

Article

Proteomic Analysis of Bovine Nucleolus

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Abstract

Nucleolus is the most prominent subnuclear structure, which performs a wide variety of functions in the eukaryotic cellular processes. In order to understand the structural and functional role of the nucleoli in bovine cells, we analyzed the proteomic composition of the bovine nucleoli. The nucleoli were isolated from Madin Darby bovine kidney cells and subjected to proteomic analysis by LC-MS/MS after fractionation by SDS-PAGE and strong cation exchange chromatography. Analysis of the data using the Mascot database search and the GPM database search identified 311 proteins in the bovine nucleoli, which contained 22 proteins previously not identified in the proteomic analysis of human nucleoli. Analysis of the identified proteins using the GoMiner software suggested that the bovine nucleoli contained proteins involved in ribosomal biogenesis, cell cycle control, transcriptional, translational and post-translational regulation, transport, and structural organization.

Key words: bovine, nucleolus, proteomics, 1D SDS-PAGE, SCX, LC-MS/MS

Introduction

Nucleolus is a dynamic subnuclear domain, which forms around the clusters of ribosomal DNA during the interphase of the cell cycle (1-6). The mammalian cell contains 1-6 nucleoli with varying sizes and numbers, depending upon the cell types and culture conditions, even within the same cell (1, 7). The nucleolus was traditionally known to be the ribosome-producing apparatus resulting from the act of building the ribosomes (8). However, research over the past decade explored its functional complexity beyond manufacturing of ribosomes (9-12). Besides

ribosome biogenesis, nucleolus plays a wide range of cellular functions, which include biogenesis of ribonucleoproteins (13, 14) and signal recognition particles (15, 16), regulation of the cell cycle (17-20), RNA editing (17, 21-23), stress response (24, 25), regulation of telomerase activity (26), tRNA processing (27, 28), aging (29), apoptosis (30), export of nuclear proteins (31) and mRNAs (32), and viral replication (33-36).

The nucleoli are formed as a result of the ribosomal gene transcription, processing of 47S pre-ribosomal RNA (rRNA) into 5.8S, 18S, and 28S rRNAs, and ribosomal subunit assembly (1, 3, 8). The nucleoli consist of three distinct sub-compartments, namely fibrillar centres (FCs), dense fibrillar centres (DFCs) and granular components (GCs) (1, 3, 11, 37, 38). The rRNAs are synthesized in the FCs, get accumulated

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and processed in the DFCs, and assemble with the ribosomal proteins and 5S rRNAs to form the ribosomal subunits in the GCs. The nucleolar assembly starts at the end of mitosis with the reinitiation of the ribosomal gene transcription and recruitment of the ribosomal processing components from the prenucleolar bodies in the nucleolar organizer regions (NORs; cluster of rDNA repeats) on different chromosomes (39, 40). These NORs associate to form one or more functional nucleoli. At the onset of the prophase, the RNA polymerase I transcription and pre-rRNA processing are repressed. The DFCs and the GCs start segregating and disappearing at the end of prophase, leading to the disassembly of the nucleolus (40, 41). The nucleolar proteins continuously shuttle between the nucleoli and the nucleoplasm (42-44). However, the proteins having the specific interactions with the nucleolar components are retained in the nucleoplasm for longer duration (45). The dynamic flux of the nucleolar proteins has also been revealed by the proteomic analysis of the nucleoli under various metabolic conditions (46). Although not enclosed by membrane, due to its unique density and the ability to retain its structural integrity following sonication, the nucleoli can be isolated by the sucrose density gradient centrifugation (2, 47-50).

Recently, the proteomic analysis of human (46, 47, 49) and plant nucleoli (51) not only has helped to gain insights into the nucleolar organization and function, but also has demonstrated the potential to analyze the composition of such a large subcellular organelle. Moreover, the bioinformatics analysis of protein domain repertoire has further helped to advance the understanding of the functional and structural diversity of the nucleolus (52). The nucleoli have evolved from the unicellular eukaryotes to higher eukaryotic organism. During the evolution, they have acquired many new structural and functional components (7, 53). Determining the structure and proteomic composition of nucleoli in different species will help to better understand the function of nucleoli. Since bovine species is evolutionary divergent from humans and plants, we performed a proteomic analysis of the bovine nucleoli by liquid chromatography tandem mass spectrometry (LC-MS/MS) and identified 311 proteins based on the search of available protein databases.

Results

Isolation of nucleoli from bovine cells

The nucleoli were isolated from Madin Darby bovine kidney (MDBK) cells and analyzed for their purity and integrity by various methods. For nucleoli isolation from MDBK cells, the addition of 0.3% NP-40 in the hypotonic lysis buffer improved the disruption of the cell membrane and separation of the cytoplasmic material from the nucleus. The nucleolar integrity was analyzed under light microscope after each sonication step to obtain the efficient nuclear disruption with minimum loss of the nucleoli. The purity of the nucleolar preparation was analyzed by Western blot. Proteins from equal amount of cytoplasmic, nucleoplasmic and nucleolar fractions were separated on 10% SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis), transferred to the nitrocellulose membrane and probed with anti-ERK2 serum, anti-Nup62 serum and anti-nucleolin/fibrillarin serum recognizing a cytoplasm, a nucleoplasm and a nucleolar specific protein, respectively. As seen in **Figure 1A**, anti-ERK2 serum recognized a specific protein in the cytoplasmic and nucleoplasmic fractions but not in the nucleolar fraction. Similarly, anti-Nup62 serum recognized a specific protein in the nucleoplasmic and cytoplasmic fractions but not in the nucleolar fraction. Anti-nucleolin serum recognized a specific protein in the nucleolar fraction but not in the nucleoplasmic and cytoplasmic fractions; however, a higher molecular weight protein could be detected in the cytoplasmic and nucleoplasmic fraction. Anti-fibrillarin serum recognized a specific protein in the nucleolar fraction but not in the nucleoplasmic and cytoplasmic fractions. These results suggest that the purified nucleolar preparation was highly enriched in nucleoli. Unlike human nucleoli (47, 49), specific enrichment of the different isoforms of the nucleolin was observed in the nucleolar fraction (60.7 kDa isoform) compared to the cytoplasmic and the nucleoplasmic fractions (121 kDa isoform). The prominent staining of the 60.7 kDa isoform in the nucleolar fraction was indicative of the significant enrichment of the nucleolar fraction. The 60.7 kDa isoform could be the alternative or processed form of nucleolin, and its specific accumulation in the nucleolar

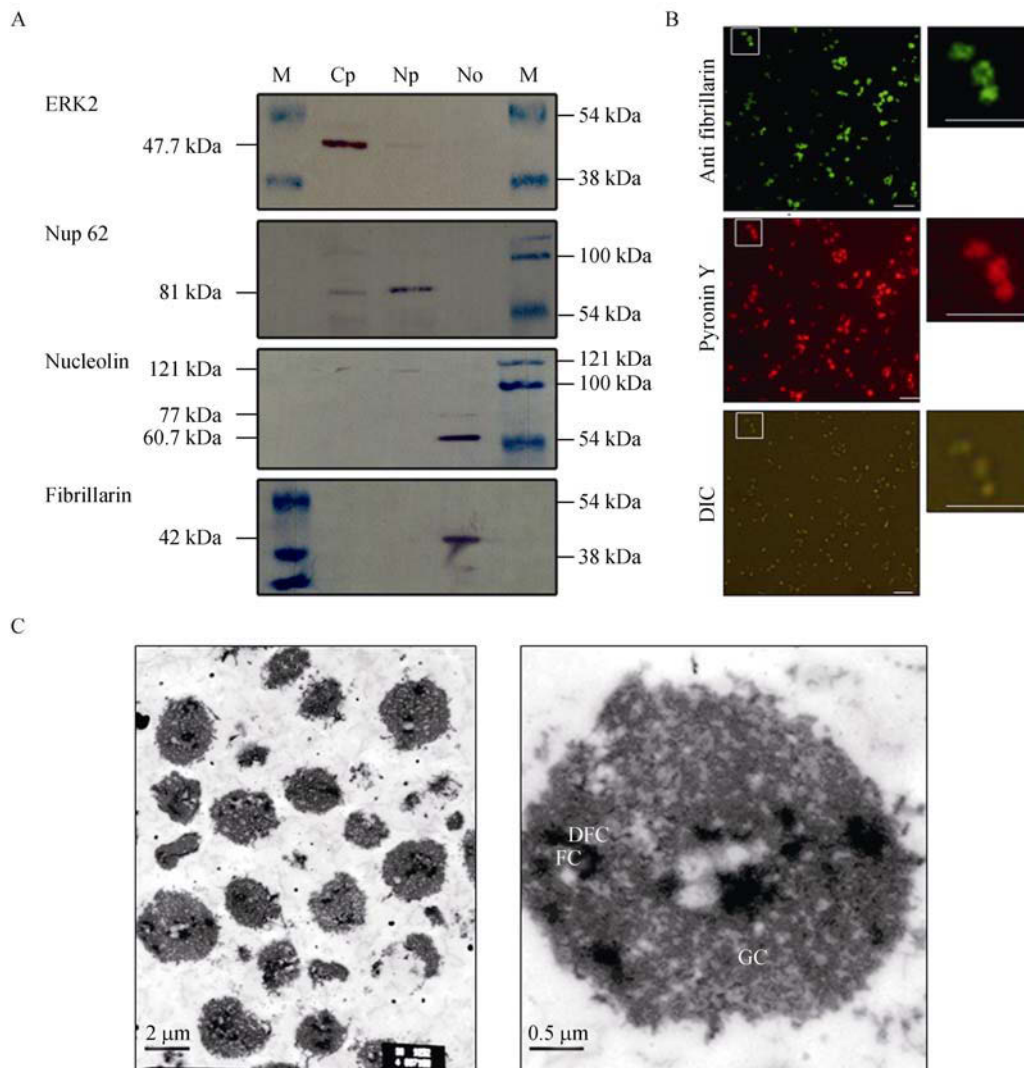


Figure 1 Analysis of the nucleoli isolated from MDBK cells. **A.** Western blot. Proteins from the cytoplasmic (lane Cp), nucleoplasmic (lane Np) and nucleolar (lane No) fractions were separated by SDS-PAGE (10%) under reducing conditions and transferred to nitrocellulose. The separated proteins were probed with anti-ERK2, anti-Nup62, and anti-fibrillarin antibodies. Pre-stained Bio-Rad molecular weight markers broad range (lane M). **B.** Immunofluorescence. Purified nucleoli were immobilized on chamber slides (observed by DIC optics; size bar=10 μ m) and immunostained with rabbit anti-fibrillarin antibody (green) and counter stained with pyronin Y (red). **C.** Electron microscopy. Left: 7,100 \times magnification; Right: 28,400 \times magnification.

fraction may suggest its differential affinity for the nucleolar components.

To further analyze the purity of the nucleolar preparations, the nucleoli were immobilized on slides and analyzed by immunostaining using anti-fibrillarin antibody after counter staining with the RNA binding dye pyronin Y. As seen in **Figure 1B**, structures observed by the differential interference contrast (DIC) optics were also stained with anti-fibrillarin serum and pyronin Y dye. These results confirmed the earlier observation that the purified nucleolar preparation

was highly enriched in nucleoli.

The integrity of the nucleoli in nucleolar preparation was analyzed by transmission electron microscopy. As seen in **Figure 1C**, the purified nucleoli maintained the integrity and showed three distinct compartments, that is, FC, DFC and GC.

Mass spectrometry analysis of the bovine nucleoli

The flowchart of the procedure used for proteomic

analysis of the bovine nucleoli is shown in **Figure 2A**. To determine the proteomic composition of the bovine nucleoli, initially, the nucleolar preparation was fractionated using one-dimensional (1D) SDS-PAGE and stained with Coomassie blue. The gel was sliced into 12 pieces and further processed by in gel-trypsin digestion method (**Figure 2B**). Secondly, the nucleolar preparation was subjected to trypsin digestion. The trypsin-digested peptides were fractionated by strong cation exchange (SCX) column and collected into 10 fractions. Both the gel separated and the SCX fractionated samples were analyzed by RPLC ESI-MS/MS.

Nucleolar protein identification by database search

To identify the proteins, the mass spectra obtained from the analysis of the 1D SDS-PAGE and SCX

fractionated samples were searched by Mascot database search using NCBI database against all entries and, by the global proteome machine (GPM) search using *Bos taurus* (ENSEMBL, NCBI unigene and NCBI genome), *Mus musculus* and *Homo sapiens* databases. The Mascot search identified 124 and 232 proteins by 1D SDS-PAGE and SCX fractionation, respectively. Similarly, the GPM database identified 137 and 242 proteins by 1D SDS-PAGE and SCX fractionation, respectively (**Figure 2C**). However, comparison of the proteins identified by 1D SDS-PAGE and SCX fractionation, using both Mascot and GPM search, identified a total of 311 proteins in bovine nucleoli (Table S1). Of the 311 proteins, 65 proteins were identified by all methods, 130 proteins identified were common between 1D SDS-PAGE and SCX fractionation, while 36 and 145 proteins were uniquely identified by each method, respectively. The

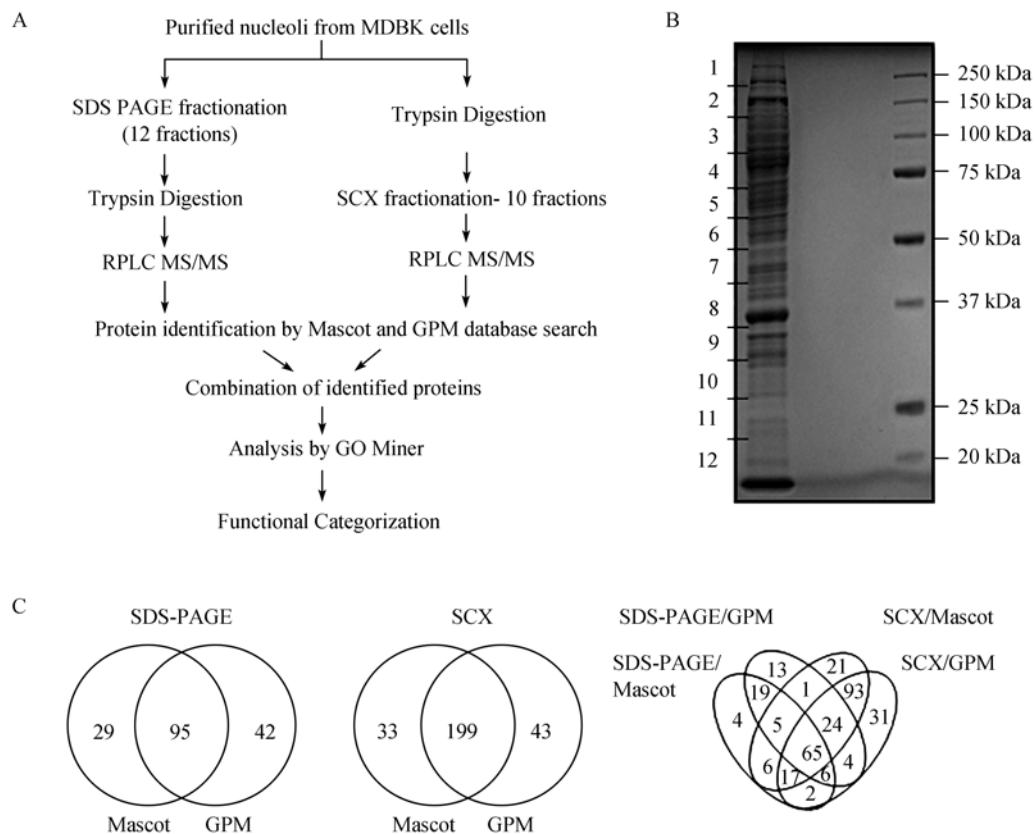


Figure 2 Mass spectrometry analysis of the bovine nucleoli. **A**. Flow chart of the procedure used for proteomic analysis of nucleoli. **B**. Proteins from the nucleolar fraction of MDBK cells were separated on 10% SDS-PAGE. **C**. Database search. Left: Number of unique and common proteins identified by 1D SDS-PAGE fractionation method using Mascot and GPM database search; Middle: Number of unique and common proteins identified by SCX fractionation method using Mascot and GPM database search; Right: Number of unique and common proteins identified by 1D SDS-PAGE and SCX methods using Mascot and GPM database search.

Mascot and the GPM database search for the 1D SDS-PAGE and SCX fractionated samples uniquely identified 4, 13, 21 and 31 proteins, respectively (Figure 2C). The false positive rate was analyzed by reverse database search using both search engines. Only 1 protein (0.26%) with single peptide matching to the validation criteria was identified by Mascot human reverse database search compared to 11 (2.91%) proteins identified by GPM reverse sequence search (Table S2). The gene names for the proteins identified from any species were assigned from the entrez gene using the gene symbols and descriptions from their respective homologs in the human or mouse genomes. Since the database search was performed with all the entries, some peptide queries retrieved the homologous proteins from different species. Such matches were assigned the same gene name and their matching peptides were compiled. It is possible that the homologous proteins identified by different accession numbers may contain protein isoforms. However, isoform analysis was not performed in this study. The list of proteins identified along with their gene name, accession number, species, molecular weight, peptide charge, and the sequence is provided in Table S2.

The bovine nucleolar proteome was also blast searched with the set of 728 proteins of human nucleolar protein database. Out of 311 proteins identified in the present study, 289 proteins had a homolog in the human nucleolar database, whereas 22 proteins did not show significant sequence homology to any of the proteins in the human nucleolar proteome (Table S3). The fragment ion spectra of the corresponding peptides identifying the 22 novel candidate proteins are given in Figure S1.

Localization of DsRed-monomer protein fusions

Selected cDNA clones of novel nucleolar proteins were individually fused inframe to DsRed-monomer gene in vector pDsRed-monomer N1 (Clontech) designed to express protein-DsRed monomer fusions from human cytomegalovirus promoter. The Vero cells were transfected with individual plasmid DNA. After 48 h post transfection, the cells were stained with anti-fibrillar serum followed by DAPI and

visualized by confocal microscopy. As seen in **Figure 3**, in addition to the localization largely restricted to the nucleolus, the fusion proteins were also localized to other nuclear or cytoplasmic structures. However, the results of the nucleolar localization of two fusion proteins (LRRC59 and AMDHD2) were not conclusive (data not shown).

Functional categorization of identified nucleolar protein

To determine the functional characteristics, the identified nucleolar proteins were analyzed by the GoMiner software. The GoMiner analysis categorized a total of 260, 242 and 271 proteins based on the molecular function, the biological process and the subcellular localization, respectively (**Table 1**).

In addition, the unique proteins were analyzed by pathway analysis program InnateDB (54). As seen in Figure S2, while most of these proteins share a relatively high degree of functional interconnectivity, they do not represent a simple pathway.

Discussion

Nucleolus plays a wide variety of functions in the cellular physiological processes (11) including the production of ribosomes, which is one of the important steps for the cell growth and reproduction. Besides ribosome synthesis, many of the important processes as discussed earlier are regulated by the nucleolar components. Previous large-scale proteomic analysis of human and plant nucleoli has provided valuable information regarding the proteomic composition and its putative role in regulating the cellular processes. Similarly, availability of structural and proteomic composition of bovine nucleolus will greatly help in understanding the structure of bovine nucleolus and its role in the biology of bovine viruses including bovine adenovirus type 3 (BAdV-3). As a first step towards achieving our goal, we performed the proteomic analysis of bovine nucleoli for the first time.

The proteomic analysis of the MDBK cell nucleoli identified a total of 311 proteins. The SCX fractionation method identified higher number of proteins compared to the 1D SDS-PAGE fractionation; however,

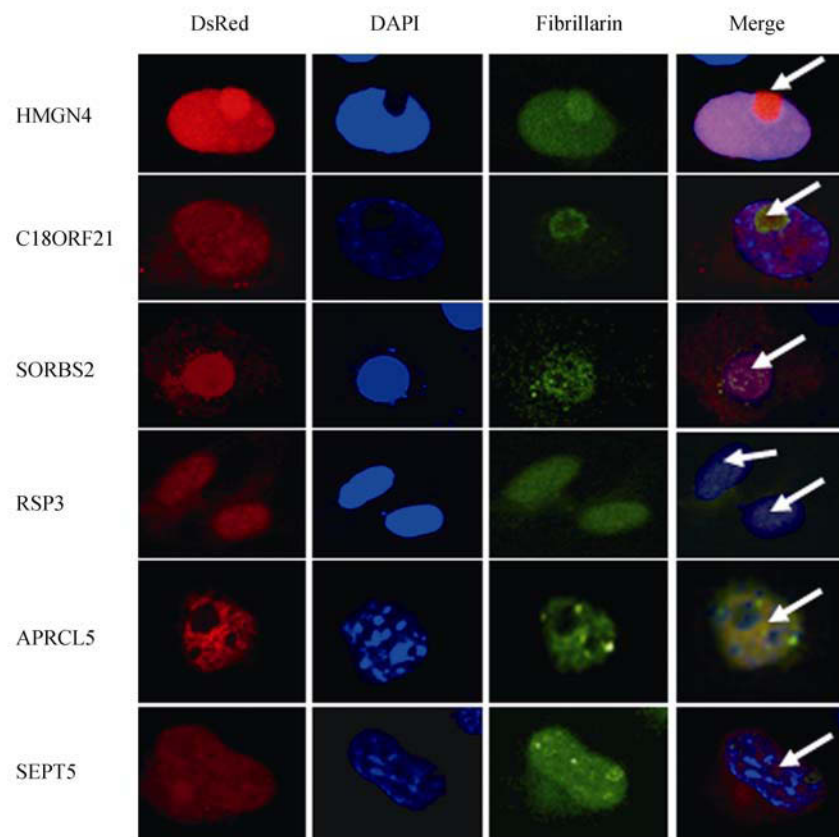


Figure 3 Subcellular localization of potential nucleolar proteins. Monolayer of Vero cells were transfected with individual plasmid DNA expressing DsRed-monomer fusion proteins. After 48 h post-transfection, the cells were fixed and stained with rabbit anti-human fibrillarin serum followed by Cy-2 conjugated goat anti-rabbit secondary antibody. Finally the cells were incubated with DAPI and visualized by confocal microscope. Arrows indicate the nucleolar localization of DsRed-monomer tagged protein.

Table 1 Categorization of the identified nucleolar proteins using GoMiner software

Molecular function	No. of genes	Biological process	No. of genes	Cellular component	No. of genes
RNA binding	127	Ribosome biogenesis/assembly	92	Nucleus	214
Protein binding	126	RNA processing	82	Cytoplasm	131
DNA binding	64	Transcription	50	Ribonucleoprotein	95
Structural molecule activity	61	Cell cycle	36	Nucleolus	83
ATP binding	43	Translation	34	Ribosome	53
NTPase activity	33	Cell communication	25	Chromosome	32
Ion binding	29	Stress response	25	Mitochondrion	32
Helicase activity	23	Transport	25	Cytoskeleton	24
Nuclease activity	19	Signal transduction	20	Nucleoplasm	22
Transferase activity	19	DNA repair	15	snoRNP	21
Transporter activity	17	RNA splicing	15	Nucleosome	13
GTP binding	10	Chromatin assembly	13	Exosome	8
Translation regulator activity	9	Apoptosis	9	Spliceosome	8
Transcription factor activity	8	DNA replication	8	Centrosome	6
Signal transducer activity	7	Ubiquitin cycle	6	hnRNP	6

both methods uniquely identified significant number of proteins thereby improving the total proteome coverage. The Mascot and the GPM database search further improved the protein identification. Although the rate of false positive protein identification was higher by the GPM search compared to the Mascot search, it was found to be complementary to each other. However, we could not make direct comparison as both methods utilized different search parameters. The number of peptides observed for each protein (Table S1) versus observable peptides shows linear relationship with the logarithm of the protein concentration (55), suggesting their relative abundance in the sample. A large fraction of the proteins were identified based on single or few peptide matches by either one or both database searches. It is possible that these proteins are in low abundance in the nucleoli or localize under specific physiological conditions. Alternatively, such proteins may be the potential contaminants sticking to the nucleoli during purification or part of the other subcellular component of the same density as nucleoli (56).

Among 311 proteins identified in the bovine nucleoli in the present study, 289 proteins (93%) showed significant sequence homology to the proteins identified in the human nucleoli (44, 45, 47). Only 22 proteins identified in the bovine nucleoli did not find their homolog in the human nucleolar proteome. These results suggest that the overall structural and functional organization of bovine and human nucleoli appears to be similar. Earlier report suggested that plant nucleoli contained a novel class of exon junction complex proteins not detected in the human nucleoli (51). However, no such proteins were observed in the proteomic analysis of bovine nucleoli.

Ribosome biogenesis is the pre-requisite process in the formation of nucleoli. About one third of the proteins identified in the bovine nucleoli are known to be implicated in the ribosomal gene transcription, rRNA processing and ribosomal subunit assembly. A significant number of proteins (93%) identified based on the molecular function appear to bind RNA, DNA, nucleotide, protein, ion, and chromatin. Since the nucleoli do not contain the membrane, their structural integrity could thus be maintained by the DNA-protein, RNA-protein and protein-protein interactions. Moreover, binding of these proteins to RNA

or nucleotides may help them in the localization or retention of these proteins in the nucleolus. The presence of RNA recognition motifs as predominant motifs in the human nucleolar proteins (52) may mediate the localization or retention of these proteins in the nucleolus (55-63). Similarly, binding of nucleostemin to GTP has been shown to regulate the nucleolar localization (59, 60). Thus, binding of proteins to RNA or nucleotide might be one of the important mechanisms for nucleolar targeting or retention of the proteins in the nucleolus.

Proteins possessing catalytic activity such as NTPases/helicases play diverse cellular functions including protein folding, assembly of macromolecular complexes, organelle biogenesis, DNA replication, recombination and repair, transcription and transport (61-63). Proteomic analysis of bovine nucleoli identified significant number of proteins possessing NTPase/helicase activity, which may play essential roles in rDNA transcription and ribosome biogenesis.

The proteins implicated in the translational regulation (DDX48, EIF2S1, EIF2S2, EEF1A1, EMG1, GTF2H2, ITGBP4, and PCBP1) were also observed in the bovine nucleoli. The consistent identification of these proteins in the human and the bovine nucleoli supports the suggestion about the possibility of their involvement in the export of pre-assembled translational machinery to the cytoplasm, mRNA surveillance (56, 64) or nucleolar/nuclear translation (65-67). Similarly, like human nucleoli (68), the identification of components involved in the ubiquitin pathways (FBXL11, POP1, RPS27A, SMURF2, UBC, and USP36) in the bovine nucleoli suggest their potential implication in ribosome biogenesis.

The nucleolus has also been known to mediate the stress response by regulating the abundance of p53 (25, 68, 69) through nucleolus-associated proteins ARF and MDM2 (70). Like human nucleoli (43, 44, 46), we could not detect the ARF and MDM2 proteins in the bovine nucleoli. It is possible that their nuclear association may vary depending upon the physiological status of the cell. Alternatively, their abundance may not be high enough to be detectable with the methods employed in our study. However, 10% of the proteins identified in the proteomic analysis of bovine nucleoli are known to be involved in the stress signaling (GoMiner analysis). These results confirm the

earlier observations and suggest that bovine nucleolus plays a crucial role in regulating the cellular response to stress. In addition, the identification of significant number of proteins in the bovine nucleoli regulating the apoptosis (4%) and cell cycle (15%) suggests the key role of bovine nucleolus in regulating these processes.

The maturation and assembly of non-ribosomal ribonucleoproteins have been known to occur in the nucleolus (13). A large fraction of ribonucleoproteins including the components of the exosome, hnRNP, spliceosomal, snoRNP, snRNP, RNase P and MRP complexes were identified in the bovine nucleoli. The exosomes are the 3'-5' exonucleases known to mediate the processing of pre-rRNAs, pre-mRNAs, pre-tRNAs and snoRNAs (71). Although the proteomic analysis of the human nucleoli reported 11 subunits of exosome (46), we only identified 8 subunits in the bovine nucleoli, including the subunits of RNase P and MRP complexes (POP1 and RPP30), which are known to be involved in tRNA and rRNA processing, respectively (72).

The components of the nucleoli are highly dynamic and their associations with the nucleoli vary depending upon the physiological conditions. The gene ontology analysis suggested that the proteins identified in the bovine nucleoli are also known to localize to other cellular components such as chromosome, mitochondria, ribonucleoprotein complexes and cytoskeleton. Since nucleoli organize around the ribosomal gene repeats, the identification of the chromosomal/nucleosomal proteins in the bovine nucleolar preparation suggests their possible role in the organization of rDNA chromatin and regulation of rDNA transcription. Besides ribosomal proteins, the other mitochondrial proteins identified in the nucleoli could play essential roles in the transport of ATP and ions or may represent potential contaminants. The proteins known to be the constituent of cytoskeleton were also identified in our preparation, suggesting their possible role in the structural organization of the nucleoli or may also represent the potential contaminants.

The nucleoli are known to play an essential role in the signal recognition particle (SRP) assembly (15, 16). Surprisingly, the components of the SRPs were not detected in bovine nucleoli. Similarly, the interaction of cajal (coiled) body with the nucleolus has been

well characterized (73, 74), whereas the cajal body marker protein p80-coilin was not detected in bovine nucleoli. However, other components known to localize in the cajal bodies, that is, fibrillarin, nucleolin, nucleolar and coiled body phosphoprotein1, nucleophosmin, pigpen, and NHP2L1 (75-80), were detected in bovine nucleoli.

Of the 311 proteins identified in the purified nucleoli by MS analysis, 22 are unique as there is no report in literature to support their localization in the nucleolus. Confocal microscopy analysis of eight unique proteins, each tagged with DsRed-monomer in transfected cells, demonstrated that six are associated with nucleoli and other cellular structures (Figure 3). Similar varied nucleolar localization patterns have been observed for novel human nucleolar proteins identified by MS (47). In addition, similar estimates have been reported for nucleolar localization of proteins as determined by analysis of MS data and YFP-tagging data (47). Absence of detection of nucleolar localization by DsRed-monomer tagging analysis could be due to interference of DsRed tag in the fusion protein or due to less sensitivity of DsRed fluorescence compared to MS. Moreover, since nucleolar localization of proteins may also vary depending upon transient/stable association with nucleolus, cell cycle stage, or the type of cell used (47), we are confident that novel proteins identified by MS are bona fide nucleolar proteins.

The proteins uniquely identified in bovine nucleoli (Table S3) have been shown to be involved in a variety of biological processes including metabolism, cell cycle, transcription, translation, apoptosis, ubiquitin dependent protein catabolism, and transport. These results suggest that the presence of different proteins in bovine nucleoli (or not yet identified in human nucleoli) belongs to similar functional categories.

Conclusion

In summary, the proteomic analysis of bovine nucleoli identified proteins that revealed significant homology (93%) to the proteins identified in human nucleoli. Furthermore, our study led to the identification of 22 novel candidate proteins, which do not show significant sequence homology to the proteins identified in

human nucleoli. Availability of the protein data will add the peptide information to the predicted bovine gene sequences and would be helpful in further analysis of the protein isoforms.

Materials and Methods

Isolation of nucleoli from bovine cells

Nucleoli from MDBK cells were isolated using a procedure adapted from published methods (47, 49). Briefly, MDBK cells were cultured at 37°C in 5% CO₂ in minimum essential medium (MEM) containing 5% fetal bovine serum (FBS). At 80% confluency, the cells were washed thrice with prewarmed phosphate buffered saline (PBS), trypsinized and collected in prewarmed MEM containing 5% FBS. The cells were immediately incubated on ice and washed thrice with ice-cold PBS. The protease inhibitor solution (PMSF 1 mM, leupeptin 1 µg/mL, pepstatin 1 µg/mL, and aprotinin 1 µg/mL) was added into each buffer prior to use. The cells were resuspended in 10 mL of hypotonic buffer (10 mM HEPES-KOH, pH 7.9, 1.5 mM MgCl₂, 10 mM KCl, 0.5 mM DTT) containing 0.3% NP-40. After 30 min incubation on ice, the cells were homogenized using Ten Broeck homogenizer (PYREX 7727-15) until >90% of the cells burst, leaving intact nuclei with various amount of cytoplasmic material attached. Finally, the nuclei were pelleted by centrifuging at 218× g for 5 min, resuspended in buffer S1 (0.25 M sucrose, 10 mM MgCl₂) and centrifuged over a gradient of buffer S2 (0.35 M sucrose, 0.5 mM MgCl₂) at 1430× g for 5 min. The nuclear pellet thus obtained was resuspended in buffer S2 and sonicated 6 times, each for 10 s on ice. The sonicated sample was layered over a gradient of buffer S3 (0.88 M sucrose, 0.5 mM MgCl₂) and centrifuged at 3000× g for 10 min. The pellet containing the nucleoli was resuspended in buffer S2 and centrifuged at 1430× g for 5 min. Finally, the nucleolar pellet was resuspended in buffer S2 and stored at -70°C until further use.

Western blot analysis of the isolated nucleoli

The enrichment of the nucleolar preparation was as-

essed by Western blot using rabbit polyclonal antibodies recognizing cytoplasmic marker extra cellular signal-regulated kinase 2 (ERK2), nucleoplasmic marker Nucleoporin 62 (Nup62) and nucleolar markers nucleolin and fibrillarin. Two micrograms of protein samples from cytoplasmic, nucleoplasmic and nucleolar fractions were separated on 10% SDS-PAGE and electrotransferred to nitrocellulose membrane. The membranes were blocked with 5% BSA and incubated with rabbit polyclonal antibodies specific to ERK2 (C-14, 1:200), Nup62 (H-122, 1:100) and fibrillarin (H-140, 1:200) (Santa Cruz) and mouse anti-nucleolin monoclonal antibody (KAM-CP100, 1:1000, Stressgen). After blocking with BSA, the membranes were washed three times with the TBS (0.05 M Tris, 0.9% NaCl, pH 7.6) containing 0.1% Tween-20 (TBST), each for 5 min. Next, the membranes reacted with the rabbit polyclonal antisera or the mouse monoclonal anti-nucleolin antibody were incubated with goat anti-rabbit or goat anti-mouse alkaline phosphatase conjugated secondary antibody, respectively. After washing in TBST for three times (5 min each), the membranes were developed with BCIP-NBT (Sigma).

Immunofluorescence staining of the isolated nucleoli

The purified nucleoli were immunostained with rabbit polyclonal anti-human fibrillarin specific antibody (Santa Cruz) as described earlier (47). Briefly, nucleoli were spotted on Lab-TekTM II chamber slide, air-dried, rehydrated in PBS for 5 min, and then incubated with anti-fibrillarin antibody (1:50). After incubation for 30 min, the nucleoli were washed three times with PBS (each for 5 min) and incubated with anti-rabbit IgG (Cy-2 conjugated, 1:400) for 30 min. Finally, the nucleoli were counter stained with 0.66 mM Pyronin Y (Sigma) for 1 min, washed three times with PBS (each for 5 min), mounted with DABCO containing Mowiol and observed by DIC optics and fluorescent microscopy (Zeiss AxioVision).

Transmission electron microscopy analysis of isolated nucleoli

The purified nucleoli were analyzed by the electron

microscopy. Briefly, the purified nucleoli were pelleted by centrifugation at $1430\times g$ and fixed with 3% glutaraldehyde in 0.1 M sodium cacodylate buffer containing 0.35 M sucrose and 0.5 mM $MgCl_2\cdot 6H_2O$. The fixatives were removed and 1% agarose was added to the pellet. After solidification, the pellet was cut off and rinsed three times with 0.1 M sodium cacodylate buffer with 0.22 M sucrose at $4^\circ C$. Samples were post fixed with 1% OsO_4 in 0.1 M sodium cacodylate buffer for 1 h at room temperature and dehydrated in 70% ethanol. After washing three times with propylene oxide, the samples were infiltrated with Epon/Araldite and polymerized at $55^\circ C$. Finally, the samples were ultrathin sectioned, stained with saturated uranyl acetate and lead citrate, and viewed on Philips 410LS TEM electron microscope.

1D SDS-PAGE fractionation followed by in gel digestion

Proteins from the nucleolar fraction were separated on 10% SDS-PAGE, stained with Coomassie blue and cut into 12 equal pieces. The gel pieces were placed in a 96-well microtitre plate (Sigma). The samples were then de-stained, reduced with dithiothreitol, alkylated with iodoacetamide, and digested with porcine trypsin (sequencing grade; Promega). The resulting peptides were extracted with 1% trifluoroacetic acid (TFA)/2% acetonitrile followed by two extractions with 0.5% TFA/50% acetonitrile, and finally transferred to 96-well PCR plates using a MassPREP protein digest station (Micromass, Manchester, UK). The digest was evaporated to dryness, resuspended in 1% aqueous TFA, and analyzed by LC-MS/MS as described below.

SCX fractionation

For SCX fractionation, 200 μg of the purified nucleolar fraction was resuspended in 0.1% RapigestTM SF (Waters, Canada), reduced with dithiothreitol, alkylated with iodoacetamide and digested with the trypsin gold (Promega) in 1:50 ratio. The tryptic digest was acidified with 0.5% TFA and fractionated by the SCX chromatography on a 200×2.1 mm Polysulpho-ethyl A column (PolyLC, Columbia, USA) preceded by a 10×2.1 mm guard column (PolyLC) as described

earlier (81). A total of 60 fractions were collected on Varian Prostar Model 330 HPLC system using a flow rate of 0.2 mL/min. The mobile phase solvents used were (1) 10 mM ammonium formate, 25% Acetonitrile pH 3.0 and (2) 500 mM ammonium formate, 25% acetonitrile, pH 6.8. After loading the sample, isocratic conditions were maintained with 100% solvent A for 10 min. The peptide separation was performed with a gradient of 0-50% solvent B for 40 min followed by 50%-100% solvent B for 10 min and 100% solvent B for 10 min. Finally, the six consecutive fractions were combined to make a total of ten fractions and analyzed by LC-MS/MS as described below.

LC-MS/MS

The LC-MS/MS analysis was performed using a capLC pump interfaced to a Q-ToF Ultima Global hybrid tandem mass spectrometer fitted with a Z-spray nanoelectrospray ion source (Waters-Micromass). The solvent A consisted of 0.2% formic acid in acetonitrile whereas solvents B and C were comprised of 0.2% formic acid in water. The trypsin-digested samples were loaded onto a C18 trapping column (SymmetryTM 300, 0.35×5 mm Opti-pak; Waters) and washed with solvent C for 3 min at a flow rate of 30 μL /min. The flow path was switched using a 10-port rotary valve before the samples were eluted onto a C18 analytical column (PepMapTM, $75\ \mu m \times 15$ cm, 3- μm particle size; LC Packings). The separations were performed using a linear gradient of 5%:95% to 40%:60% A:B in the case of gel separated proteins, and a linear gradient of 5%:95% to 35%:75% A:B for SCX fractionated peptides over 100 min. The composition was then changed to 80%:20% A:B and held for 10 min to flush the column before re-equilibrating for 7 min at 5%:95% A:B. Mass calibration of the Q-ToF instrument was performed using a product ion spectrum of Glu-fibrino peptide B acquired over the m/z range of 50 to 1900. LC-MS/MS analysis was carried out using Data Dependent Acquisition, during which peptide precursor ions were detected by scanning from m/z 400 to 1900 in TOF MS mode. Multiply charged (2+, 3+, or 4+) ions rising above predetermined threshold intensity were automatically selected for TOF MS/MS analysis. Low energy collision in-

duced dissociation of the peptides was performed in the collision cell with argon as the collision gas and varying the collision energy with charge state recognition. Product ion spectra were acquired over the m/z range of 50 to 900. LC-MS/MS data were processed using ProteinLynx software v4.0 (Waters-Micromass) to generate .pkl files or Mascot Distiller v2.1.0 (Matrixscience) to generate .mgf files.

Data analysis

The MS/MS data files were searched against the NCBI non-redundant database for all entries using Mascot v2.1 (Matrixscience) with parameter setting as follows: (1) trypsin as the specific enzyme allowing one missed cleavage, (2) peptide window tolerance of ± 0.6 Da, (3) fragment mass tolerance of ± 0.4 Da, (4) oxidation of methionine (fixed and variable) and (5) carbamidomethylation of cysteine (fixed and variable). Protein hits matching more than one peptides with individual ion score ≥ 30 and at least one peptide with p value < 0.05 or the proteins matching single peptide with an ion score ≥ 50 and p value < 0.05 were considered as valid protein match. Only first-rank peptides were considered and the protein hits identified by at least one unique peptide were included for further analysis. The MS/MS data were further searched using the Mascot search engine against the reverse sequence (NCBI human_rev) database to analyze the rate of false positive protein identifications. The data were also analyzed using the GPM open source software (57) against bovine (ENSEMBL, NCBI genome and NCBI unigene database), human and mouse database using parameter setting as follows: (1) parent ion mass error < 100 ppm, (2) fragment mass error < 0.4 Da, (3) trypsin as a specific enzyme allowing maximum missed cleavage of 1, (4) $\log(e)$ value < -2 (p value < 0.01), (5) carbamido-methylation of cysteine, and (6) oxidation of methionine along with the reversed sequence search. The GPM search also included the refinement modifications, which were oxidation and dioxidation of methionine and tryptophan, deamidation of asparagine and glutamine, and acetylation of lysine. The keratins being considered as potential contaminants were removed. The proteins identified by the Mascot and the GPM database search were combined, analyzed by the GoMiner

software (<http://discover.nci.nih.gov/gominer/>) (58), and categorized based on the molecular function, biological process and cellular component. The bovine nucleolar proteins were further blast searched with the dataset of 728 proteins of human nucleolar database (kindly provided by Anthony K.L. Leung, Massachusetts Institute of Technology, USA and Angus I. Lamond, University of Dundee, UK).

Construction of DsRed monomer-nucleolar fusion proteins

cDNA clones containing bovine HMGN4 (8059446), APRC5L (8253815), RPS3 (7945675), C18ORF21 (8425238), SorBS2 (8387106), SEPT5 (8226403), LRRC9 (8279478) and AMDHD2 (8558625) were obtained from Open biosystems (www.openbiosystems.com). The cDNA clones and the junctions of protein-DsRed-monomer fusions were verified by DNA sequencing.

Subcellular localization of novel nucleolar proteins

The Vero cells were seeded in four-well Lab-Tek chamber slides. After 18 h, the cells were transfected with 2 to 5 μg individual plasmid DNA using lipofectamine as per manufacturer's instructions (Invitrogen). After 48 h post-transfection, the cells were fixed in 4% paraformaldehyde for 15 min, washed four times with PBS and permeabilized with 0.5% Triton X-100 for 20 min. The cells were stained with anti-human fibrillar serum (Santa Cruz) followed by Cy-2 labeled secondary antibody. Finally, the cells were washed three times with PBS, mounted in DABCO mounting media (Fluka) containing DAPI and analyzed by confocal microscopy.

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Authors' contributions

AKP conducted the experiments, collected the datasets, conducted data analyses, and prepared the manuscript. DO and SKT supervised the project and co-wrote the manuscript. All authors read and approved the manuscript.

Competing interests

The authors have declared that no competing interests exist.

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Supplementary Material

Tables S1-S3; Figures S1 and S2

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