montecino MA, Lian JB, van Wijnen AJ, Imbalzano AN, Stein GS, Stein JL. Phenotypic transcription factors epigenetically mediate cell growth control. *Proc Natl Acad Sci U S A*. 2008; 105:6632–7. [PubMed: 18445650]
- Ali SA, Zaidi SK, Dobson JR, Shakoori AR, Lian JB, Stein JL, van Wijnen AJ, Stein GS. Transcriptional corepressor TLE1 functions with Runx2 in epigenetic repression of ribosomal RNA genes. *Proc Natl Acad Sci U S A*. 2010; 107:4165–9. [PubMed: 20160071]
- Bacalini MG, Pacilli A, Giuliani C, Penzo M, Trere D, Pirazzini C, Salvioli S, Franceschi C, Montanaro L, Garagnani P. The nucleolar size is associated to the methylation status of ribosomal DNA in breast carcinomas. *BMC Cancer*. 2014; 14:361. [PubMed: 24884608]
- Belin S, Beghin A, Solano-Gonzalez E, Bezin L, Brunet-Manquat S, Textoris J, Prats AC, Mertani HC, Dumontet C, Diaz JJ. Dysregulation of ribosome biogenesis and translational capacity is associated with tumor progression of human breast cancer cells. *PLoS One*. 2009; 4:e7147. [PubMed: 19779612]
- Bell SP, Learned RM, Jantzen HM, Tjian R. Functional cooperativity between transcription factors UBF1 and SL1 mediates human ribosomal RNA synthesis. *Science*. 1988; 241:1192–7. [PubMed: 3413483]
- Bowman LH. rDNA transcription and pre-rRNA processing during the differentiation of a mouse myoblast cell line. *Dev Biol*. 1987; 119:152–63. [PubMed: 3641762]
- Datta PP, Wilson DN, Kawazoe M, Swami NK, Kaminishi T, Sharma MR, Booth TM, Takemoto C, Fucini P, Yokoyama S, Agrawal RK. Structural aspects of RbfA action during small ribosomal subunit assembly. *Mol Cell*. 2007; 28:434–45. [PubMed: 17996707]
- Ghoshal K, Majumder S, Datta J, Motiwala T, Bai S, Sharma SM, Frankel W, Jacob ST. Role of human ribosomal RNA (rRNA) promoter methylation and of methyl-CpG-binding protein MBD2 in the suppression of rRNA gene expression. *J Biol Chem*. 2004; 279:6783–93. [PubMed: 14610093]
- Grummt I. Different epigenetic layers engage in complex crosstalk to define the epigenetic state of mammalian rRNA genes. *Hum Mol Genet*. 2007; 16(Spec No 1):R21–7. [PubMed: 17613545]
- Hamdane N, Stefanovsky VY, Tremblay MG, Nemeth A, Paquet E, Lessard F, Sanij E, Hannan R, Moss T. Conditional inactivation of Upstream Binding Factor reveals its epigenetic functions and the existence of a somatic nucleolar precursor body. *PLoS Genet*. 2014; 10:e1004505. [PubMed: 25121932]
- Harris RA, Wang T, Coarfa C, Nagarajan RP, Hong C, Downey SL, Johnson BE, Fouse SD, Delaney A, Zhao Y, Olshen A, Ballinger T, Zhou X, Forsberg KJ, Gu J, Echipare L, O'Geen H, Lister R, Pelizzola M, Xi Y, Epstein CB, Bernstein BE, Hawkins RD, Ren B, Chung WY, Gu H, Bock C, Gnirke A, Zhang MQ, Haussler D, Ecker JR, Li W, Farnham PJ, Waterland RA, Meissner A, Marra MA, Hirst M, Milosavljevic A, Costello JF. Comparison of sequencing-based methods to profile DNA methylation and identification of monoallelic epigenetic modifications. *Nat Biotechnol*. 2010; 28:1097–105. [PubMed: 20852635]
- Hayashi Y, Kuroda T, Kishimoto H, Wang C, Iwama A, Kimura K. Downregulation of rRNA transcription triggers cell differentiation. *PLoS One*. 2014; 9:e98586. [PubMed: 24879416]
- Hernandez-Verdun D, Roussel P, Thiry M, Sirri V, Lafontaine DL. The nucleolus: structure/function relationship in RNA metabolism. *Wiley Interdiscip Rev RNA*. 2010; 1:415–31. [PubMed: 21956940]
- Jantzen HM, Admon A, Bell SP, Tjian R. Nucleolar transcription factor hUBF contains a DNA-binding motif with homology to HMG proteins. *Nature*. 1990; 344:830–6. [PubMed: 2330041]
- Kelly TK, Liu Y, Lay FD, Liang G, Berman BP, Jones PA. Genome-wide mapping of nucleosome positioning and DNA methylation within individual DNA molecules. *Genome Res*. 2012; 22:2497–506. [PubMed: 22960375]
- Kuo BA, Gonzalez IL, Gillespie DA, Sylvester JE. Human ribosomal RNA variants from a single individual and their expression in different tissues. *Nucleic Acids Res*. 1996; 24:4817–24. [PubMed: 8972871]
- Larson DE, Xie W, Glibetic M, O'Mahony D, Sells BH, Rothblum LI. Coordinated decreases in rRNA gene transcription factors and rRNA synthesis during muscle cell differentiation. *Proc Natl Acad Sci U S A*. 1993; 90:7933–6. [PubMed: 8396256]

- Learned RM, Learned TK, Haltiner MM, Tjian RT. Human rRNA transcription is modulated by the coordinate binding of two factors to an upstream control element. *Cell*. 1986; 45:847–57. [PubMed: 3708692]
- Li J, Langst G, Grummt I. NoRC-dependent nucleosome positioning silences rRNA genes. *EMBO J*. 2006; 25:5735–41. [PubMed: 17139253]
- Marcel V, Catez F, Diaz JJ. Ribosomes: the future of targeted therapies? *Oncotarget*. 2013; 4:1554–5. [PubMed: 24145310]
- McKeown PC, Shaw PJ. Chromatin: linking structure and function in the nucleolus. *Chromosoma*. 2009; 118:11–23. [PubMed: 18925405]
- Merrill AE, Sarukhanov A, Krejci P, Itoni B, Camacho N, Estrada KD, Lyons KM, Deixler H, Robinson H, Chitayat D, Curry CJ, Lachman RS, Wilcox WR, Krakow D. Bent bone dysplasia-FGFR2 type, a distinct skeletal disorder, has deficient canonical FGF signaling. *Am J Hum Genet*. 2012; 90:550–7. [PubMed: 22387015]
- Moss T, Langlois F, Gagnon-Kugler T, Stefanovsky V. A housekeeper with power of attorney: the rRNA genes in ribosome biogenesis. *Cell Mol Life Sci*. 2007; 64:29–49. [PubMed: 17171232]
- Neben CL, Itoni B, Salva JE, Tuzon CT, Rice JC, Krakow D, Merrill AE. Bent bone dysplasia syndrome reveals nucleolar activity for FGFR2 in ribosomal DNA transcription. *Hum Mol Genet*. 2014; 23:5659–71. [PubMed: 24908667]
- Ornitz DM, Marie PJ. FGF signaling pathways in endochondral and intramembranous bone development and human genetic disease. *Genes Dev*. 2002; 16:1446–65. [PubMed: 12080084]
- Panov KI, Friedrich JK, Russell J, Zomerdijs JC. UBF activates RNA polymerase I transcription by stimulating promoter escape. *EMBO J*. 2006; 25:3310–22. [PubMed: 16858408]
- Panov KI, Friedrich JK, Zomerdijs JC. A step subsequent to preinitiation complex assembly at the ribosomal RNA gene promoter is rate limiting for human RNA polymerase I-dependent transcription. *Mol Cell Biol*. 2001; 21:2641–9. [PubMed: 11283244]
- Poortinga G, Hannan KM, Snelling H, Walkley CR, Jenkins A, Sharkey K, Wall M, Brandenburger Y, Palatsides M, Pearson RB, McArthur GA, Hannan RD. MAD1 and c-MYC regulate UBF and rDNA transcription during granulocyte differentiation. *EMBO J*. 2004; 23:3325–35. [PubMed: 15282543]
- Quarles LD, Yohay DA, Lever LW, Caton R, Wenstrup RJ. Distinct proliferative and differentiated stages of murine MC3T3-E1 cells in culture: an in vitro model of osteoblast development. *J Bone Miner Res*. 1992; 7:683–92. [PubMed: 1414487]
- Sanij E, Hannan RD. The role of UBF in regulating the structure and dynamics of transcriptionally active rDNA chromatin. *Epigenetics*. 2009; 4:374–82. [PubMed: 19717978]
- Sanij E, Poortinga G, Sharkey K, Hung S, Holloway TP, Quin J, Robb E, Wong LH, Thomas WG, Stefanovsky V, Moss T, Rothblum L, Hannan KM, McArthur GA, Pearson RB, Hannan RD. UBF levels determine the number of active ribosomal RNA genes in mammals. *J Cell Biol*. 2008; 183:1259–74. [PubMed: 19103806]
- Santoro R, Grummt I. Molecular mechanisms mediating methylation-dependent silencing of ribosomal gene transcription. *Mol Cell*. 2001; 8:719–25. [PubMed: 11583633]
- Schlesinger S, Selig S, Bergman Y, Cedar H. Allelic inactivation of rDNA loci. *Genes Dev*. 2009; 23:2437–47. [PubMed: 19833769]
- Stefanovsky V, Langlois F, Gagnon-Kugler T, Rothblum LI, Moss T. Growth factor signaling regulates elongation of RNA polymerase I transcription in mammals via UBF phosphorylation and r-chromatin remodeling. *Mol Cell*. 2006; 21:629–39. [PubMed: 16507361]
- Sunters A, McCluskey J, Grigoriadis AE. Control of cell cycle gene expression in bone development and during c-Fos-induced osteosarcoma formation. *Dev Genet*. 1998; 22:386–97. [PubMed: 9664690]
- Trainor PA, Merrill AE. Ribosome biogenesis in skeletal development and the pathogenesis of skeletal disorders. *Biochim Biophys Acta*. 2014; 1842:769–78. [PubMed: 24252615]
- Wang D, Christensen K, Chawla K, Xiao G, Krebsbach PH, Franceschi RT. Isolation and characterization of MC3T3-E1 preosteoblast subclones with distinct in vitro and in vivo differentiation/mineralization potential. *J Bone Miner Res*. 1999; 14:893–903. [PubMed: 10352097]

- Watanabe-Susaki K, Takada H, Enomoto K, Miwata K, Ishimine H, Intoh A, Ohtaka M, Nakanishi M, Sugino H, Asashima M, Kurisaki A. Biosynthesis of ribosomal RNA in nucleoli regulates pluripotency and differentiation ability of pluripotent stem cells. *Stem Cells*. 2014; 32:3099–111. [PubMed: 25187421]
- Xie W, Ling T, Zhou Y, Feng W, Zhu Q, Stunnenberg HG, Grummt I, Tao W. The chromatin remodeling complex NuRD establishes the poised state of rRNA genes characterized by bivalent histone modifications and altered nucleosome positions. *Proc Natl Acad Sci U S A*. 2012; 109:8161–6. [PubMed: 22570494]
- Xue S, Barna M. Specialized ribosomes: a new frontier in gene regulation and organismal biology. *Nat Rev Mol Cell Biol*. 2012; 13:355–69. [PubMed: 22617470]
- Young DW, Hassan MQ, Pratap J, Galindo M, Zaidi SK, Lee SH, Yang X, Xie R, Javed A, Underwood JM, Furcinitti P, Imbalzano AN, Penman S, Nickerson JA, Montecino MA, Lian JB, Stein JL, van Wijnen AJ, Stein GS. Mitotic occupancy and lineage-specific transcriptional control of rRNA genes by Runx2. *Nature*. 2007; 445:442–6. [PubMed: 17251981]
- Zahradka P, Larson DE, Sells BH. Regulation of ribosome biogenesis in differentiated rat myotubes. *Mol Cell Biochem*. 1991; 104:189–94. [PubMed: 1921999]
- Zhang Q, Shalaby NA, Buszczak M. Changes in rRNA transcription influence proliferation and cell fate within a stem cell lineage. *Science*. 2014; 343:298–301. [PubMed: 24436420]

Highlights

- Osteoinduction triggers repression of rRNA gene transcription.
- Phasic nucleolar depletion, first of UBF1 and then of RNA Pol I, precedes rRNA gene silencing during osteoblast differentiation.
- Silencing of rRNA transcription is mediated by a methylation-independent mechanism that involves UBF1.
- A reduction in rRNA gene transcription leads to decreased ribosome biogenesis, and subsequently, protein synthesis.

Fig. 1. rRNA expression fluctuates during osteoblast differentiation

(A) Levels of 45S precursor rRNA relative to β -actin in MC3T3-E1 cells, detected by qPCR, were reduced 27% at day 3 compared to day 0 and elevated 1.4-fold at day 6 compared to day 0 ($n = 4$). (B) Transcript levels of *Runx2*, *Collagen I*, and *Osteocalcin*, detected by qPCR, were up-regulated upon osteoinduction in MC3T3-E1 cells ($n = 4$). Error bars represent SD. ** $P < 0.01$.

Fig. 2. Nucleolar localization of UBF1 and POLR1D is altered following osteoinduction
Immunofluorescent detection of UBF1 and the RNA Pol I subunit POLR1D in MC3T3-E1 cells during osteoinduction. (A) At day 0, prior to osteoinduction, UBF1 (green, A) and POLR1D (red, A') were highly enriched and colocalized (yellow, A'') in mature nucleoli. (B) On day 2 of osteoinduction, the number and intensity of UBF1 nucleolar foci was reduced (B), while nucleolar POLR1D localization remained high (B'). Thus, colocalization of UBF1 and POLR1D was reduced (B''). (C) By day 3, differentiating osteoblasts showed a reduction in the number and intensity of POLR1D nucleolar foci (C') compared to day 0. Nucleolar localization of UBF1 remained depleted (C). The remaining colocalization of UBF1 and POLR1D was confined to nucleoli that were much reduced in size compared to their undifferentiated counterparts at day 0 (C''). (D) ChIP showed that UBF1 was significantly reduced at the rRNA gene UCE and 5'-ETS at day 3 of osteoinduction compared to day 0 ($n = 3$). Upstream enhancer element (UCE), 5'-external transcribed spacer (5'-ETS), and 28S transcribed region (28S). Error bars represent SD. * $P < 0.05$.

Fig. 3. CpG methylation and the positioning of the promoter-bound nucleosome are altered during osteoblast determination

NOME was used to identify endogenous CpG methylation sites (black circles) as well as accessible GpC dinucleotides (teal circles) in MC3T3-E1 rRNA gene promoter clones. The pink bar represents the promoter-bound nucleosome. **(A)** At day 0, 11% of the 18 clones analyzed were partially methylated. GpC accessibility indicated that the TSS was accessible in 61% of these 18 clones. **(B)** On day 2 of osteoinduction, 11% of the 18 clones analyzed were partially methylated and 5.5% were hypermethylated. The percentage of clones with TSS accessibility reduced to 39%. **(C)** By day 3, 6.3% of clones were partially methylated and 6.3% were hypermethylated. Of these 16 clones, 62% were accessible at the TSS.

Fig. 4. Translation potential is decreased following osteoinduction

(A) Polysome profiling of MC3T3-E1 cells showed decreased synthesis of the 40S (11%) and 60S subunits (32%), as well as the abundance of the 80S ribosomes (18%) and polysomes (21%) at day 3 compared to day 0 ($n = 3$). (B) Protein synthesis, as measured by O-propargyl-puromycin incorporation, was reduced 27% by day 3 of osteoinduction compared to day 0 ($n = 3$). Error bars represent SD. ** $P < 0.01$.