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Ly-1 Antibody Reactive Clone Is an Important Nucleolar Protein for Control of Self-Renewal and Differentiation in Embryonic Stem Cells

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ABSTRACT

Embryonic stem cells (ESCs) possess the capacity to self-renew and differentiate into all cell types of an organism. It is essential to understand how these properties are controlled for the potential usage of their derivatives in clinical settings and reprogramming of differentiated somatic cells. Although transcriptional factors, such as Oct4, Sox2, and Nanog, have been considered as a part of the core regulatory circuitry, a growing body of evidence suggests that additional factors exist and contribute to the control of ESC self-renewal and differentiation. Here, we report that Ly-1 antibody reactive clone (LYAR), a zinc finger nucleolar protein highly expressed in undifferentiated ESCs, plays a critical role in maintaining ESC identity. Its downregulation significantly reduces the rate of ESC growth and increases their apoptosis. Moreover, reduced expression of LYAR in ESCs impairs their differentiation capacity, failing to rapidly silence pluripotency markers and to activate differentiation genes upon differentiation. Mechanistically, LYAR forms a complex with another nucleolar protein, nucleolin, and prevents its self-cleavage, maintaining a normal steady-state level of nucleolin protein in undifferentiated ESCs. Interestingly, the downregulation of *nucleolin* is detrimental to the growth of ESCs and increases the rate of apoptosis, similarly to the knockdown of LYAR. Thus, our data emphasize the fact that other genes besides Oct4 and Nanog are uniquely required for ESC self-renewal and differentiation and demonstrate that LYAR functions to control the stability of nucleolin protein, which in turn is essential for maintaining the self-renewal of ESCs. STEM CELLS *2009;27:1244–1254*

Disclosure of potential conflicts of interest is found at the end of this article.

INTRODUCTION

Embryonic stem cells (ESCs), derived from the inner cell mass of blastocyst-stage embryos, have the potential to differentiate into all cell types of the three embryonic germ layers and to grow indefinitely in culture [1, 2]. These properties make their derivatives an attractive cell source for future regenerative medicine, as well as an excellent in vitro model system for the study of early mammalian development. However, the mechanisms by which these properties are regulated are still largely undefined, which could limit the usage of their derivatives in clinical settings.

Transcriptional factors highly expressed in undifferentiated ESCs, such as Oct4 [3, 4], Sox2 [5], and Nanog [6, 7], have been considered as master genes in the control of pluripotency and self-renewal in ESCs and have been intensively studied [8–10]. However, growing evidence suggests that additional factors, different from canonical transcriptional circuit, also contribute to the control of ESC properties. Recently, Dejosez et al. [11] reported Ronin as a novel type of pluripotency factor, which acts broadly through an epigenetic mechanism. This is in contrast to Oct4 and Nanog, which regulate the expression of specific genes for particular lineages or functions. Obviously, identification of novel factors or pathways is necessary for future application of ESC derivatives in regenerative medicine and for reprogramming of differentiated somatic cells. Of note, nucleolar proteins have been implicated in the control of cell growth [12, 13]. For example, nucleostemin, a newly discovered nucleolar protein, has been demonstrated to participate in controlling cell proliferation in adult stem cells and cancer cells [14, 15]. Some important nucleolar proteins, including nucleophosmin 1 (NPM1) [16] and nucleolin [17, 18], have been shown to be highly expressed in undifferentiated ESCs [19], although their functions in ESCs are poorly characterized. Previously, we showed that NPM1 is essential for ESC growth [20]. Recently, we have studied another nucleolar protein, Ly-1

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antibody reactive clone (LYAR). The cDNA of LYAR was initially isolated from a mouse T-cell leukemia line more than a decade ago [21]. It encodes a polypeptide consisting of 388 amino acid residues with a zinc finger motif and three copies of nuclear localization signals. The study showed that LYAR may function as a nucleolar oncoprotein to regulate cell growth. Recently, microarray data showed that LYAR is highly expressed in undifferentiated human ESCs and is significantly downregulated upon differentiation [22]. Thus, LYAR appears to be a likely candidate for the study of the involvement of nucleolar mechanisms in controlling the self-renewal of ESCs.

In the present study, we have generated stable ESC lines in which the expression of small interference RNAs (siRNAs) specifically targeting LYAR can be induced by addition of tetracycline (Tc) to the cell culture. Using the cell lines, we demonstrate that LYAR is an important nucleolar protein for the control of self-renewal and differentiation of ESCs. Mechanistically, we show that LYAR forms complexes with another nucleolar protein, nucleolin, and inhibits its autocleavage. Furthermore, our data indicate that ESC growth is severely compromised and the rate of cell apoptosis increases when *nucleolin* expression is knocked down. These findings establish a previously unrecognized nucleolar mechanism in controlling the self-renewal and differentiation of ESCs.

Materials and Methods

Plasmids and Cell Lines

The information on plasmids and cell lines used in this study is provided in the supporting information.

Cell Transfection

To generate stable cell lines, F9 embryonic carcinoma (EC) and CGR8-TetR host ESCs described previously [20] were transfected with 40 μg plasmids by electroporation and then selected with puromycin (Sigma-Aldrich, St. Louis, MO, http://www.sigmaaldrich.com) at 1 μg/ml or zeocin (Invitrogen, Carlsbad, CA, http://www.invitrogen.com) at 30 μg/ml. 293T cells were transiently transfected using a standard calcium phosphate method.

Quantitative Real-Time Polymerase Chain Reaction

Total RNA was isolated with TRIzol (Invitrogen) and transcribed into cDNA using oligo $(dT)_{15}$ and ReverTra Ace reverse transcriptase (Toyobo, Osaka, Japan, http://www.toyobo.co.jp). The primer sequences for different genes are listed in supporting information Table S1. Quantitative real-time polymerase chain reaction (qPCR) was performed as described previously [23].

Western Blot Analysis

Western blot analysis was carried out as previously described [20]. The antibodies used are described in the supporting information.

Nuclear Extract Preparation and Protein Complex Purification

The nuclear extract (NE) was extracted from F9 cells transfected with Flag-LYAR/pPyCAG or vector as described previously [24]. Four milligram of the NE was incubated with 50 μl of packed M_2-agrose beads at 4°C for 6 hours and washed with buffer. The protein complexes were electrophoretically resolved and stained with Coomassie blue. Peptide bands specific to Flag-LYAR-expressed samples were subjected to mass spectrometric analysis as previously described [24].

Immunoprecipitation and Glutathione *S*-Transferase Pull-Down Assays

The experiments were conducted as previously described [25] and were repeated at least three times.

Immunofluorescence Staining

Cells were stained as previously described [20]. Fluorescence signals were detected using a fluorescence microscope or a Leica TCS SP2 confocal scanning microscope.

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium Bromide Assay

Cells at a density of 2×10^3 cells per well were seeded onto 24-well plates (or 300 cells per well for 96-well plates). At the indicated time, 50 μl (10 μl for 96-well plates) of 5 mg/ml 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) per well was added. MTT assays were performed as previously described [20].

Teratoma Formation

Six-week-old male nude mice were grouped randomly and given water with or without Tc (1.5 mg/ml). One week later, 1.2×10^6 cells were injected subcutaneously into each mouse. One month after injection, teratomas were dissected. During this period, the drinking water was changed every 2 days.

Terminal Deoxynucleotidyl Transferase-Mediated dUTP Nick End Labeling Assay

CGR8 cells were trypsinized and fixed in 4% paraformaldehyde for 1 hour at room temperature and then permeabilized in 0.2% Triton X-100 for 15 minutes. Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) staining was performed according to the manufacturer's instructions (In Situ Cell Death Detection Kit, POD; Roche Diagnostics, Basel, Switzerland, http://www.roche-applied-science.com). The stained cells were analyzed using a BD FACSAria Cell Sorter (BD Biosciences, San Diego, CA, http://www.bdbiosciences.com).

Alkaline Phosphatase Staining

Cells were seeded at a density of 2,000 per 6-cm dish. After 7 days, the colonies were fixed with 50% acetone and 50% methanol at room temperature for 2 minutes and stained using an alkaline phosphatase (ALP) staining kit (Vector Laboratories, Burlingame, CA, http://www.vectorlabs.com) according to a standard protocol.

Embryoid Body Formation

CGR8 ESCs were plated onto petri dishes and cultured in suspension without leukemia inhibitory factor (LIF) for the indicated time.

Statistical Analysis

All values are expressed as means ± SD. The paired Student's t test was used to determine the significance of differences in comparisons. Values of $p < .05$ were considered statistically significant.

RESULTS

LYAR Is a Nucleolar Protein Highly Expressed in Undifferentiated ESCs

To verify and extend our understanding of the LYAR expression pattern at a protein level, we raised and affinity-purified a polyclonal antibody against LYAR. The specificity of this antibody is shown in supporting information Figure S1. Using this specific antibody, we found that, among different organs/tissues of adult mice, LYAR proteins were detected at the highest level in testis and at a moderate level in spleen, liver, and lung, but were very low or absent in other organs/tissues tested (Fig. 1A). Next, the mRNA and protein levels of LYAR in various cell lines were compared. The data from qPCR analysis showed that the mRNA level of LYAR was the highest in undifferentiated ESCs (CGR8) among all cell lines examined. In contrast, its expression in differentiated cell lines, including fibroblast cells (NIH3T3, mouse embryonic fibroblast [MEF], and L929) and myoblast cells (myoblast C2), was lower than 50% of that in ESCs (Fig. 1B). Interestingly, the transcript level of LYAR was also low in some tumor cells, such as hepatocellular carcinoma cells (Hepa1–6), though it was previously proposed as an "oncoprotein" [21]. A similar expression pattern was obtained when Western blot analysis was used to assess the protein levels of LYAR in these cell lines, with the highest level in the undifferentiated E14T ESC line (Fig. 1B). The results clearly demonstrate that LYAR is highly expressed in pluripotent ESCs and in the organ containing pluripotent cells.

When cultured in suspension in the absence of LIF, ESCs can spontaneously form aggregates called embryoid bodies (EBs), in which cells differentiate into heterogeneous cell types representing all three embryonic germ layers. Therefore, EBs have become the most often used in vitro model for studying ESC differentiation and early mammalian development [26]. To learn whether the expression of LYAR in ESCs is regulated by differentiation, the LYAR mRNA level in spontaneously formed EBs was determined. As shown in Figure 1C, the expression of LYAR was quickly decreased at the 4th day of EB formation and remained at a low level until the 16th day. This finding is consistent with the reported microarray data gathered from human ESCs [22]. Simultaneously, the expression of other developmental markers was examined (supporting information Fig. S2). As expected, expression of the pluripotency marker (Oct4) was significantly decreased, and expression of differentiation markers (Cdx2, Gata6, Brachyury, Fgf5, and Nestin) was rapidly increased during EB formation, confirming the occurrence of differentiation process in these EBs. In addition, Western blot analysis showed that the LYAR protein level was gradually reduced by treatment of ESCs with retinoid acid (RA), a strong ESC differentiation inducer (Fig. 1D). Therefore, our results indicate that LYAR expression is downregulated during ESC differentiation.

Su et al. [21] reported LYAR to be localized in the nucleolus of MEF and COS-7 cells transiently transfected with LYAR cDNA. Here, we further examined the subcellular localization of endogenously expressed LYAR in ESCs. As illustrated in Figure 1E, LYAR proteins were detected

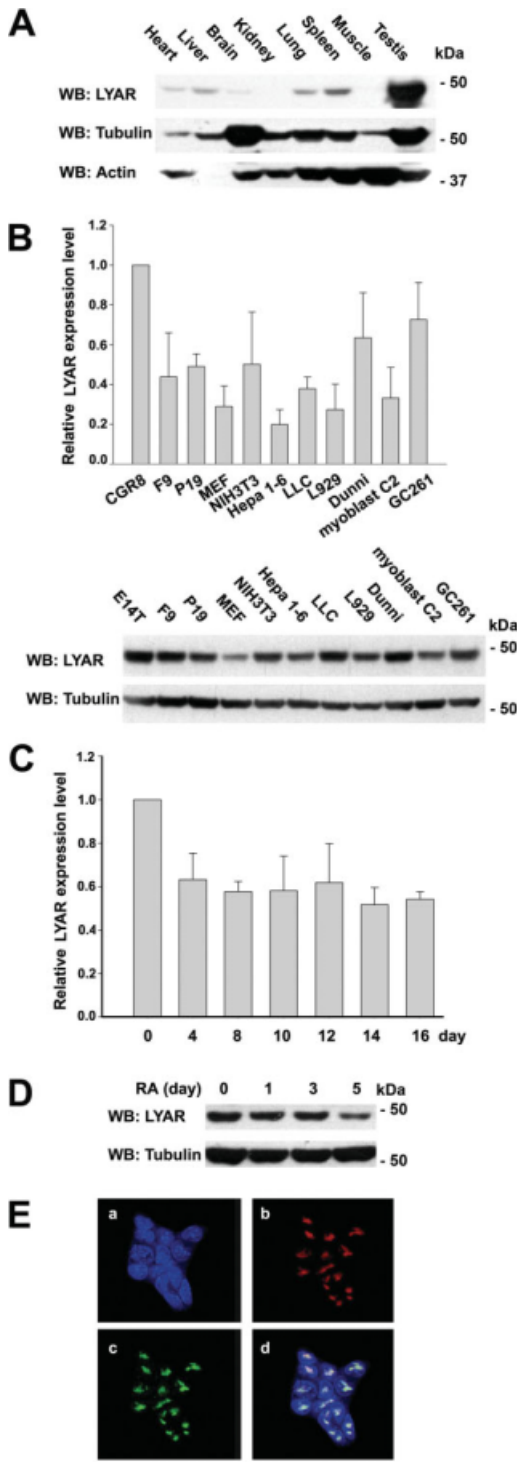

Figure 1. LYAR is highly expressed in undifferentiated embryonic stem cells (ESCs) and mouse testis. **(A):** LYAR expression pattern in various organs of adult mice as determined by WB analysis. **(B):** Levels of LYAR mRNA and protein in different cell lines detected by quantitative real-time polymerase chain reaction (qPCR) and WB analysis, respectively. **(C):** The expression level of LYAR decreases during embryoid body (EB) formation. CGR8 ESCs were cultured in the form of EB and the LYAR mRNA level at the indicated time points was determined using qPCR. **(D):** The protein level of LYAR decreases when ESCs are induced to differentiate by retinoid acid treatment. **(E):** Nucleolar localization of endogenous LYAR in CGR8 ESCs. **(a):** 4,6-Diamino-2-phenylindole staining highlighting the nuclei (blue); **(b):** Nucleophosmin 1 (1:1,000) staining (red); **(c):** LYAR (1:1,600) staining (green); **(d):** a composite image of **(a)**–**(c)**. Abbreviations: LLC, Lewis lung cancer; LYAR, Ly-1 antibody reactive clone; MEF, mouse embryonic fibroblast; WB, Western blot.

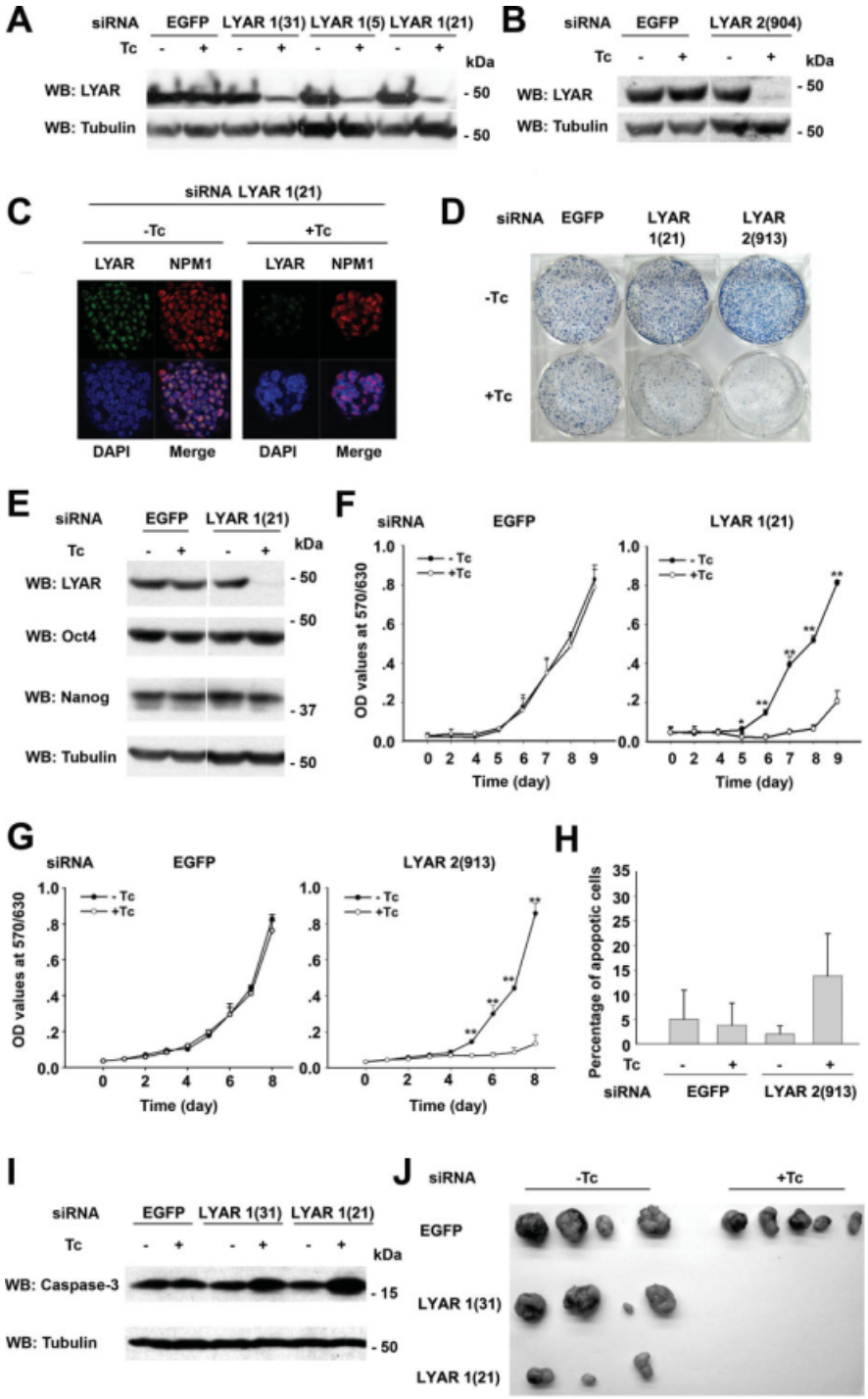

Figure 2. Embryonic stem cell (ESC) self-renewal is impaired when LYAR expression is downregulated. **(A, B):** Tc treatment efficiently knocks down the LYAR protein level in selected ESCs integrated with Tc-inducible siRNA LYAR 1 (**A**) and siRNA LYAR 2 (**B**) but not in siRNA enhanced green fluorescent protein (EGFP) expressing cells. The cells were cultured in the presence or absence of Tc for 96 hours. **(C):** Immunofluorescence staining of LYAR and NPM1 in CGR8 ESCs of siRNA LYAR 1 cultured with or without Tc. **(D):** Downregulation of LYAR expression impairs the self-renewal of CGR8 ESCs. The ESCs of siRNA LYAR 1 and siRNA LYAR 2 were seeded at a low density, treated with or without Tc for 7 days, and the colonies were stained with alkaline phosphatase. **(E):** The protein levels of pluripotency markers (Nanog and Oct4) were not affected by the knockdown of LYAR. The lysate was collected after the cells were treated with or without Tc for 4 days. **(F):** The growth rate of ESCs of siRNA EGFP or siRNA LYAR 1 [21] was measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays. *, $p < .05$; **, $p < .01$; the data are mean values of three independent experiments. **(G):** The growth rate of ESCs of siRNA EGFP or siRNA LYAR 2 (clone 913) was measured by MTT assays. **(H):** Cell apoptosis increases when LYAR expression is downregulated, as determined by terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling assays conducted after the cells were treated with or without Tc for 4 days. Data from three independent experiments are presented. **(I):** Caspase-3 is activated when LYAR is downregulated. The cells were treated with or without Tc for 60 hours before collection. **(J):** Teratoma formation is abrogated in CGR8 cells expressing siRNA LYAR. Teratomas collected from the nude mice injected with ESCs of siRNA EGFP and LYAR 1 (clones 21 and 31) are shown. Abbreviations: DAPI, 4,6-diamino-2-phenylindole; EGFP, enhanced green fluorescent protein; LYAR, Ly-1 antibody reactive clone; NPM1, nucleophosmin 1; OD, optical density; siRNA, small interference RNA; Tc, tetracycline; WB, Western blot.

exclusively in the nucleoli of ESCs, a fact that was highlighted on staining the nucleolar protein NPM1 [16].

Taken together, our data reveal that LYAR is a nucleolar protein highly expressed in the undifferentiated mouse ESCs and in the organ containing pluripotent cells. Importantly, its expression is downregulated by the differentiation process, implying its role in maintaining ESC identity.

LYAR Is Required for Self-Renewal of ESCs

To explore the role of LYAR in ESCs, we established ESC lines that were stably integrated with Tc-inducible siRNAs targeting the LYAR sequence. For the specificity of the siRNA, two sequences (siRNA LYAR 1 and siRNA LYAR 2) targeting LYAR coding and the 5′ untranslated region (UTR) sequences, respectively, were designed, and multiple single clones were selected for each sequence. Stable ESC lines of siRNA enhanced green fluorescent protein (EGFP) were also generated for a Tc-treatment control. As shown in Figures 2A and 2B, Tc treatment remarkably reduced the protein levels of LYAR in all siRNA LYAR-expressing ESC lines examined, but it did not affect LYAR protein levels in siRNA EGFP-expressing cells, demonstrating that the downregulation of LYAR protein levels in siRNA LYAR-expressing ESCs is due to the specific RNA interference of LYAR but not due to the presence of Tc. Furthermore, indirect immunofluorescence staining in ESCs was conducted. As illustrated in Figure 2C, both LYAR and NPM1 proteins were readily detected in the nucleoli of ESCs before addition of Tc. However, Tc treatment dramatically diminished the LYAR signal without affecting that of NPM1, further confirming the efficiency and specificity of siRNA LYAR.

Using these established ESC lines, we first performed a colony forming assay, which is often used to evaluate the self-renewal ability of ESCs [7, 27]. Strikingly, the number of ALP-positive colonies declined drastically in both siRNA LYAR 1- and siRNA LYAR 2-expressing cells in the presence of Tc, when compared with that in the absence of Tc (Fig. 2D). In addition, the size of colonies from siRNA LYAR-expressing cells was much smaller, although these colonies were ALP positive. In contrast, treatment with Tc altered neither the number nor the size of ALP-positive colonies in siRNA EGFP-expressing ESCs (Fig. 2D). Of note, siRNA LYAR-expressing cells remained morphologically undifferentiated in the presence of Tc, suggesting that the knockdown of LYAR in ESCs might impair their self-renewal ability but not promote differentiation. In addition, the markers for undifferentiated ESCs, Oct4 and Nanog, were expressed at a normal level when LYAR protein level was substantially reduced (Fig. 2E), implying that the reduction in the colony number and the size was not due to an abnormality in protein levels of Oct4 and Nanog.

It is generally considered that the ability of ESCs to self-renew is maintained through promotion of proliferation and prevention of differentiation and cell death [28]. Because it appeared that the knockdown of LYAR did not promote differentiation, we were interested to know whether its knockdown would affect cell growth. Therefore, MTT assays were performed to analyze the cell growth rate [29]. As illustrated in Figures 2F and 2G and supporting information Figure S3, cell lines expressing siRNA LYAR, including siRNA LYAR 1 (clones 21 and 31) and siRNA LYAR 2 (clone 913), displayed a significantly slower growth rate in the presence of Tc, when compared with that in the absence of Tc. This result indicates a requirement for LYAR in ESC growth. In contrast, the knockdown of LYAR did not produce significant effects on the cell growth rate after ESCs were induced into differentiation by RA treatment (supporting information Fig. S4), implying that LYAR may function primarily in undifferentiated cells. To determine whether the observed reduction in cell growth was caused by increased cell apoptosis, TUNEL assays were conducted to assess the extent of apoptosis. We found that the knockdown of LYAR increased apoptosis in the ESC lines expressing siRNA LYAR 2 (Fig. 2H) and LYAR 1 (data not shown). To further confirm the phenomenon, we examined the level of the activated caspase-3. As shown in Figure 2I, the level of activated caspase-3 at a molecular weight of 17 kDa was clearly elevated in the cells in which LYAR was downregulated. Therefore, the reduction in the cell growth rate observed when LYAR was downregulated could be, at least partially, explained by the increased cell apoptosis.

Taking advantage of the fact that the Tc induction system can be manipulated in vivo by administering Tc in the drinking water of mice [30], we investigated whether LYAR was also required for ESC growth in vivo using a teratoma formation assay. We found that ESCs of siRNA LYAR 1, from both clones (31 and 21; Fig. 2J, left panel), and siRNA LYAR 2 (clone 913, data not shown) produced teratomas when Tc was absent. However, these cells did not generate any visible teratomas (Fig. 2J, right panel) or produced significantly smaller ones (data not shown) when Tc was present to knock down LYAR. In contrast, teratomas of comparable size were observed in ESCs of siRNA EGFP, regardless of the presence or absence of Tc in the drinking water (Fig. 2J, the first row; supporting information Fig. S5). Failure to form teratomas from ESCs expressing siRNA LYAR could be due to their reduced capacity to self-renew. Therefore, our data strongly argue that normal LYAR expression is required for self-renewal of ESCs both in vitro and in vivo.

Downregulation of LYAR Expression Impairs ESC Differentiation

Another unique characteristic of ESCs is their ability to differentiate into all cell types of an organism. We wanted to know whether LYAR is involved in the control of ESC differentiation processes. For this purpose, we examined the expression pattern of differentiation markers in the EBs formed from ESCs of siRNA LYAR 1. The data from qPCR showed that the expression of germ layer markers, including Fgf5 (primitive ectoderm), Nestin (ectoderm), Gata6 (endoderm), and MHox (mesoderm), was evidently induced by differentiation in the absence of Tc. However, when LYAR expression was downregulated, the expression of these differentiation markers was substantially less activated (Fig. 3A). Moreover, the expression of the marker of undifferentiated ESCs, Rex1, was quickly reduced during EB formation in the absence of Tc. In contrast, the reduction in Rex1 expression was noticeably slower in the presence of Tc. Thus, the downregulation of LYAR expression severely impairs induction of differentiation markers and delays silencing of the undifferentiation gene during EB formation.

We were also interested in knowing whether the knockdown of LYAR expression at different stages of EB formation would affect their further differentiation. Thus, ESCs integrated with siRNA LYAR sequence were cultured in suspension without Tc for different lengths of time (0–14 days) as indicated in Figure 3B. These EBs were continuously cultured for an additional 6 days in the presence or absence of Tc. The analysis of expression levels of differentiation markers indicates that the knockdown of LYAR expression markedly impaired the induction of differentiation markers, including Cdx2 (trophectoderm), Gata6, Brachyury (mesoderm), and Nestin, no matter at which stage Tc was added. It further appeared that the earlier the Tc was added to EBs, the larger was the difference in the gene expression levels between the

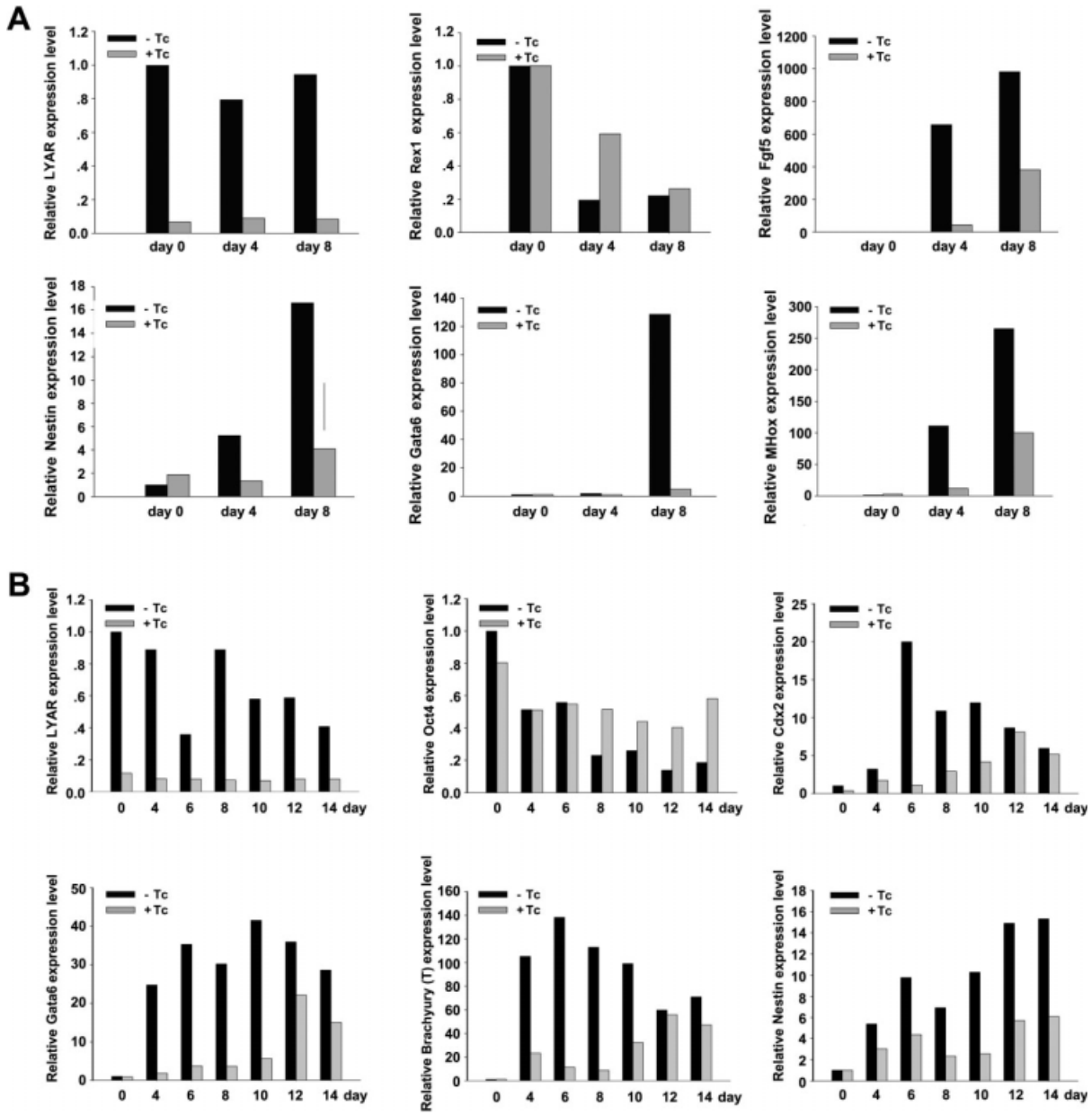

Figure 3. Downregulation of LYAR expression blocks embryonic stem cell (ESC) differentiation. **(A):** Quantitative real-time polymerase chain reaction (qPCR) analysis was performed to estimate the expression levels of the pluripotency marker (*Rex1*) and the differentiation markers (*Fgf5, Nestin, MHox*, *Gata6*) in embryoid bodies (EBs) derived from ESCs of small interference RNA (siRNA) LYAR 1 (clone 21). The cells were pretreated with or without Tc for 2 days and then suspended to form EB for the indicated time in the presence or absence of Tc. The representative results of two independent experiments are shown. The expression level of genes in the cells untreated with Tc at day 0 is defined as 1. **(B):** The knockdown of LYAR at different stages of EB formation affects further ESC differentiation. After the EBs derived from the ESCs of siRNA LYAR 1 (clone 21) were suspended in the absence of Tc for the indicated time, they were treated with or without Tc for additional 6 days. The expression levels of genes were detected using qPCR analysis. The representative results of two independent experiments are shown. Abbreviations: LYAR, Ly-1 antibody reactive clone; Tc, tetracycline.

presence and absence of Tc. On the other hand, the knockdown of LYAR expression blocked further downregulation of the pluripotency marker (Oct4).

To further verify the role of LYAR for ESC differentiation, we performed immunofluorescence staining on EBs attaching to culture dishes. We found that a variety of differentiated cells grew out of control EBs. In contrast, few differentiated cells were observed in EBs expressing siRNA LYAR. Moreover, immunofluorescent signals for antibodies against Gata4 (endoderm), Vimentin (mesoderm), and Nestin (ectoderm) were easily detected in control EBs. However, there were no specific signals in EBs expressing siRNA LYAR (supporting information Fig. S6; data not shown). These results indicate that a proper expression level of LYAR is required for both initiation and maintenance of a normal ES differentiation process.

LYAR Associates with Nucleolin

Having demonstrated that LYAR is an important regulator for ES self-renewal both in vitro and in vivo, we attempted to

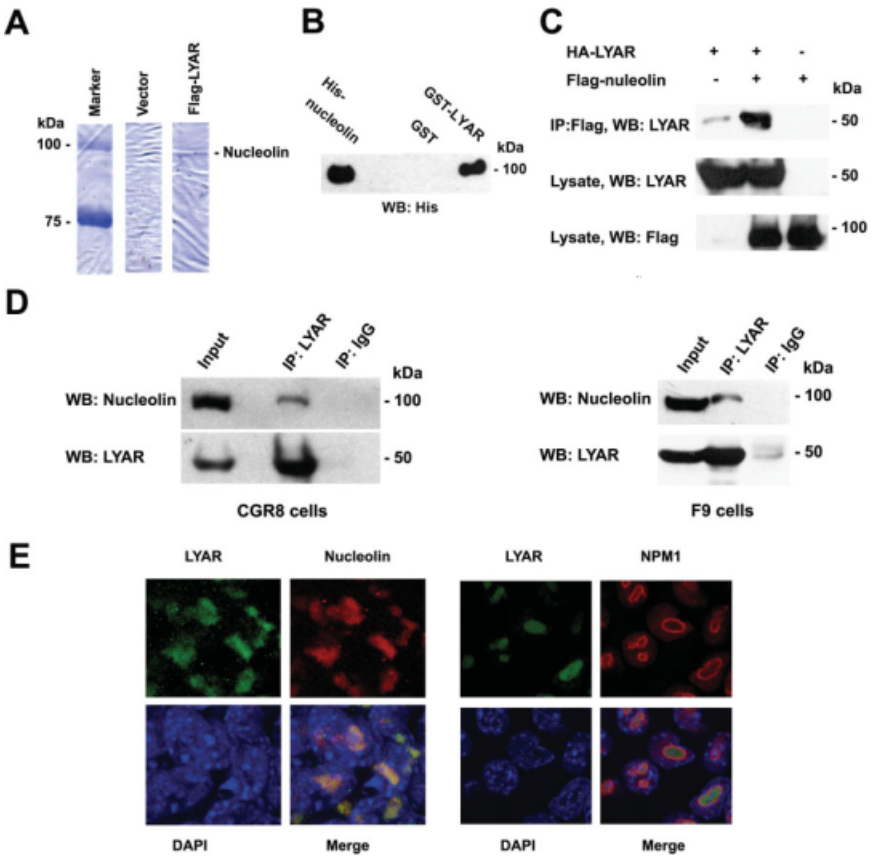

Figure 4. Identification of nucleolin as a LYAR-associated protein. **(A):** Purification by affinity chromatography of nucleolin from the nuclear extract of F9 cells stably expressing Flag-LYAR. Coomassie blue staining of SDS-polyacrylamide gel electrophoresis gel is shown. **(B):** Direct interaction of His-nucleolin with GST-LYAR is determined by GST pull-down experiments. Proteins pulled down by glutathione-sepharose 4B beads were analyzed by WB analysis. **(C):** The interaction between exogenously expressed HA-LYAR and Flag-nucleolin in HEK 293T cells was detected by CoIP and WB analysis. **(D):** The interaction between endogenous LYAR and nucleolin in CGR8 embryonic stem cells (left panel) and F9 embryonic carcinoma cells (right panel) is shown. **(E):** Colocalization of LYAR with nucleolin, but not with NPM1. Blue color represents DAPI staining, green staining represents LYAR protein, and red staining represents nucleolin (1:30) or NPM1 protein. Abbreviations: DAPI, 4,6-diamino-2-phenylindole; GST, glutathione *S*-transferase; HA, hemagglutinin; IP, immunoprecipitation; LYAR, Ly-1 antibody reactive clone; NPM1, nucleophosmin 1; WB, Western blot.

investigate the underlying mechanism, beginning with a search for other proteins associated with LYAR in pluripotent stem cells. To this end, the NE prepared from F9 EC cells stably transfected with Flag-tagged LYAR cDNA sequence was used in affinity chromatography. As shown in Figure 4A, one band at a molecular weight of approximately 100 kDa was found only in the Flag-LYAR NE-containing column, and therefore, it was excised for mass spectrometric analysis, which identified nucleolin, a major nucleolar protein of exponentially growing eukaryotic cells. This protein has been the focus of many studies since its first description 35 years ago [31] because it plays a role in many pathways and functions [17, 32, 33]. Thus, we expected that the exploration of the link between LYAR and nucleolin would provide novel insights into the molecular details of LYAR-mediated functions in ESCs.

The interaction between LYAR and nucleolin was further verified by glutathione *S*-transferase (GST) pull-down assays. As shown in Figure 4B, GST-LYAR fusion protein was able to pull down His-nucleolin, indicating a direct interaction between these two nucleolar proteins. Specificity of the interaction was confirmed by the failure of GST alone to pull down His-nucleolin. Next, the specific interaction between LYAR and nucleolin in mammalian cells was verified by coimmunoprecipitation (CoIP) experiments using lysates of 293T cells transiently expressing HA-tagged LYAR and Flag-tagged nucleolin (Fig. 4C, row 1). Used as a negative control, LYAR was not detected in the immunoprecipitates of cells expressing either Flag-nucleolin or HA-LYAR. Lastly, CoIP experiments were conducted between endogenously expressed LYAR and nucleolin. As shown in Figure 4D, anti-LYAR antibody, but not rabbit control IgG, was capable of coimmunoprecipitating nucleolin from the NE of ES (left panel) and F9 (right panel) cells, demonstrating the existence of endogenous LYAR-nucleolin protein complexes in embryonic pluripotent stem cells.

The nucleolus consists of three main components: the fibrillar centers (FCs), the dense fibrillar component (DFC), and the granular component (GC) [13]. It has been reported that NPM1 is located in GC [13], whereas nucleolin is found mainly in DFC around FCs [34]. We reasoned that if nucleolin was a physiologically relevant partner of LYAR, these two proteins should reside within the same nucleolar components. Therefore, two-color immunofluorescence staining was carried out in CGR8 ESCs. As illustrated in Figure 4E, LYAR staining (green) coincided almost perfectly with the nucleolin staining (red) in the nucleoli. In contrast, NPM1 staining (red) was distributed more peripherally in the nucleoli. Therefore, it is likely that LYAR mainly resides in DFC, as does nucleolin. These data clearly illustrate that LYAR specifically colocalizes with nucleolin within the same nucleolar subregion.

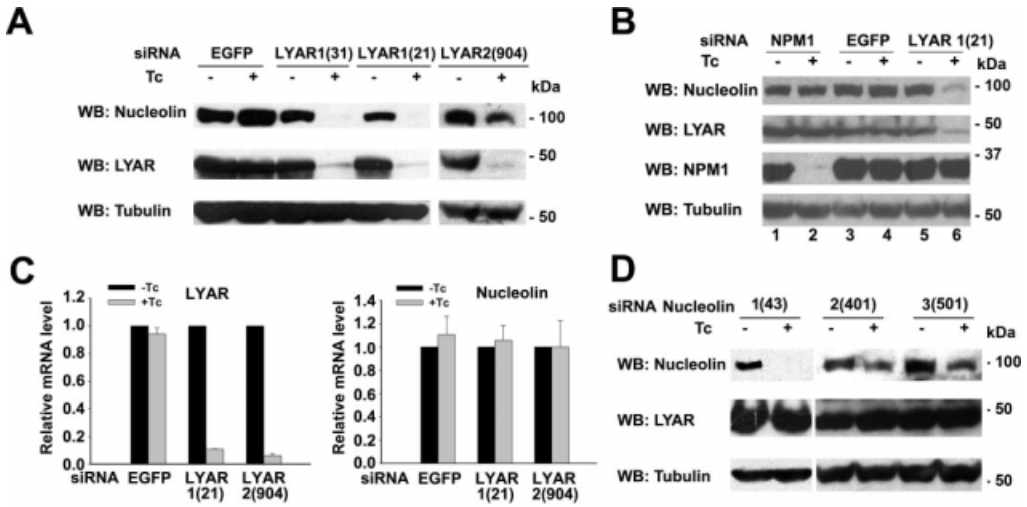

Figure 5. The downregulation of LYAR specifically decreases the steady-state level of nucleolin protein. **(A):** The steady-state level of nucleolin protein decreases when LYAR is knocked down. The cell lysate was collected for WB analysis after treatment with or without Tc for 4 days. **(B):** LYAR does not regulate the steady-state level of NPM1 protein and down-regulation of NPM1 does not affect the protein level of nucleolin. The embryonic stem cells (ESCs) of siRNA NPM1, LYAR1, and EGFP were treated with or without Tc for 4 days before collection. **(C):** LYAR regulates nucleolin at a post-transcriptional level. The mRNA level of *nucleolin* in the ESCs of siRNA EGFP or siRNA LYAR was examined using quantitative real-time polymerase chain reaction. The cells were treated with or without Tc for 4 days before collection. **(D):** The downregulation of nucleolin does not affect the protein level of LYAR. The ESCs of siRNA *nucleolin* 1 (clone 43), siRNA *nucleolin* 2 (clone 401), and siRNA *nucleolin* 3 (clone 501) were treated with or without Tc for 3 days. Abbreviations: EGFP, enhanced green fluorescent protein; LYAR, Ly-1 antibody reactive clone; NPM1, nucleophosmin 1; siRNA, small interference RNA; Tc, tetracycline; WB, Western blot.

LYAR Controls the Steady-State Level of Nucleolin Protein

Given that LYAR and nucleolin form complexes within the same nucleolar component, it became necessary to establish the functional consequence of their association. Thus, we examined the steady-state level of nucleolin protein when LYAR expression was suppressed by its siRNA in ESCs. Strikingly, the endogenous nucleolin protein level substantially declined when LYAR expression was knocked down by both siRNA LYAR 1 and 2 (Fig. 5A). It has been reported that the protein level of nucleolin is cell proliferation dependent [35–37]. This finding led to the question of whether the reduced nucleolin protein level observed in siRNA LYAR-expressing ESCs resulted from reduced cell growth or directly from downregulation of LYAR expression itself. To clarify this issue, we examined the steady-state level of nucleolin protein in the ESC line stably expressing siRNA NPM1, in which we had previously shown that the growth of ESCs significantly slowed when NPM1 was downregulated [20]. Unlike siRNA LYAR, the expression of siRNA NPM1 did not produce any detectable change in the nucleolin protein level, although NPM1 protein level was evidently decreased by Tc treatment (Fig. 5B, lane 2). Notably, the knockdown of LYAR did not alter the NPM1 protein level (lane 6), although nucleolin protein level decreased evidently in the same population of cells. These observations provide clear evidence that the influence of LYAR on the nucleolin protein level is specific and not generalized to nucleolar proteins. Next, we determined whether the regulatory effect of LYAR on the nucleolin protein level occurs at a transcriptional or post-transcriptional level. The qPCR results showed that the knockdown of LYAR did not produce any discernible alteration in the mRNA level of *nucleolin* in the cells expressing either siRNA LYAR 1 or siRNA LYAR 2 (Fig. 5C), indicating that LYAR may regulate the nucleolin level post-transcriptionally. Finally, we examined whether there is a mutual regulation between LYAR and nucleolin in ESCs. Therefore, Tc-inducible ESC lines expressing siRNA *nucleolin* targeting three different regions of *nucleolin* coding sequence (siRNA *nucleolin* 1, siRNA *nucleolin* 2, and siRNA *nucleolin* 3) were generated and the steady-state level of LYAR protein in these cell lines was evaluated. Unlike nucleolin, the LYAR protein level remained steady when the nucleolin protein level was downregulated by Tc treatment (Fig. 5D), revealing that nucleolin did not exert a regulatory effect upon LYAR protein level in ESCs.

Nucleolin Is Indispensable for ESC Growth

Although nucleolin has been implicated in controlling cell growth and apoptosis [33, 34], its role in ESC growth remained unexplored. We predicted that the downregulation of *nucleolin* expression would produce a phenomenon similar to that seen in the knockdown of LYAR in ESCs if the effect of LYAR on ESC growth was primarily mediated through nucleolin. To test this hypothesis, the cell growth rate and apoptosis were examined for ESCs expressing siRNA *nucleolin* in the presence or absence of Tc. As expected, induction of siRNA *nucleolin* expression considerably reduced the cell growth rate in all three cell lines tested (Fig. 6A) and apoptosis was increased (Fig. 6B). Thus, our data demonstrate for the first time that nucleolin is indispensable for normal ESC growth and survival.

LYAR Is a Putative Proteolytic Inhibitor of the Self-Cleavage Activity of Nucleolin

A previous report indicated that the precise control of the level of nucleolin depends upon an enzymatic self-cleavage activity of nucleolin and upon a putative proteolytic inhibitor present only in the nuclei of actively dividing cells to prevent the self-cleavage [36]. Based upon this report and upon our observation that the knockdown of LYAR dramatically

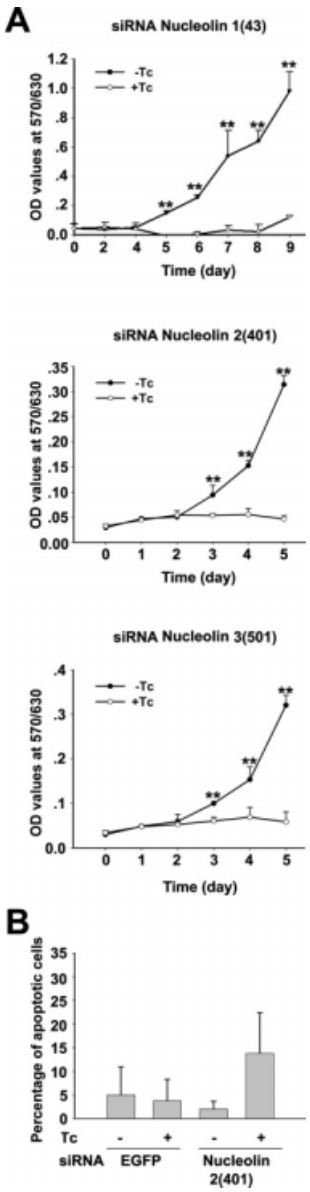

Figure 6. Nucleolin is indispensable for embryonic stem cell (ESC) growth and survival. **(A):** The growth rate of ESCs of siRNA *nucleolin* 1 (clone 43), siRNA *nucleolin* 2 (clone 401), and siRNA *nucleolin* 3 (clone 501) in the presence or absence of tetracycline was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assays. **, $p < .01$; the data are mean values of three independent experiments. **(B):** Cell apoptosis in the ESCs of siRNA EGFP and siRNA *nucleolin* 2 (401) was determined using terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling assays. Abbreviations: EGFP, enhanced green fluorescent protein; OD, optical density; siRNA, small interference RNA.

decreased the steady-state level of nucleolin protein but not its transcript level, we deduced that LYAR could be the proteolytic inhibitor of nucleolin which exists in the undifferentiated ESCs and so we undertook verification of this deduction. In line with a previous work from Chen et al. [36], we found that the major species of His-nucleolin with a molecular weight >100 kDa purified from bacteria was cleaved when incubated at 37°C in vitro for the indicated time; simultaneously, the cleaved fragment of nucleolin accumulated (Fig. 7A). This observation further confirmed that nucleolin is a labile protein and autocatalyzes its own degradation. Notably, the most significant observation from this work is that the addition of GST-LYAR fusion proteins to the incubation mixture apparently prevented the proteolysis of purified His-nucleolin in that the full-length nucleolin became relatively stable and a less cleaved fragment was produced in the presence of GST-LYAR (Fig. 7B, lanes 3, 5, and 7). In contrast, the cleaved fragment was readily detected without LYAR (Fig. 7B, lanes 4, 6, and 8). To further confirm the self-cleavage phenomenon of nucleolin and the inhibitory role of LYAR, Flag-nucleolin was immunoprecipitated from HEK 293T cells transiently expressing Flag-nucleolin and was incubated in vitro. As shown in Figure 7C, a significant portion of full-length Flag-nucleolin was cleaved and multiple cleaved fragments were observed. Again, the presence of GST-LYAR profoundly reduced the self-cleavage of nucleolin (Fig. 7D, lanes 3, 5, and 7), whereas incubation with GST alone could not prevent the cleavage (Fig. 7D, lanes 4, 6, and 8). Therefore, we conclude that LYAR functions to maintain the stability of nucleolin protein and is a candidate for the proliferation-dependent proteolytic inhibitor proposed previously by Chen et al. [36].

Discussion

This study, for the first time, defines a role for the nucleolar protein in controlling ESC self-renewal and differentiation. Our data indicated that the downregulation of nucleolar protein LYAR leads to reduced cell growth rate and increased apoptosis. Furthermore, when ESCs are induced to differentiate in suspension culture, activation of differentiation markers and inactivation of markers of undifferentiated cells are severely impaired by the knockdown of LYAR expression. Recently, Galan-Caridad et al. [38] reported the roles of the transcription factor Zfx in ESC functions. Similar to knockdown of LYAR in ESCs, *Zfx*-deleted ESCs showed increased apoptosis and reduced cell growth. Unlike knockdown of LYAR, however, the differentiation capacity of *Zfx*-deleted ESCs was intact, suggesting that different molecular mechanisms are associated with these two factors, although they are all required for ESC self-renewal. On the other hand, defects in ESC differentiation have been described in several cases of gene knockout models. Although each knockout model has its unique characteristics, they share defects in silencing the stem cell program and in inducing or maintaining differentiation in ESCs. For example, *Dicer*-null ESCs did not express differentiation markers during EB formation and stopped growing after 8 days in suspension culture [39]. Similarly, *Dgcr8*-knockout in ESCs resulted in defective differentiation. Various markers of differentiation cells were either absent or severely reduced in the *Dgcr8* knockout EBs [40]. These observations point to an essential role of microRNA processing in the control of ESC differentiation potential. A similar phenomenon was also reported for *Mbd3*-deficient ESCs, which failed to commit to developmental lineages [41]. In addition, the Dnmt3a and Dnmt3b DNA methyltransferases and the G9a histone methyltransferase have been implicated in the maintenance of differentiation in ESCs after RA treatment [42]. These observations further highlight an important role for epigenetic silencing in the cell fate commitment of ESCs. Here, we demonstrate that the nucleolar protein LYAR is required for proper silencing of pluripotency markers and for induction of markers of differentiated cells in the context of EBs. Our results add not only a new member to the group of global regulators of ESC differentiation but also a new tier to the complex regulatory network of ESC pluripotency.

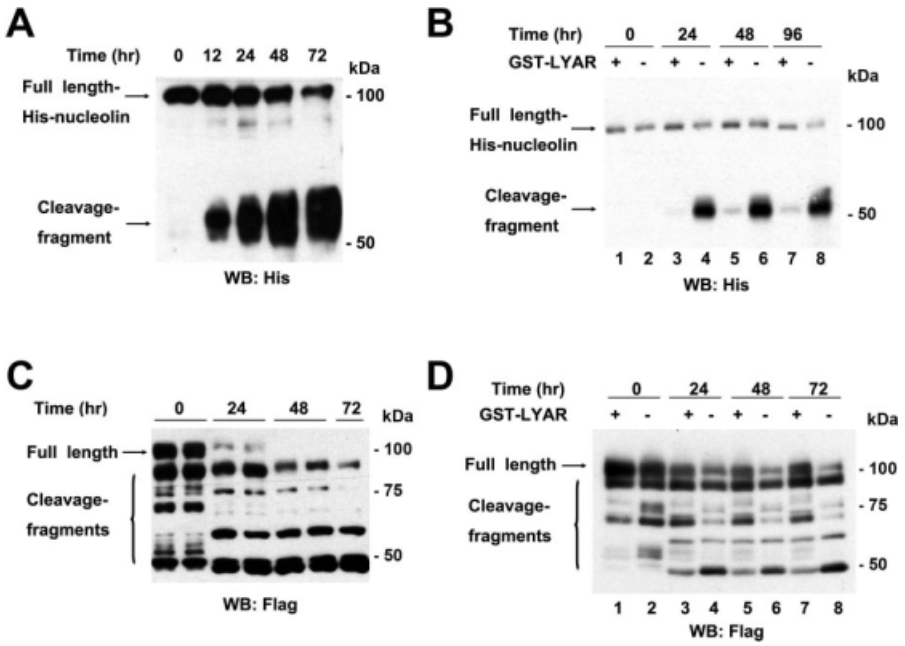

Figure 7. Ly-1 antibody reactive clone (LYAR) exhibits an inhibitory effect on nucleolin autocleavage activity. **(A):** His-nucleolin undergoes autocleavage in vitro. Recombinant His-nucleolin was incubated in vitro at 37°C for the indicated time, and WB analysis revealed the pattern of His-nucleolin autocleavage. **(B):** LYAR inhibits His-nucleolin autocleavage in vitro. His-nucleolin was incubated with GST-LYAR or GST for the indicated time. **(C):** Flag-nucleolin undergoes autocleavage in vitro. Flag-nucleolin purified from HEK 293T cells was incubated for the indicated time. WB analysis revealed the pattern of Flag-nucleolin autocleavage. Duplicate samples were analyzed except for a sample of 72 hours of incubation. **(D):** LYAR inhibits Flag-nucleolin autocleavage in vitro. Flag-nucleolin was incubated with GST-LYAR or GST alone for the indicated time. Abbreviations: GST, glutathione *S*-transferase; hr, hour; WB, Western blot.

Another significant advance produced by this study was the identification of a biochemical, physical, and functional association of LYAR with nucleolin, a major nucleolar protein of rapidly growing cells. We proposed that the reduction in the ability of self-renewal by downregulation of LYAR expression is secondary to the reduced nucleolin protein level, which is in turn required for normal ESC self-renewal. As an abundant multifunctional phosphoprotein of proliferating and cancerous cells, nucleolin is fundamental to the survival and growth of cells. It activates and represses gene transcription as well as regulating RNA metabolism [17, 33–35]. Many new nucleolin-binding proteins that may mediate its functions have been identified in recent years [32]. Although the molecular details determining nucleolin's functions in ESCs are not known, it is possible that nucleolin acts through regulating the expression of genes critical for ESC self-renewal. It was previously reported that nucleolin acts as a subunit of the transcription factor LR1, which activates the expression of *c-myc* gene [43, 44]. Nucleolin is also known to inhibit p53 expression through a post-transcriptional mechanism [45]. Further investigation is in process in our laboratory to elucidate the molecular mechanisms underlying its functions in ESCs.

Nucleolin has been known to have an intrinsic protease activity utilized in its autodegradation. Further investigation suggested that there is a putative proteolytic inhibitor present only in the nuclei of actively dividing cells to prevent the self-cleavage [36, 37]. Given that (a) the steady-state protein level, but not the mRNA level, of nucleolin is controlled by LYAR, (b) LYAR interacts and colocalizes with nucleolin in the nucleoli of undifferentiated ESCs, (c) the expression patterns of these two proteins are parallel during ESC differentiation, and (d) LYAR can efficiently prevent nucleolin autocleavage in vitro, it is conceivable that LYAR may be a putative proteolytic inhibitor existing in undifferentiated ESCs to block nucleolin self-cleavage. Our in vitro study provided direct biochemical evidence that LYAR maintains the stability of nucleolin by preventing its self-cleavage.

The molecular mechanisms underlying the role of LYAR in ESC differentiation remain unclear. It seems unlikely that LYAR controls ESC differentiation through stabilization of nucleolin because the knockdown of *nucleolin* does not block ESC differentiation (Yang et al., unpublished data). The identification of other nucleolar proteins associated with LYAR may help to explain how it controls ESC differentiation. *LYAR* knockout mice are under construction in our laboratory, which will help us to elucidate the specific mechanism for this important nucleolar protein.

Summary

In summary, our work demonstrate an important role for the nucleolar protein LYAR in controlling ESC self-renewal and differentiation. The disclosure of the association between LYAR and nucleolin stability opens up new avenues for our understanding of the molecular control of self-renewal in ESCs and may contribute to the development of safer stem cell therapies.

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Disclosure of Potential Conflicts of Interest

The authors indicate no potential conflicts of interest.

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