We examined the functional implication of nucleolin in the mouse first molar development. Both the nucleolin mRNA and protein expressions were demonstrated in the odontogenic epithelial cells in the early stage and in the inner enamel epithelial layer in the late stage. The expression pattern of nucleolin corresponded to the proliferating cells in the tooth germ, thus showing that nucleolin could possibly be related to cell proliferation. No in situ signal of nucleolin was found in the primary enamel knot (PEK). Furthermore, nucleolin protein was demonstrated in the PEK by immunohistochemistry. The existence of nucleolin protein in the PEK may possibly be related to the apoptosis in the PEK cells. An inhibition assay using the hemagglutinating virus of Japan-liposome containing nucleolin antisense phosphorothioated oligonucleotide (AS S-ODN) in cultured mouse mandibles at embryonic day (E) 11.0 showed a marked growth inhibition of tooth germ. Moreover, no developmental arrest was found in the cultured tooth germ at E15.0 treated with nucleolin AS S-ODN. Real time PCR was performed to examine the mRNA expression of nucleolin-related genes, and a significant reduction in the midkine mRNA expression was thus observed in the mouse mandible after being treated with nucleolin AS S-ODN. This inhibition assay indicated that nucleolin could thus be involved in the early stage of tooth germ initiation and morphogenesis, possibly by regulating the midkine expression.

A multistep and complex process of the gene expressions are involved in the early stage of tooth development (1). There have been many reports regarding the expression of various kinds of genes that are related to tooth morphogenesis (2). However, the precise signaling pathway, which might be related with the initiation, growth, and differentiation of tooth germ, has not yet been fully disclosed.

We previously performed cDNA subtraction between the mandibles of mice at embryonic day (E) 10.5 and 12.0. Thirty five highly expressed positive clones were obtained from the E10.5 mandible by means of colony array screening. 47 highly expressed positive clones were also obtained from the E12.0 mandible (3). We have already reported that the expressions of several genes among them were closely associated with the developing tooth germ (4–6). Nucleolin was one of the highly expressed genes in the E10.5 mandible. Nucleolin is a major nucleolar phosphoprotein that belongs to a large family of RNA-binding proteins (7, 8). Nucleolin is thought to play a role in the pre-rRNA transcription and ribosome assembly that is implicated in the early stage of preribosomal ribonucleoprotein assembly and processing (9, 10). The amount of nucleolin mRNA fluctuates in parallel with the status of DNA synthesis (11). The intact 110-kDa nucleolin molecule is the major species in actively dividing cells, and degraded forms are relatively abundant in nondividing cells (12). In addition, nucleolin has been known to participate in the packaging and shuttling of the ribosome between the nucleus and cytoplasm (13). There have also been numerous reports regarding the functional roles of nucleolin by forming large molecular complexes with other related factors, such as casein kinase (CK) II, c-Myb, midkine (MK), histone H1, nucleophosmin, p53, and protein phosphatase 1 (PP1) (14–20), and then either directly or indirectly playing a role in the regulation of cell proliferation and growth, cytokinesis, replication, embryogenesis, and nucleogenesis (14, 21–24). In addition to the many known functions of nucleolin, it may also function as a low affinity receptor of extracellular growth factor on the cell surface (25).

Here we examined the detailed in situ and immunohistochemical expression patterns of nucleolin in developing tooth germ. To further analyze the functional role of nucleolin, man-
dibles at E11.0 and tooth germs at E15.0 were cultured with nucleolin antisense phosphorothioated oligonucleotide (AS S-ODN) by using the hemagglutinating virus of Japan (HVI)-liposome transfection method. We performed a histological analysis and real time PCR to estimate the effect of the nucleolin AS S-ODN on the formation of tooth germ and the mRNA expression of nucleolin-related gene in nucleolin AS S-ODN-treated tissue specimens, respectively. Nucleolin may be involved in the early stage of tooth germ initiation and morphogenesis, possibly by regulating the MK expression.

**EXPERIMENTAL PROCEDURES**

Animals—Embryos of BALB/c mice at 10.5, 11.0, 12.0, 14.0, 15.0, 16.0, 17.0, and 18.0 days after gestation were used in this study. Adult BALB/c mice were obtained from Charles River Japan Inc. (Yokohama, Japan). All mice were bred in an air-conditioned clean room with a 12:12-h light-dark cycle and were provided standard laboratory food and water *ab libitum*. All manipulations of mice were performed in accordance with the guidelines of the Animal Center of Kyushu University. Female BALB/c mice (10–30 weeks) were caged together with male mice. After 3 h, successful insemination was determined based on the presence of a post-copulatory plug in the vagina, and the embryonic day was defined as E0 after such a plug was recognized.

In Situ Hybridization—The details of the section preparation, the probe labeling, the specificity of the digoxigenin-labeled *in situ* RNA probes, and *in situ* hybridization methods have been shown in our previous studies (4–6). Three embryos at each embryonic day were removed from the pregnant mice under ether anesthesia. The removed embryos were fixed in 4% paraformaldehyde in diethyl pyrocarbonate-treated phosphate-buffered saline (PBS, pH 7.4) for 12 h at 4 °C and embedded in Tissue-Tek OCT (Miles Inc., Elkhart, IN). Serial cryosections were cut at a thickness of 8 μm and then were mounted on silane-coated glass slides for *in situ* hybridization. All sections were washed four times in PBS, for 2 h at room temperature. After rinsing with PBS five times in PBS, the sections were incubated with 4′,6-diamino-2-phenylindole (DAPI, 0.5 μg/ml) (Wako, Osaka, Japan) for 15 min at room temperature. For the negative control, the application of the primary antibody was omitted from the above described procedure. These sections were examined under a fluorescence microscopy Olympus IX71 (OLYMPUS, Tokyo, Japan), and immunofluorescent images were acquired using an Olympus DP70 camera.

**Double Staining for Nucleolin Immunohistochemistry and TUNEL**—The tissue sections were prepared in the same method as mentioned above and double-stained by immunohistochemistry for nucleolin and the terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick-end labeling method (TUNEL) for apoptosis using the *in situ* apoptosis detection kit (Takara Bio Inc., Shiga, Japan). Briefly, the sections were treated with the permeabilization buffer for 5 min at 4 °C. To block nonspecific immunoreaction, the sections were preincubated with 5% chicken serum and 5% donkey serum (Cosmo Bio) in PBS for 30 min, respectively, and then incubated with the C23 antibody (Santa Cruz Biotechnology, diluted 1:200) for 90 min at room temperature. After washing in PBS, the sections were incubated with the mixture of reaction solution containing the terminal deoxynucleotidyltransferase and Alexa 568-labeled anti-goat IgG (Invitrogen) for 90 min at 37 °C. The sections were counterstained with DAPI. In a negative control incubated without the terminal deoxynucleotidyltransferase, no fluorescent products were observed.

Quantitative Real Time PCR—Total RNA was isolated from the mandibles removed from E10.5 and E12.0 mouse embryos and 6-day cultured mandible explants using the SV total RNA isolation system (Promega). The cDNA was prepared by a reverse transcription reaction using the SuperScript III first strand synthesis system (Invitrogen) according to the manufacturer’s instructions. Real time PCR was performed in 10 μl of mixture consisting of 5 μl of SYBR Premix Ex Taq (Takara Bio Inc.) containing TaqDNA polymerase, oligonucleotide primers (0.2 μM each), and 1 μl of template cDNA. The amplification consisted of a two-step procedure as follows: denaturation at 95 °C for 10 s, and then annealing/elongation at 60 °C for 30 s by using ABI PRISM 7000 sequence detection systems (Applied Biosystems, Foster City, CA). The glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene was used as an endogenous control. The primer sequences were designed using the Primer Express software version 1.0 (Applied Biosystems). The gene-specific forward (fw) and reverse (rv) primers for nucleolin and GAPDH were as follows: nucleolin fw, 5′-AAG CAG CAC CTC GGA AAA AAC G-3′, and nucleolin rv, 5′-TCT GAG CCT TCT ACT TTC TGT TTC-3′; and GAPDH fw, 5′-GAA CAT CAT CCC TTC TGA GAT TTG-3′, and GAPDH rv, 5′-CCA GTG AGC TTC CCG CTC A-3′.

**Western Blot Analysis**—A Western blot analysis for the nucleolin level was performed from the cytosolic fraction of the homogenate of E10.5 and E12.0 mandibles and 6-day cultured mandible explants. The tissue specimens were lysed in RIPA buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 1% Triton X-100, 1 mM EDTA, pH 8.0, 0.1% SDS), supplemented with protease inhibitor mixture (50 μM), lactacystin (20 μM),

Implication of Nucleolin in Tooth Development

EXPERIMENTAL PROCEDURES
β-glycerophosphate (25 mM), and sodium orthovanadate (1 mM). Protein samples (20 μg) were separated by 12% SDS-polyacrylamide gel and transferred to Immobilon® polyvinylidene difluoride membrane (Bio-Rad). The membrane was probed with antibody C23 (Santa Cruz Biotechnology) against nucleolin for 1 h at room temperature, and incubated for 1 h with secondary rabbit anti-mouse IgG conjugated with horseradish peroxidase (Amersham Biosciences). The membrane was developed using the enhanced chemiluminescence (ECL) system (Amersham Biosciences). Emitted light was detected using a cooled CCD camera (LAS-1000, Fujifilm, Tokyo, Japan).

Preparation of S-ODN—The HVJ-liposomes were prepared as described elsewhere (26, 27). In brief, egg yolk phosphatidylcholine (Sigma), cholesterol (Sigma), and bovine brain phosphatidylserine (Sigma) were each dissolved in chloroform, mixed in a weight ratio of 1:2:4.8, and then dried with a rotary evaporator (Eyela, Tokyo, Japan). Purified HVJ (kindly donated by Professor Katsuo Sueishi, Kyushu University) adjusted to 15,000 hemagglutinating units/ml was inactivated by ultraviolet light. Thereafter, 10 mg of liposomes mixture was fused with 1 ml of HVJ and 20 nmol of S-ODNs in 6 ml of balanced salt solution (140 mM NaCl, 5.4 mM KCl, 10 mM HEPES, 5 mM MgCl₂, and 0.5% polyvinylidene difluoride membrane (Bio-Rad). The membrane was developed using the enhanced chemiluminescence (ECL) system (Amersham Biosciences). Emitted light was detected using a cooled CCD camera (LAS-1000, Fujifilm, Tokyo, Japan).

TRANSLATION ARREST BY NUCLEOLIN AS S-ODN AND ORGAN CULTURE—For the translation arrest of nucleolin, we designed AS S-ODN as 5'-CTC GGA AGA GCT CAG CCG AA-3', corresponding to nucleotides 118–137 of murine nucleolin (GenBank™ accession number AK031606). The corresponding sense (SE) S-ODN (5'-TTC CGC TGA GCT CTT CCG AG-3') and random sequence (RS) S-ODN (5'-AGT CGA CGC AAG TGC GCG AA-3') were used for the control experiments.

The protocol for the organ culture of the tooth germs was almost identical to that used in our previous studies (28). Briefly, the mandibles and tooth germs of the lower first molar were dissected out from E11.0 and E15.0 embryos, respectively. These explants were supported by a filter (0.8 μm pore size, Millipore, MA) mounted on metal mesh, and then were incubated in the presence of ascorbic acid (Invitrogen), and 100 μg/ml ascorbic acid (Invitrogen), and 100 unit/ml penicillin/streptomycin (Invitrogen) in a 5% CO₂ atmosphere at 37 °C (29). The final concentration used in the culture medium included 1.25 μM each of ODN, 2.4 mg/ml for the HVJ-liposome complex, and 1 mM CaCl₂. The culture media containing HVJ-liposome S-ODNs were changed every 24 h.

Tissue Preparation and Histological Analysis of Cultured Explants—The cultured mandibles and tooth germs were fixed with 4% paraformaldehyde for 12 h at 4 °C at the 2nd, 4th, 5th, 6th, 7th, 8th, and 10th day after cultivation. At least three (up to 14) mandibles and tooth germs explants were used on each culture day for histological analysis. Five-μm-thick sections were cut in an antero-posterior direction, and hematoxylin and eosin double staining was then performed, and the sections were examined under light microscopy.

Measurement of Nucleolin Suppression and Statistical Analysis—The effects of the AS S-ODNs on blocking the translation of nucleolin was confirmed by real time PCR and Western blot analysis. Each experiment was performed independently three times. The density for the blotting signals was measured using NIH Image software version 1.37. Unpaired Student’s t test was used to determine the significance of differences. Differences with a probability value of less than 0.05 were considered to be significant.

Effects of Nucleolin Suppression on Gene Transcription—Real time PCR was performed to estimate the subsequent expression of selected genes after nucleolin transcription was arrested in 6-day cultured E11.0 mandibles. The following primers were used in the present study: MK fw, 5'-CTC TCC CAC AGG CCC AAG A-3', and MK rv, 5'-AGG ACA GGC GTG ATT GAC GAC AGA-3'; CKI-II fw, 5'-CAA TAT GAT GTC AGG GAT TTC TTC A-3', and CKI-II rv, 5'-GCT GAG AGC GGC AAT CAC-3'; histone H1 fw, 5'-TGG GTG AGA ACG CCA ACT C-3', and histone H1 rv, 5'-ACC CCT TTG GT TGC TTG AGA-3'; nucleophosmin fw, 5'-GAA ATG TGT TC-3', and nucleophosmin rv, 5'-GAC GAT CTG TTG C-3', and nucleophosmin rv, 5'-GTC CAT ATC CAT CGA GTC TTC CA-3'; p53 fw, 5'-GCT GGT AGG ATG ATT GAC AGA-3', and p53 rv, 5'-CTG ATG GAC GAT CTG TTG-3'; PP1 fw, 5'-AGT GAC TTG CCC TTC ACT TG-3'; CKII fw, 5'-TCC ACC ACG ATC GAG GCC TTC ACT TG-3'; PKI fw, 5'-GCT GGC AGG GAG CTG CTT TT-3', and PKI rv, 5'-TCC ACC ACG ATC TGA GGT TTG T-3'; c-Myc fw, 5'-ATG CAA AAT GGA GAA ATG TGT TC-3', and c-Myc rv, 5'-TCT ATT GCC CCC TGA CAC AGA-3'.

Quantitative real time PCR was also performed to estimate the expressions of amelogenin (AMGN), ameloblastin (AMBN), dentin sialophosphoprotein (DSP), and dentin matrix protein 1 (DMP-1) after nucleolin transcription was arrested in 6-day cultured E11.0 mandibles. The following primers were used in this study: AMGN fw, 5'-CCC CTT CCC CCA TTG TT, and AMGN rv, 5'-ACT TCT TCC CGC TTG GTC TTG; AMBN fw, 5'-TGC AGG AAG GAG AGC TGA TAG C, and AMBN rv, 5'-CAG GTG TTG GTG TTG TCT GC; DSP fw, 5'-TGG GCA TGA CAG TTA CGA GTT C, and DSP rv, 5'-TGT CGT CAC TCC CGT TAG ATT CG; DMP1 fw, 5'-TGC CTT CCC AGT TGC CAG AT, and DMP1 rv, 5'-GTG GAG CCA GCC ACC AAA TCA TC.

RESULTS

DIFFERENT EXPRESSION OF NUCLEOLIN BETWEEN E10.5 AND E12.0—
Real time PCR and Western blotting analyses were performed to estimate the different expression of nucleolin between the mandibles at E10.5 and E12.0. The expression of nucleolin was 2.6-fold higher for mRNA (p < 0.01) and 1.8-fold higher for protein (p < 0.01) in the mandible at E10.5 than in the mandible at E12.0, and the difference was confirmed to be significant (Fig. 1, A–C).

Expression of Nucleolin mRNA and Protein during Odontogenesis—Membrane hybridization was carried out to confirm the specificity of the digoxigenin-labeled in situ RNA probes. The details of the assay have been described in our previous study (6). The binding activity of antisense nucleolin...
Implication of Nucleolin in Tooth Development

Figure 1. Different expression of nucleolin was detected in E10.5 and E12.0 mandibles. A, real time PCR data were normalized with GAPDH as an endogenous control. The expression of nucleolin mRNA was 2.6-fold higher in the E10.5 mandible than that in E12.0 (*, p < 0.01). B, Western blot assay demonstrated the different expression of nucleolin protein between E10.5 and E12.0 mandibles. C, data analysis of Western blotting showed the expression of nucleolin protein to be significantly higher (1.8-fold) in the mandible of E10.5 than that of E12.0 (*, p < 0.01).

At the initiation stage of E10.5, the expression of nucleolin mRNA and protein in the lower first molar were analyzed by in situ hybridization (Fig. 2A) and immunohistochemical method (Fig. 2B) from the initiation stage (E10.5) to the bell stage (E18.0) in a series of serial sections. Other special terms in the developmental stages of tooth germ have been referred to in previous studies (3–6).

At the initiation stage of E10.5, the expression of nucleolin mRNA was observed in the oral epithelium and mesenchyme at the site where the presumptive molar tooth germ was estimated to be formed. At E12.0, both nucleolin in situ signal and immunofluorescence were detected in the thickening oral epithelium. At the bud stage of E14.0, the expression of nucleolin mRNA and protein was mainly detected in the epithelial cells of the tooth bud. The immunolocalization of nucleolin was demonstrated in the whole enamel organ at the cap stage of E15.0, but no in situ signal was found in the primary enamel knot (PEK). The immunofluorescence reaction of nucleolin protein was observed in the cytoplasm and nucleus as a dot-like appearance (Fig. 2C). At the early bell stage of E16.0, in situ signal and immunofluorescence staining patterns were presented in the inner enamel epithelium, cervical loop, and in part of the outer enamel epithelial cells. The in situ signal and staining patterns of immunofluorescence were restricted in the inner enamel epithelial cells at the subsequent E17.0 and E18.0 (Fig. 2, A and B).

The expression of mRNA thus generally coincided with that of protein. In particular, both mRNA and protein were concomitantly expressed in the early bud stage of E12.0 and the bell stage at E16.0–E18.0 during tooth germ development. How-
ever, several differences were also detected in the expression patterns between mRNA and protein. At the bud stage of E14.0, the expression intensity of protein in the dental papilla appeared stronger than that of mRNA. The expression of mRNA was absent in the PEK in the tooth germ at E15.0; meanwhile, a strong expression of immunoreaction was found in this structure. Double staining for nucleolin protein and TUNEL showed that the fluorescence images of nucleolin and apoptosis were not merged (Fig. 2D).

**Arrest of Nucleolin Translation by AS S-ODN**—Based on the in situ hybridization and immunohistochemistry results for nucleolin, we examined the role of nucleolin in early tooth germ development and in tooth mineralization at the later stage using AS S-ODN in the cultured mandible at E11.0 and tooth germ at E15.0, respectively.

Real time PCR was performed to examine the effect of nucleolin AS S-ODN on mouse E11.0 mandible explants cultured for 6 days by using HVJ-liposome transfection method. After performing a statistical analysis for the nucleolin expressions, more than 30% reduction of nucleolin mRNA expression (Fig. 3A) and 70% reduction of nucleolin protein (Fig. 3, C and D) were found in the nucleolin AS S-ODN-treated samples in comparison with control explants cultured with nucleolin SE or RS S-ODN (Fig. 3A). Regarding the toxicity assay, no significant difference of nucleolin mRNA expression was detected between control samples cultured without ODN and samples cultured with HVJ-liposome containing SE S-ODN, RS S-ODN, or HVJ-liposome only (Fig. 3B).

**Implication of Nucleolin in Tooth Development**

**Arrest of Tooth Germ Development by the Nucleolin AS S-ODN in E11.0 Mandible Culture**—Preliminary experiments of organ culture for mandibles showed normal development of tooth germ to the cap stage by the 8th day of culture. However, no further development of the tooth germ was observed after the 8th day (supplemental Fig. 1A). The mandible explants removed from E11.0 mouse embryos were used and cultured with nucleolin SE S-ODN, RS S-ODN, or without ODNs as controls. After culturing for 8 days, the morphogenesis of the enamel organ could be detected, and most of enamel organs showed normal cap-like tooth germ (Fig. 4A). At 4, 6, and 8 days of culture, the tooth germ in the ODN-untreated mandibles further developed (Fig. 4A), thus demonstrating features of the early and late cap stage. On the other hand, although the bud-like tooth germ treated with AS S-ODN had increased in size at day 6 (Fig. 4A), the epithelial buds were smaller than those treated without ODNs (Fig. 4A). The epithelial bud at 8 days cultured and treated with AS S-ODN showed a similar size in comparison with 6 days of treatment with AS S-ODN and after 4 days without the ODN treatment (Fig. 4A). Almost all of the tooth germs in the mandible treated with AS S-ODN did not show differentiation into the early cap stage (p < 0.05; Table 1). However, some (15%) of the tooth bud developed an early cap stage similar to that in the control mandibles.

**No Effect of Nucleolin AS S-ODN on the Differentiation of Enamel Organ Cells in E15.0 Tooth Germ Culture**—Tooth germs were removed micro-surgically from E15.0 mouse embryos and cultured for periods of up to 10 days. Preliminary experiments of organ culture for tooth germs showed mineralization by the 10th day of culture. However, no further development of the tooth germ was observed after the 10th day (supplemental Fig. 1B). After 6 days of culture, both the tooth germs treated with or without ODNs showed differentiation into the early bell stage (data not shown). The inner enamel epithelial cells were well differentiated to high columnar cells with polarized nucleus. The mesenchymal cells, adjacent to the cells of the inner enamel epithelium, were also differentiated into odontoblasts. After culturing for 8 days, the ameloblasts and odontoblasts became more mature in S-ODN-untreated tooth germ (Fig. 5A), and thin
layers of dentin and enamel matrices were seen in the interface between ameloblastic and odontoblastic layers. The tooth germs cultured with AS, SE, and RS S-ODN also showed a formation of matrices. However, a slight delay in development was observed in comparison with the tooth germs without S-ODN treatment. After 10 days of culture, the untreated tooth germ and those treated with AS, SE, or RS S-ODN showed further development and a normal structure with a typical odontoblastic and ameloblastic differentiation (Fig. 5B). The deposition of enamel and dentin matrices was also detected in both S-ODN-treated and untreated tooth germs (data not shown), thus confirming that the formation of extracellular matrix and the differentiation of ameloblast and odontoblast were not influenced by the treatment of AS-ODN.

Down-regulation of the Transcription of Midkine by the Arrest of Nucleolin Expression—Based on the findings of the nucleolin inhibition assay, we also performed real time PCR on CKII, c-Myb, MK, histone H1, nucleophosmin, p53, and PP1 to

**TABLE 1**
The effects of AS S-ODN on the enamel organ development in cultured mandible

Development of enamel organs at day 8 of culture was significantly inhibited.

| Stages | Untreated | AS | SE | RS |
|--------|-----------|----|----|----|
|        | Bud | Cap | Bud | Cap | Bud | Cap | Bud | Cap |
| Sample no. | 2 (14%) | 12 (86%) | 11 (85%) | 2 (15%) | 2 (25%) | 6 (75%) | 2 (33%) | 4 (67%) |

* Values are p < 0.05.
detect the possible different expressions between AS S-ODN-treated groups and the others. Mouse GAPDH was used as an internal control to standardize the mRNA expression of selected genes. After performing the data analysis, a 50% reduction of MK mRNA expression was detected in AS S-ODN-treated E11.0 mandible after 6 days of culture (*, p < 0.05). Meanwhile, no significant difference was found for CKII, histone H1, c-Myb, p53, PP1, and nucleophosmin (data not shown).

No Down-regulation of the Transcription of AMGN, AMBN, DSPP, and DMP-1 by the Arrest of Nucleolin Expression—The results of real time PCR on AMGN, AMBN, DSPP, and DMP-1 showed no significant decrease of the expression levels of these genes by the treatment of nucleolin AS S-ODN (supplemental Fig. 2).

DISCUSSION

In this study, we described the temporal and spatial expression pattern of nucleolin during the developmental process of the mouse lower first molar, and a possible functional role of this gene in the tooth germ development. This is the first study to show a close relationship between the expression of nucleolin and the development and differentiation of the dental tissue in the mouse mandible.

Strong expressions of nucleolin protein in the early stage of tooth germ development (E12.0–E14.0) were mainly detected in the oral epithelium and thus closely corresponded to the localization of proliferating cells as demonstrated by the 5-bromo-2′-deoxyuridine incorporation method in our previous study (30). A previous report demonstrated that the nucleolin protein expression is coupled to the cellular growth rate, with proliferating cells having a >3-fold higher nucleolin protein levels in comparison to quiescent cells (31). The increased expression of nucleolin mRNA has been reported in the early stage of embryogenesis of Xenopus laevis (23). The increase of nucleolin protein was also demonstrated in the regenerative liver after partial hepatectomy (32). Moreover, nucleolin was definitely implicated in ribosome biogenesis (9, 10). Based on both our findings and those of previous reports, the expression of nucleolin is thus suggested to be closely related to the cell proliferation (33), and as a result, it is thus considered to participate in the tooth germ morphogenesis.

Nucleolin has already been identified as an apoptosis-associated protein involved in different apoptosis process through multiple mechanisms (34). We previously reported that many apoptotic cells and immuno-expression of activated caspase-3 were demonstrated in the PEK cells (30, 35). Therefore, nucleolin protein in the PEK seems to be related to the occurrence of apoptosis in the PEK cells. Nucleolin protein has a molecular mass of 110-kDa when present in the proliferating cells (12), whereas nucleolin protein is cleaved in the apoptotic cells resulting in a molecular mass of 80 kDa (20). The results of double staining for nucleolin protein and TUNEL showed that the fluorescence image of nucleolin and apoptosis had not merged, and therefore it was difficult to decide whether the nucleolin protein in the PEK was intact or degraded and whether the presence of nucleolin protein in the PEK was the result of the apoptosis or the cause of the apoptosis. Based on our present data, it is also difficult to speculate that the nucleolin protein in the PEK actually participated in the occurrence of apoptosis in the PEK. Further examinations are thus required to elucidate this issue.

The in situ expression patterns of nucleolin closely corresponded to the localization of protein detected by the immuno-fluorescence method. However, there were several differences between the expression of mRNA and localization of protein. The reasons why these differences occurred are still unknown. Interestingly, nucleolin mRNA was absent in the PEK, whereas the protein was strongly expressed in this area. A previous report demonstrated the half-life of nucleolin mRNA to be 3.2 h in peripheral blood mononuclear cells stimulated by phorbol 12-myristate 13-acetate (12), whereas the nucleolin protein was maintained after 3 days in normal prostate cells exposed to cycloheximide, which inhibited the translation (36). It therefore seems likely that the nucleolin protein in the PEK thus be a residue of previously formed protein (before E15.0) in the tooth germ.

An inhibition assay for nucleolin expression in cultured mandibles at E11.0 revealed an arrest of tooth germ development. Furthermore, the expression of MK mRNA was also found to be markedly decreased by nucleolin translation arrest. This result may indicate that the nucleolin is related to the direct or indirect regulation of the transcription of the MK gene. MK is a multifunctional heparin-binding growth factor expressed in various cell types during embryogenesis (37). Enamel organ formation and cell differentiation were both inhibited in cultured tooth germ by adding the neutralizing antibodies for MK to the culture media (38), thus showing the MK to be an indispensable factor for tooth germ development. Previous reports indicated that the cell surface localized nucleolin proteins act as the receptor of MK (16, 25). No other functional role of nucleolin on the transcriptional regulation of MK has been reported. There has so far been no report describing the interaction of nucleolin and MK during the signal transduction and gene expression. However, our present study showed that the reduction of MK mRNA level because of the suppression of nucleolin transcription by the application of nucleolin antisense probe
Implication of Nucleolin in Tooth Development

was about 50%. This phenomenon seems to be caused by following several reasons as follows: 1) transcriptional reduction of nucleolin was not complete even by the antisense probe application, probably because the nucleolin was a ubiquitously expressed gene in cells; 2) it is unknown whether the transcriptional regulation of MK by nucleolin is direct or indirect, and there may be another transcriptional pathway for the MK expression besides nucleolin (39, 40); 3) translational disturbance of nucleolin induced by adding antisense probe may influence the stabilization of MK mRNA as shown in previous reports (41, 42). It therefore seems important to disclose the molecular interaction between nucleolin and MK in the cellular biology of tooth germ development.

The nucleolin mRNA and protein were concomitantly expressed in the inner enamel epithelial layer at the bell stage, thus suggesting that this gene is related to the proliferation, differentiation, and function of ameloblast. However, nucleolin translation arrest by adding AS-ODN in the culture media did not show any effects on the differentiation of ameloblast and hard tissue formation by ameloblast and odontoblast. The results of reverse transcription-PCR regarding the expression of AMBN, AMGN, DSPP, and DMP-1 did not show any significant differences after the treatment of nucleolin AS-ODN in the cultured tooth germ at E15.0. These results may indicate that nucleolin plays no effective role in the differentiation of ameloblasts and the odontoblasts.

Recently, nucleolin was reported to be related to the expression of laminin and MMP-9 (43, 44). Both laminin and MMP-9 are known to be related to the modulation of basement membrane between the dental epithelium and the underlying dental mesenchyme, and it thus plays a role in the matrix formation of enamel and dentin (45, 46). Although the function of nucleolin in the ameloblasts is unknown at present, nucleolin may also be related to the modulation of basement membrane. Further investigations are needed to disclose the function of the nucleolin in the ameloblast.

In conclusion, this study proves that the nucleolin is a ubiquitously expressed gene in cells; 2) it is unknown whether the transcriptional regulation of MK by nucleolin is direct or indirect, and there may be another transcriptional pathway for the MK expression besides nucleolin (39, 40); 3) translational disturbance of nucleolin induced by adding antisense probe may influence the stabilization of MK mRNA as shown in previous reports (41, 42). It therefore seems important to disclose the molecular interaction between nucleolin and MK in the cellular biology of tooth germ development.

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