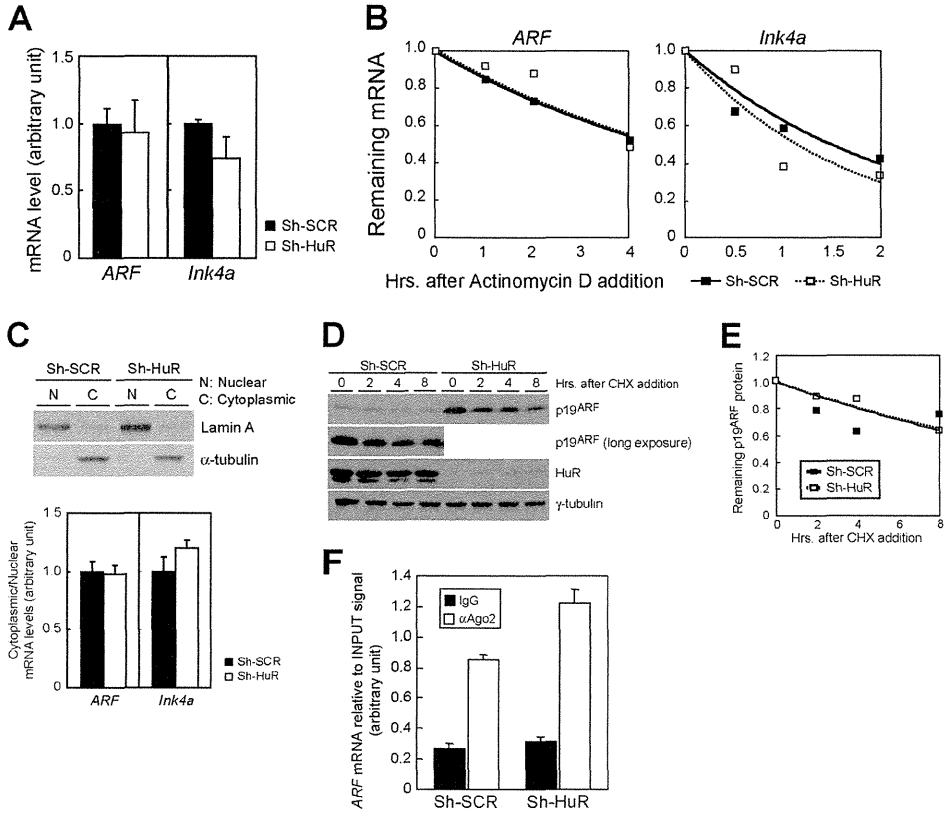

FIG 4 HuR does not affect *ARF* mRNA transcription, stability, nuclear export, or $p19^{ARF}$ turnover. (A) The expression of *ARF* and *Ink4a* mRNA in control and sh-HuR MEFs was analyzed by real-time PCR. mRNA in each sample was normalized to 18S rRNA. (B) Control or sh-HuR retrovirus-infected MEFs were treated with actinomycin D for the indicated periods. Total RNA was extracted at each time point, and *ARF* and *Ink4a* mRNA levels relative to the 18S rRNA level were analyzed by real-time PCR. (C) Nuclear and cytoplasmic fractions were prepared from control and sh-HuR MEFs. (Upper blot) Samples were analyzed by immunoblotting using Lamin (for the nuclear marker) and α-tubulin (for the cytoplasmic marker) antibodies. (Lower blot) RNA was isolated from these fractions. *ARF* and *Ink4a* mRNA levels were quantified by real-time PCR and normalized to the 18S rRNA level in each fraction, and the ratios of cytoplasmic mRNA to nuclear mRNA were determined. (D) Wild-type MEFs infected with control or sh-HuR retroviruses were treated with cycloheximide (CHX) for the indicated periods. Cell lysates were prepared, and $p19^{ARF}$ levels were analyzed by immunoblotting. (E) The intensity of the $p19^{ARF}$ band in each sample was determined using ImageJ and plotted. (F) HuR does not affect Ago2 association with *ARF* mRNA in mouse cells. Lysates of MEFs expressing sh-SCR or sh-HuR were immunoprecipitated using Ago2 or a control antibody. RNA recovered from the immune complex was analyzed using real-time PCR. Error bars represent SEM of results from triplicate samples.

HuR does not affect ARF mRNA or protein stability. The above results indicate that, unlike in HDFs, HuR regulates the expression of $p19^{ARF}$ but not of $p16^{Ink4a}$ in MEFs. To gain insights into how HuR regulates $p19^{ARF}$ expression, we first compared *ARF* mRNA levels in control and sh-HuR MEFs. Real-time PCR analysis revealed no increase in *ARF* or *Ink4a* mRNA levels in the presence or absence of HuR (Fig. 4A), implying that HuR was not involved in the transcriptional regulation of these genes. We next checked whether HuR could affect the stability of these mRNAs. Cells were treated with actinomycin D to block *de novo* mRNA synthesis, and the remaining mRNA was chased by real-time PCR. Although HuR has been shown to negatively regulate *Ink4a* mRNA stability in human fibroblasts (25), there was no significant difference in the levels of stability of *ARF* mRNA in MEFs (Fig. 4B). Likewise, we observed no difference in the ratios of cytoplasmic to nuclear *ARF* and *Ink4a* mRNA between these cells; therefore, it is unlikely that HuR regulates the nuclear export of these mRNAs (Fig. 4C). We also compared levels of protein stability in these cells with a cycloheximide chase but did not observe changes in $p19^{ARF}$ stability (Fig. 4D and E).

In human cells, HuR has been shown to destabilize *Ink4a* mRNA by recruiting RISC to it (25). We therefore wished to determine if this was also the case with *ARF* regulation in MEFs. Lysates from sh-SCR and sh-HuR MEFs were immunoprecipitated using Ago2 antibodies, and RNAs recovered from immune complexes were subjected to real-time PCR analysis for *ARF*. *ARF* mRNA was enriched in the Ago2 immune complex from sh-SCR cells, suggesting that RISC is also involved in *ARF* mRNA regulation (Fig. 4F). Nonetheless, we did not observe any decrease in the RISC-*ARF* mRNA interaction in HuR-depleted cells. Thus, unlike in human cells, RISC is not involved in HuR-mediated *ARF* mRNA regulation.

HuR translationally regulates $p19^{ARF}$ expression. Next, we checked the possibility that HuR affects the translation of *ARF* mRNA since it has been well established that HuR regulates the translation of its target mRNAs (13, 40). MEFs were infected with GFP or GFP fused to ribosomal protein L10a (GFP-L10a) together with sh-SCR or sh-HuR retroviruses. Cytoplasmic lysates were immunoprecipitated using GFP antibody to purify ribosome-mRNA complexes (32, 42). Immunoblotting confirmed that GFP-

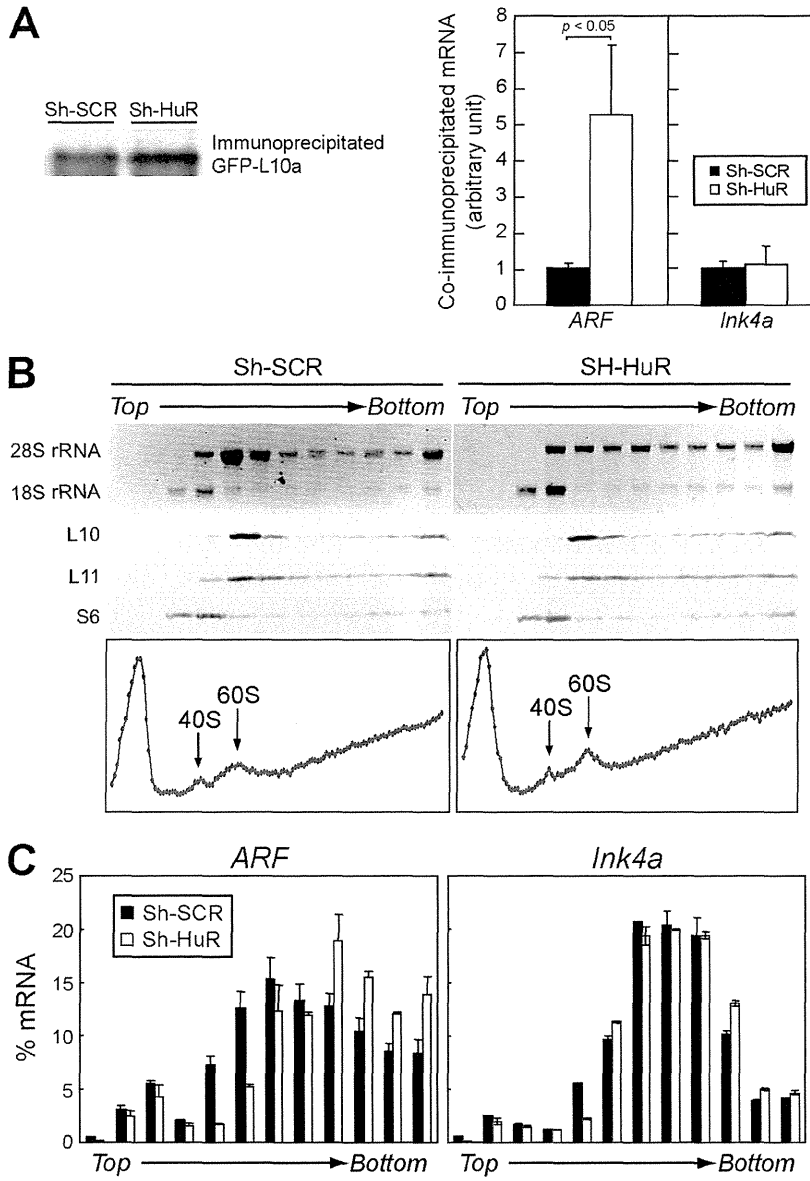

FIG 5 HuR regulates the translation of *ARF* mRNA. (A) Wild-type MEFs were infected with sh-SCR or sh-HuR retroviruses together with GFP or GFP-L10 retroviruses. Cytoplasmic lysates were immunoprecipitated using GFP antibody. Immunoblotting using GFP antibody indicated that equal amounts of GFP-L10 protein were immunoprecipitated. RNAs were extracted from immune complexes and subjected to real-time PCR analysis. Amounts of *ARF* or *Ink4a* mRNA in each sample were normalized to 18S rRNA in the complex. Data are representative of three independent experiments. Error bars represent SEM of results from triplicate samples. (B) Cytoplasmic lysates prepared from MEFs infected with sh-SCR or sh-HuR retroviruses were fractionated by sucrose density gradient centrifugation. Samples were manually separated into 120 fractions, and the relative values of optical densities at 254 nm were plotted (graphs). Ten fractions were pooled, and 28S and 18S rRNAs and ribosomal proteins (L10, L11, and S6) were visualized by ethidium bromide staining and immunoblotting, respectively. (C) The amount of *ARF* or *Ink4a* mRNA in each fraction was analyzed using real-time PCR.

L10a proteins were specifically enriched in immunoprecipitated complexes, and equivalent amounts of GFP-L10a were obtained from control and sh-HuR cells (Fig. 5A). RNAs were then recovered from immune complexes and subjected to real-time PCR analysis. *ARF* mRNA was significantly enriched in the ribosome complex in sh-HuR cells; the amount of ribosome-associated *ARF* mRNA was more than five times higher than that of the control, while no change in ribosome association with *Ink4a* mRNA was observed under these conditions. To further validate the *ARF* mRNA-ribosome association, cytoplasmic lysates were fractionated into polysome/nonpolysome fractions by sucrose gradient sedimentation (Fig. 5B). RNAs were recovered from each fraction, and *ARF* and *Ink4a* mRNAs were analyzed by real-time PCR. As in the GFP-L10a immunoprecipitation experiment (Fig. 5A), we observed more *ARF* mRNA in the polysome fractions of HuR-depleted cells than in the nonpolysome fractions (Fig. 5C). Together, these results indicate that HuR specifically represses p19^{ARF} expression by inhibiting mRNA-ribosome association.

We next sought to investigate if HuR affects *ARF* mRNA localization. To this end, *ARF* mRNA, including both its 5′- and its

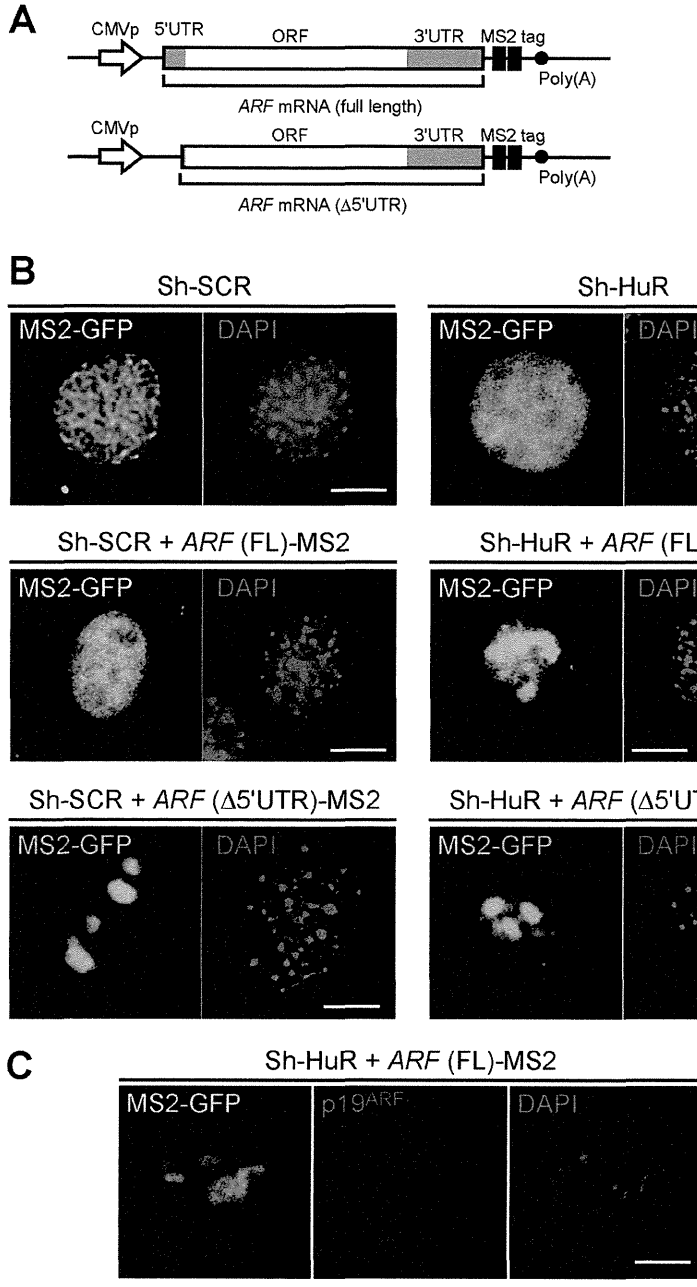

FIG 6 Loss of HuR relocalizes *ARF* mRNA to nucleoli. (A) DNA constructs for the expression of MS2-tagged *ARF* mRNA (full length or Δ5′UTR). CMVp, cytomegalovirus promoter. (B) Sh-SCR- or sh-HuR-expressing *ARF p53* DKO cells were transiently transfected with MS2-GFP-NLS plasmids. Where indicated, cells were cotransfected with MS2-tagged *ARF* (full length or Δ5′UTR)-expressing plasmids. Three days later, cells were fixed in paraformaldehyde and stained with DAPI. Bars, 10 μm (C) Sh-HuR-expressing *ARF p53* DKO cells were transiently transfected with MS2-GFP-NLS together with MS2-tagged *ARF* (full length)-expressing plasmids. Cells were stained using $p19^{ARF}$ antibody and DAPI.

3′UTR, was conjugated to tandem MS2-binding sequences (MS2 tag in Fig. 6A) (43) and coexpressed with MS2-EGFP fusion protein with a nuclear localization signal (MS2-EGFP-NLS) in *ARF* and *p53* double-knockout (DKO) MEFs expressing sh-SCR or sh-HuR (30). In the absence of *ARF* mRNA, the GFP signal was observed only in the nucleus, irrespective of HuR status (Fig. 6B). In cells expressing MS2-tagged *ARF* mRNA, the GFP signal was also observed in the nucleus, indicating that the majority of *ARF* mRNA remains in the nucleus. Interestingly, we observed that *ARF* mRNA (full length) specifically accumulated in a subnuclear compartment when HuR was depleted. This subnuclear compartment represented nucleoli, since the GFP signal colocalized with $p19^{ARF}$ (Fig. 6C). Thus, HuR also regulates the nuclear trafficking of *ARF* mRNA, which may contribute to translational regulation (44).

HuR associates with ARF mRNA in living cells. Since our re-

sults suggest that HuR regulates the translation of *ARF* mRNA, we next sought to determine if HuR associates with *ARF* mRNA. Wild-type MEFs were infected with control (GFP) or HA-HuR expression retroviruses, and HA-HuR complexes were immunoprecipitated using HA antibody. As previously reported, β-*actin*, c-*myc*, and *p53* mRNAs were specifically enriched in HuR complexes (Fig. 7A) (16, 20, 26). Under these conditions, we could not detect *ARF* mRNA in the HuR immune complex. We performed similar experiments with endogenous HuR proteins and failed to detect binding of HuR to *ARF* mRNA (Fig. 7B). We next employed a UV cross-linking and immunoprecipitation (CLIP) assay, which is a more sensitive method to detect protein-RNA interaction. MEFs were irradiated with UV to covalently cross-link protein-RNA complexes prior to lysate preparation and immunoprecipitated using HuR or control antibodies. Although we could not detect the HuR and *ARF* mRNA interaction with the standard RNA immunoprecipitation protocol (Fig. 7A and B), *ARF* mRNA was slightly enriched in HuR immune complexes following UV cross-linking (Fig. 7C). Hence, it is likely that *ARF* mRNAs form an extremely fragile or transient complex in living cells, unlike other HuR ligands.

HuR regulates the translation of ARF mRNA through its 5′UTR. To find the region responsible for HuR in *ARF* mRNA, we expressed exogenous *ARF* mRNA that included the open reading frame (ORF) and both the 5′- and 3′UTRs (full-length *ARF*), the ORF and 5′UTR (Δ3′UTR *ARF*), or the ORF and 3′UTR (Δ5′UTR *ARF*) in NIH 3T3 cells (*ARF* and *Ink4a* null) expressing sh-SCR or sh-HuR. These cells expressed comparable amounts of exogenous *ARF* mRNA (Fig. 8A). Under these conditions, $p19^{ARF}$ levels were increased in the absence of HuR expression, and this effect was more prominent in full-length *ARF* mRNA cells and in Δ3′UTR cells than in Δ5′UTR cells (Fig. 8B). $p19^{ARF}$ expression from Δ5′UTR *ARF* mRNA was also slightly increased in HuR-depleted cells. However, this likely reflects the larger amount of *ARF* mRNA in these cells (Fig. 8A), since the effect of HuR knockdown was diminished when $p19^{ARF}$ levels were normalized to *ARF* mRNA levels in each sample (Fig. 8C). Consistently with the above results, we observed more ribosome association with full-length and Δ3′UTR *ARF* mRNAs than with Δ5′UTR mRNA in the absence of HuR (Fig. 8D). Furthermore, CLIP analysis revealed that the 5′UTR is required for HuR association (Fig. 8E). Together, these results strongly suggest that HuR regulates the translation of *ARF* mRNA through its 5′UTR. Consistently with this notion, *ARF* mRNA localized to nucleoli irrespective of HuR status when the 5′UTR was deleted (Fig. 6B, lowest panels). However, this region by itself did not respond to HuR when it was conjugated to the luciferase reporter (data not shown), suggesting that the ORF region also contributes to regulation or that there are more-stringent requirements for the RNA secondary structure. We also performed similar experiments using full-length *Ink4a* mRNA or *Ink4a* mRNA lacking both the 5′ and 3′UTRs (ORF). Consistently with the above results indicating that HuR does not increase $p16^{Ink4a}$ levels in MEFs and that *ARF* mRNA does not share the 5′UTR with *Ink4a*, knockdown of HuR did not affect $p16^{Ink4a}$ expression from these mRNAs, further confirming that the effect of HuR is specific to *ARF* in this locus (Fig. 9).

Nucleolin interacts with ARF mRNA in nucleoli and is required for $p19^{ARF}$ expression in HuR knockdown cells. We next sought a possible mediator of $p19^{ARF}$ expression in HuR knockdown cells. The nucleolar RNA-binding protein nucleolin has

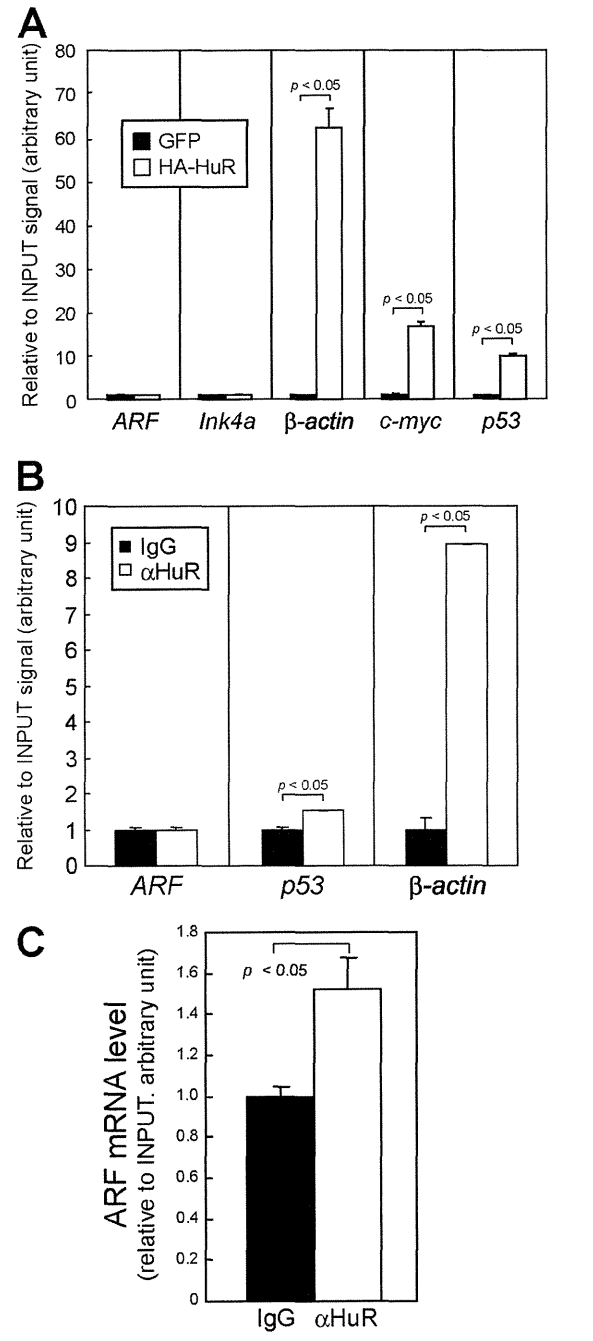

FIG 7 HuR weakly associates with *ARF* mRNA in living cells. (A) Lysates of (wild-type) MEFs expressing HA-tagged HuR proteins were immunoprecipitated using HA antibody. RNA extracted from the immune complex was analyzed by real-time PCR. Error bars represent SEM ($n = 3$). (B) Lysates prepared from wild-type MEFs were immunoprecipitated using control (IgG) or HuR antibodies. RNAs were extracted from immune complexes and subjected to real-time PCR analysis. *p53*, c-*myc*, and β-*actin* were used as positive controls. (C) Lysates were prepared from UV-cross-linked MEFs and immunoprecipitated using control or HuR antibodies. RNAs were recovered from the immune complex following proteinase K treatment and analyzed by real-time PCR for *ARF* mRNA levels. Error bars represent SEM of results from triplicate samples.

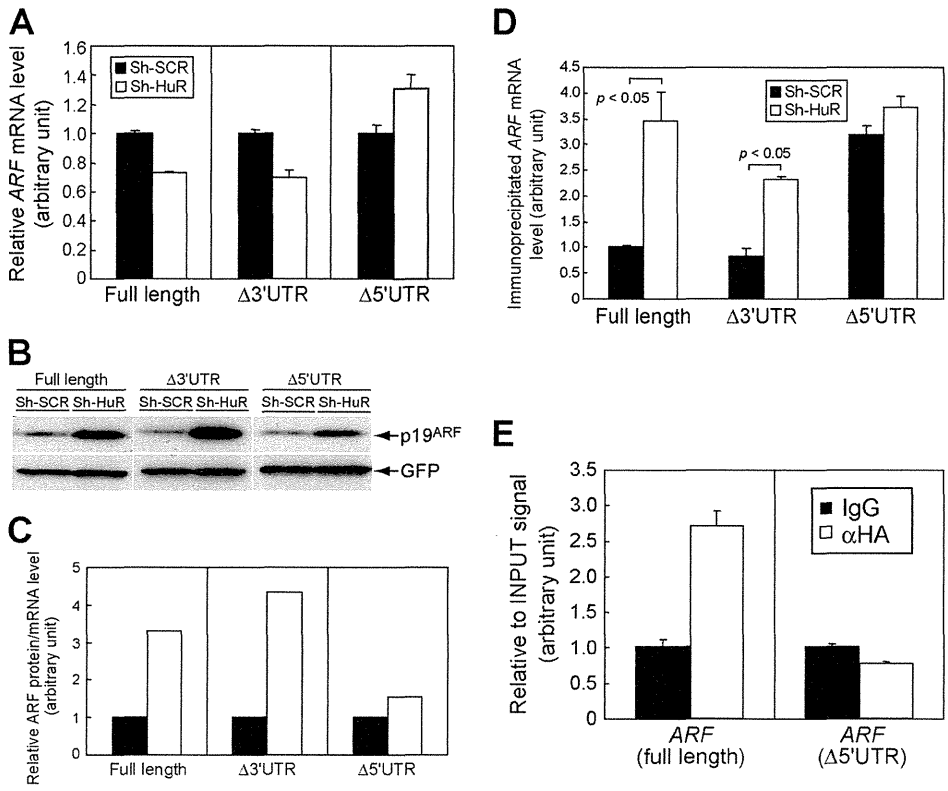

FIG 8 HuR regulates $p19^{ARF}$ expression through the 5′UTR of *ARF* mRNA. (A) NIH 3T3 cells expressing sh-SCR or sh-HuR were transfected with plasmids bearing full-length *ARF*, including the 5′- and 3′UTRs (full length), *ARF* lacking the 3′UTR (Δ3′UTR), or *ARF* lacking the 5′UTR (Δ5′UTR) together with GFP expression plasmids. Three days later, total RNA was extracted and exogenous *ARF* expression was analyzed by real-time PCR. Values were normalized to *GFP* mRNA levels in each sample. (B) The cells from panel A were analyzed by immunoblotting for expression of $p19^{ARF}$ and GFP. (C) $p19^{ARF}$ levels in panel B were quantified using ImageJ, and the $p19^{ARF}$ level and *ARF* mRNA level in each sample were calculated. (D) NIH 3T3 cells expressing sh-SCR or sh-HuR were transfected with *ARF* expression plasmids (full length, Δ3′UTR, or Δ5′UTR) together with GFP-L10 plasmids. Three days later, cytoplasmic lysates were prepared and immunoprecipitated using GFP antibody to purify RNA-protein complexes, including GFP-L10. RNAs were recovered from immune complexes and subjected to real-time PCR analysis for *ARF* mRNA. Values were normalized to input signals in each sample. (E) 293T cells were transfected with *ARF* expression plasmids that express full-length or mutant *ARF* mRNA that lacks the 5′UTR (Δ5′UTR) together with HA-HuR expression plasmids. Forty-eight hours later, cells were subjected to UV cross-linking and immunoprecipitated using control or HA antibodies. Recovered RNA was analyzed by real-time PCR. Error bars represent SEM of results from triplicate samples.

been shown to bind to several mRNAs involved in the cellular stress response, and the binding of nucleolin enhances the translation of their target mRNAs (45). Moreover, microarray analysis of mRNA in the nucleolin complex has revealed that *CDKN2A* (*Ink4a* and *ARF*) mRNA physically associates with nucleolin in HeLa cells (45). Because *ARF* mRNA localized to nucleoli upon HuR depletion, we tested if nucleolin interacts with the nucleolar *ARF* mRNA in HuR knockdown cells. While no *ARF* mRNA was detected in the nucleolin complex of control cells, it was significantly enriched in the absence of HuR expression (Fig. 10A). The interaction of nucleolin with *ARF* mRNA does not require 5′UTR; therefore, relocalization of *ARF* mRNA to the nucleolus seems sufficient for the interaction (Fig. 10B). Thus, HuR impedes the nucleolar localization of *ARF* mRNA by binding to its 5′UTR, thereby inhibiting the interaction of *ARF* mRNA with nucleolin. Next, we examined whether nucleolin is required for $p19^{ARF}$ expression in HuR knockdown cells. For this purpose, siRNA targeting *nucleolin* mRNA was transfected into sh-SCR- or sh-HuR-expressing MEFs. Although the effect of siRNA on nucleolin level was limited, $p19^{ARF}$ induction was suppressed to basal levels in HuR knockdown cells (Fig. 10C), suggesting that nucleolin is required for $p19^{ARF}$ induction in HuR-depleted cells.

Loss of HuR inhibits adipocytic differentiation in a $p19^{ARF}$-dependent manner. A recent report by Minamino and colleagues has shown that senescence in adipose tissue results in decreased insulin sensitivity, thereby leading to type 2 diabetes mellitus (46). Hence, we investigated whether HuR-mediated $p19^{ARF}$ regulation had any effect on adipocyte function. To this end, we first tested whether loss of HuR could affect adipocyte differentiation *in vitro*. Wild-type MEFs expressing sh-SCR or sh-HuR were differentiated into adipocytes in the presence of insulin, dexamethasone, and 3-isobutyl-1-methylxanthine (IBMX). Oil Red O staining revealed that HuR depletion suppressed adipocytic differentiation in wild-type MEFs (Fig. 11A). It has been reported that HuR directly binds to *C/EBPβ* mRNA to regulate its expression (47). We therefore checked if HuR depletion affected the expression of genes required for adipocyte differentiation. As shown in Fig. 11B, C/EBPβ expression was slightly diminished in HuR-depleted cells. Nonetheless, levels of expression of its downstream C/EBPα and PPARγ genes were still comparable to those in control cells, suggesting that defects in adipogenesis in the absence of HuR were

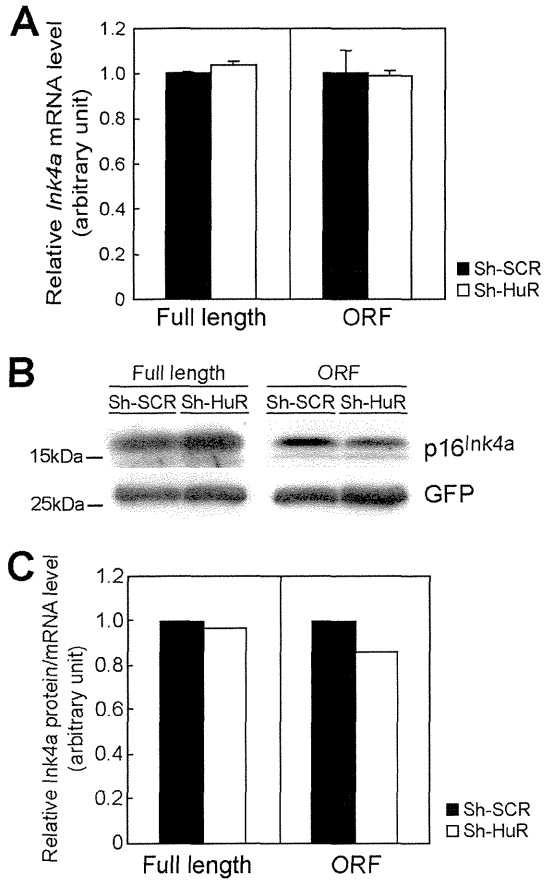

FIG 9 HuR does not affect *Ink4a* translation. (A) NIH 3T3 cells expressing sh-SCR or sh-HuR were transfected with plasmids bearing full-length *Ink4a*, including its 5′- and 3′UTRs (full length), or *Ink4a* lacking its 5′- and 3′UTRs (ORF) together with GFP expression plasmids. Three days later, RNAs were extracted and the expression of exogenous *Ink4a* mRNA was analyzed by real-time PCR. Values were normalized to *GFP* expression levels in each sample. (B) The expression of $p16^{Ink4a}$ and GFP was analyzed by immunoblotting. (C) $p16^{Ink4a}$ levels were quantified and normalized to *Ink4a* expression levels. Error bars represent SEM of results from triplicate samples.

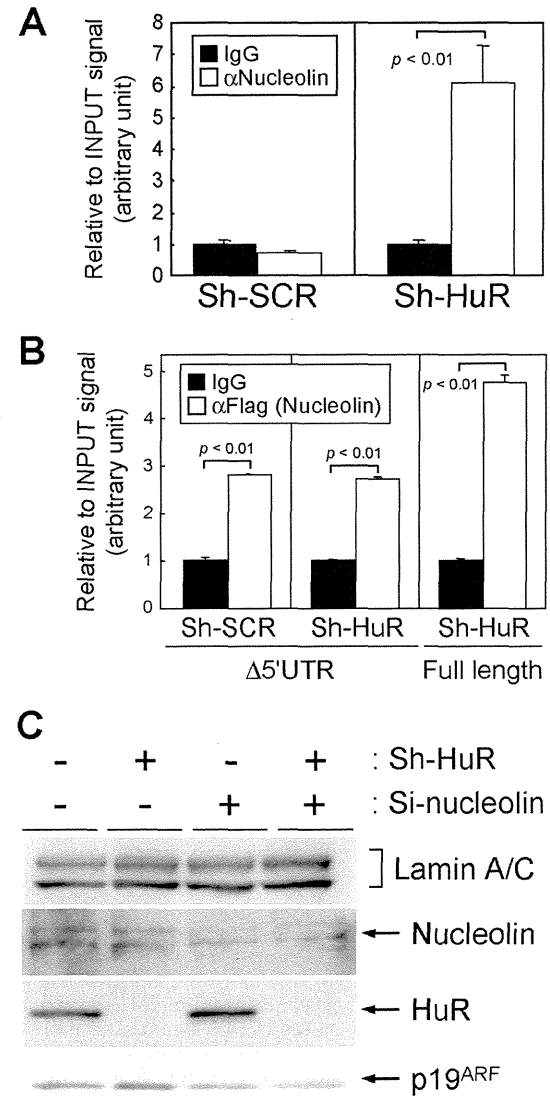

FIG 10 Nucleolin associates with *ARF* mRNA and mediates the $p19^{ARF}$ induction in HuR knockdown cells. (A) Lysates prepared from MEFs expressing sh-SCR or sh-HuR were immunoprecipitated using control (IgG) or nucleolin antibodies. RNAs were recovered from the immune complexes and analyzed by real-time PCR. (B) NIH 3T3 cells expressing sh-SCR or sh-HuR were transiently transfected with *ARF* (Δ5′UTR or full length)-expressing plasmids together with Flag-tagged nucleolin-expressing plasmids. Two days later, lysates were prepared and immunoprecipitated using control or Flag tag (M2) antibodies. RNAs in the immune complexes were analyzed by real-time PCR. Error bars represents SEM of results from triplicate samples. (C) MEFs (P2) expressing sh-SCR or sh-HuR were transfected with siRNA targeting *nucleolin*. Two days later, lysates were prepared and the expression of the indicated proteins was analyzed by immunoblotting. Lamin A/C was used as a loading control.

not attributed to altered expression of adipocyte-related genes. We then checked whether the adipocyte phenotype was dependent on *ARF*. In sharp contrast, HuR knockdown had virtually no effect on adipocyte differentiation in *ARF* knockout MEFs (Fig. 11C).

Given that $p19^{ARF}$ activates the p53-dependent cell cycle checkpoint, we were prompted to check the possibility that $p19^{ARF}$ affects clonal expansion during the initial stage of adipogenesis. Cells were stimulated to differentiate and were pulse-labeled with 5-ethynil-2′-deoxyuridine (EdU) to assess cell cycle reentry. EdU staining showed a significant reduction in cell cycle reentry in HuR-depleted wild-type MEFs (Fig. 11D). In contrast, S-phase entry was not affected by sh-HuR in the absence of *ARF* (Fig. 11E). These results suggest that defective adipogenesis in HuR-depleted cells can be attributed to $p19^{ARF}$-dependent cell cycle arrest or senescence.

Adipose-specific HuR knockout accelerates age-dependent insulin resistance. Our above results indicated that the loss of HuR enhanced the translation of *ARF* mRNA, thus inducing $p19^{ARF}$-dependent cellular senescence, and that HuR may affect adipocyte function through $p19^{ARF}$. To explore the impact of HuR-mediated translational regulation of the *ARF* gene *in vivo*, we generated adipose tissue-specific *HuR* knockout mice ($HuR^{fl/fl}$; AP2-CRE) (Fig. 12A and B) (10). *ARF* mRNA levels were low in the adipose tissue of young animals (1 to 3 months old) of both genotypes but significantly increased in older animals (6 to 9 months old) (Fig. 12C), which is consistent with previous reports

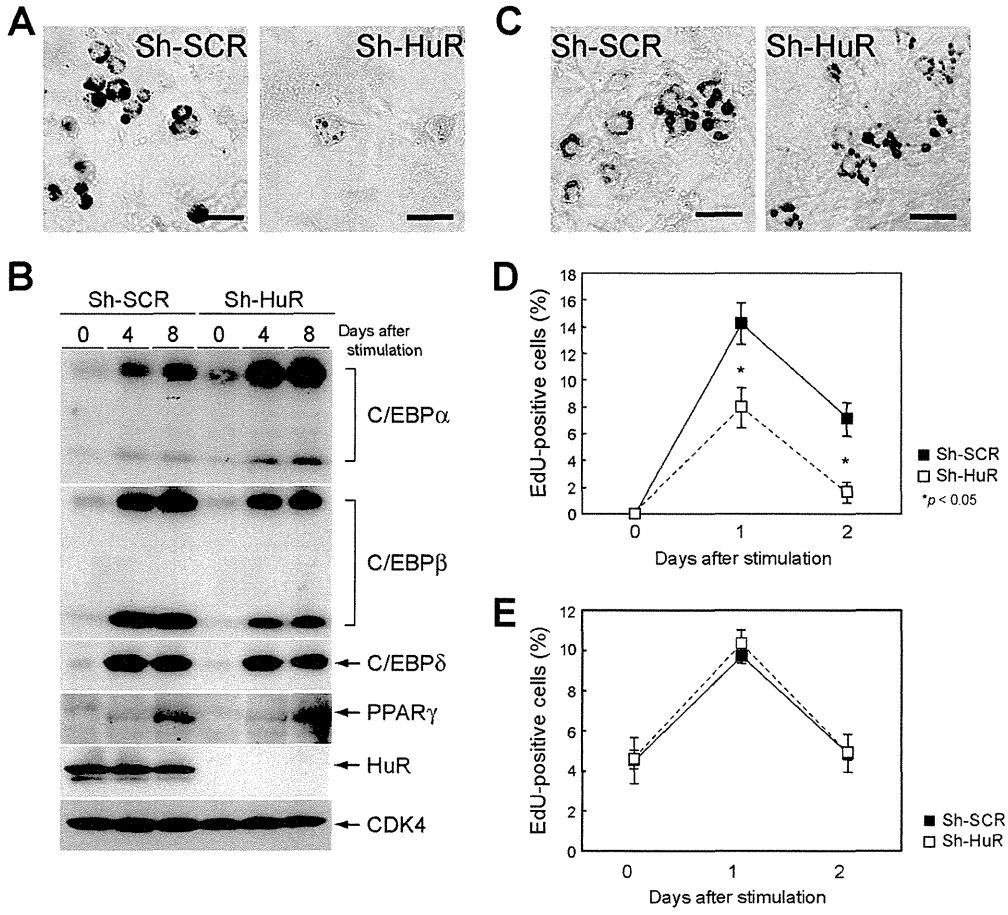

FIG 11 Adipogenesis is impaired in HuR-depleted wild-type MEF. (A) Wild-type MEFs were infected with sh-SCR or sh-HuR retroviruses. Selected cells were stimulated to differentiate them into adipocytes in the presence of insulin, 3-isobutyl-1-methylxanthine (IBMX), and dexamethasone for 10 days and stained with Oil Red O. Bars, 50 μm. (B) Wild-type MEFs with sh-SCR or sh-HuR were cultured in adipocyte differentiation medium for the indicated periods. The expression of C/EBPα, -β, -δ, and PPARγ were analyzed by immunoblotting. (C) *ARF*-null MEFs with sh-SCR or sh-HuR were stimulated to differentiate them for 10 days and stained with Oil Red O. Bars, 50 μm. (D and E) Wild-type (D) and *ARF*-null (E) MEFs expressing sh-SCR or sh-HuR were stimulated to differentiate them for 0, 1, and 2 days. Cells were pulse-labeled with EdU for 45 min and stained for EdU. EdU-positive and -negative cells in microscopic fields were counted. Data are representative of two independent experiments. Error bars represent SEM of results from five microscopic fields.

indicating that *ARF* expression increases in many tissues as animals age (48). $p19^{ARF}$ was still hardly detectable in the adipose tissue of older $HuR^{fl/fl}$ mice (Fig. 12D). However, we detected $p19^{ARF}$ in a certain population of older $HuR^{fl/fl}$; AP2-CRE mouse adipose tissue (Fig. 12E and F). Changes in SA-β-Gal activity were difficult to detect; however, *PAI-1* levels were significantly increased in *HuR* knockout adipose tissue (Fig. 12G). We subsequently tested if HuR loss in adipose tissue affected insulin-mediated glucose homeostasis, which is one of the major functions of this tissue. There was little difference in insulin sensitivity among both genotypes when animals were at a young age; however, in older animals, adipose-specific *HuR* deletion significantly accelerated insulin resistance (Fig. 13A). Similar results were obtained by the glucose tolerance test (Fig. 13B). So far, we have not been able to confirm that this effect is *ARF* dependent, because *ARF*-null animals develop tumors by this age (49). However, the timing of the onset of insulin resistance correlates well with that of $p19^{ARF}$ appearance in adipose tissue. Hence, these results suggest that HuR is required to repress $p19^{ARF}$ expression in adipose tissue, thereby inhibiting adipose senescence, which can lead to insulin resistance.

DISCUSSION

Our data show that HuR is downregulated in senescent mouse fibroblasts and that decreases in HuR contribute to senescence-associated growth arrest. It has been shown that HuR levels decline in human diploid fibroblasts during cellular senescence (22); therefore, the role of HuR in cellular senescence is likely to be evolutionally conserved. How HuR expression is controlled during senescence is unclear, but in human cells, it is attributed, at least in part, to increased expression of miR-519 during senescence (24). Whether HuR is regulated by miRNA in mouse senescence is unknown, but we did not observe a significant change in *HuR* mRNA levels in senescent MEFs (data not shown). Therefore, such posttranscriptional regulation may also contribute to the control of HuR levels in mouse cells.

Although HuR is implicated in senescence in both human and mouse cells, the mechanisms underlying them are different. The

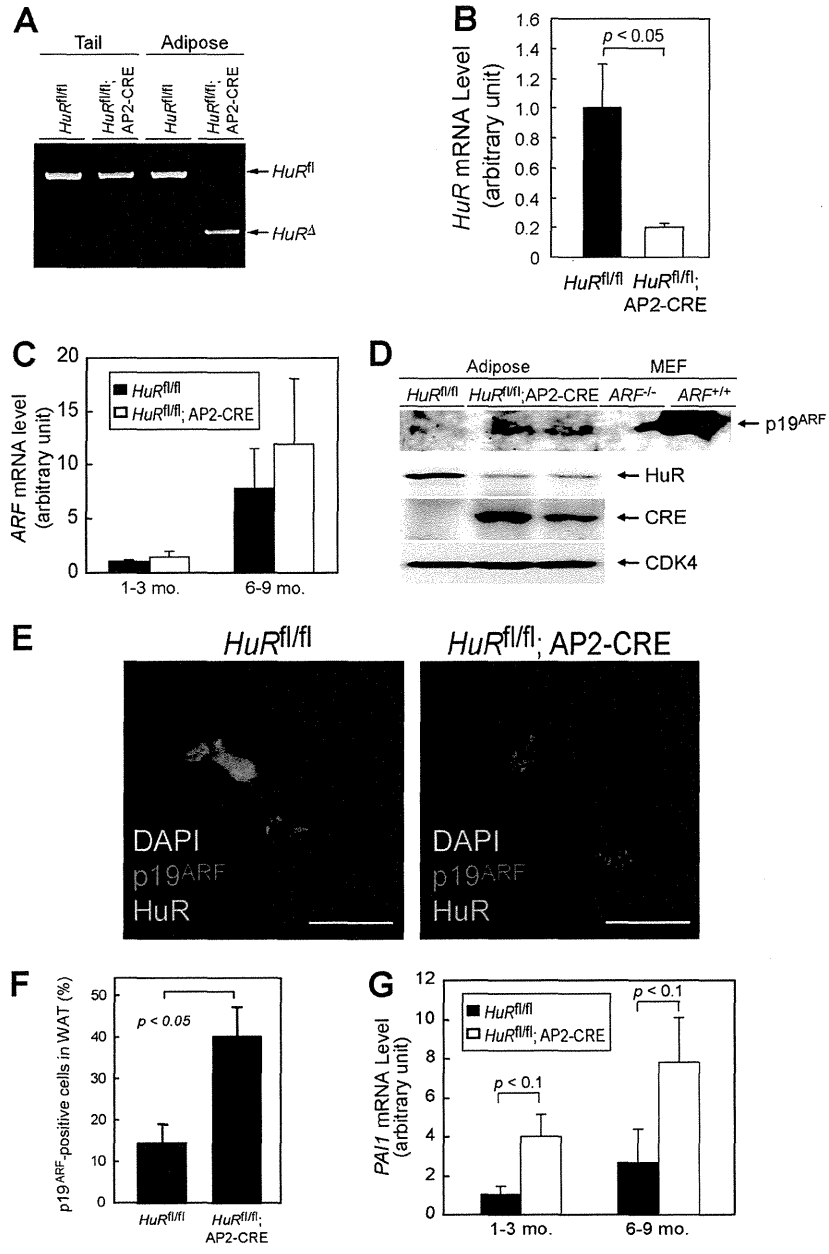

FIG 12 Adipose-specific HuR deletion accelerates the senescence of adipocytes. (A) Genotyping of adipose- and tail-derived genomic DNA. (B) *HuR* mRNA levels in adipose tissue from mice with the indicated genotypes were analyzed by real-time PCR. Values were normalized to 18S rRNA in each sample. (C) *ARF* mRNA was analyzed by real-time PCR. (D) Lysates were prepared from the adipose tissue of $HuR^{fl/fl}$ and $HuR^{fl/fl}$; AP2-CRE mice. Expression of the indicated proteins was analyzed by immunoblotting. CDK4 was used as a loading control. Testis lysate from $ARF^{-/-}$ and $ARF^{+/+}$ animals was used as the negative and positive controls for $p19^{ARF}$, respectively. (E) Frozen sections of adipose tissue of $HuR^{fl/fl}$ and $HuR^{fl/fl}$; AP2-CRE mice (9 months old) were immunostained using $p19^{ARF}$ and HuR antibodies. Sections were counterstained with DAPI. Bars, 20 μm. (F) Rates of $p19^{ARF}$-positive cells in panel E were plotted. WAT, white adipose tissue. (G) *PAI-1* mRNA levels were analyzed using real-time PCR.

$p16^{Ink4a}$-Rb pathway plays pivotal roles in cell cycle arrest during cellular senescence in human cells. In contrast, it has been well established that the $p19^{ARF}$-p53 pathway is essential and that $p16^{Ink4a}$ is dispensable in the senescence of mouse cells. Consistently with these concepts, our results show that loss of HuR leads to increased expression of $p19^{ARF}$, but not $p16^{Ink4a}$, levels in MEFs. We further demonstrated that senescence caused by HuR loss can be abrogated by either *ARF* or *p53* deletion. Therefore, under normal conditions in which cells express sufficient amounts of HuR protein, $p19^{ARF}$ expression is suppressed, thereby protecting cells from undergoing p53-dependent replicative senescence. Additionally, it has been proposed that HuR positively regulates the expression of Mdm2, which is a major E3 ligase for p53 protein, and is negatively regulated by $p19^{ARF}$ (50). Thus, HuR suppresses p53 activity by modulating the expression of multiple targets integrated into the p53 pathway. Although we do not formally ex-

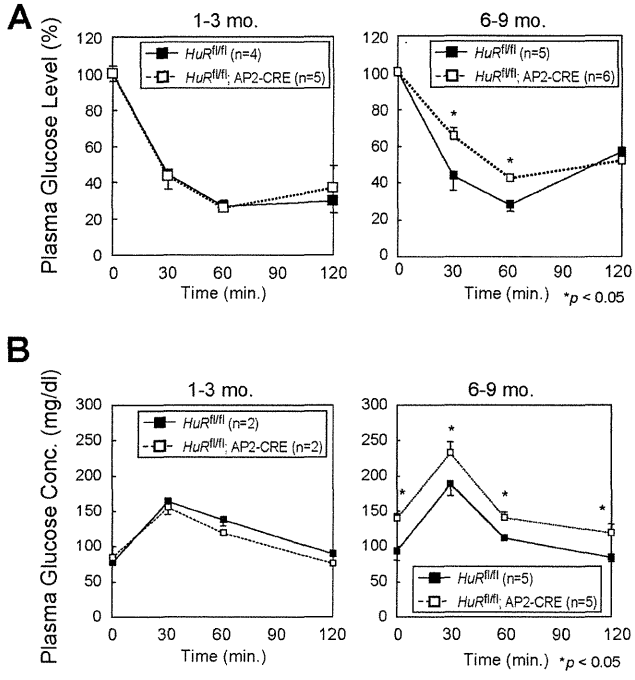

FIG 13 Adipose-specific HuR deletion accelerates age-dependent insulin resistance. Insulin tolerance tests (A) and glucose tolerance tests (B) were performed with $HuR^{fl/fl}$ and $HuR^{fl/fl}$; AP2-CRE mice.

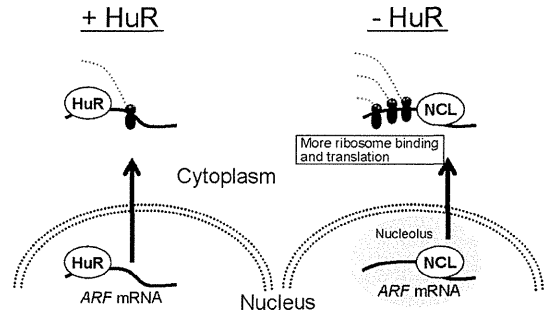

FIG 14 Model for *ARF* regulation by HuR and nucleolin. In the presence of HuR, *ARF* mRNA binds to HuR through the 5′UTR, and the mRNA localizes mainly to the nucleoplasm. The HuR-bound *ARF* mRNA is less efficiently translated. In the absence of HuR, *ARF* mRNA localizes to the nucleolus, where it associates with nucleolin (NCL). The nucleolin association facilitates ribosome binding, thereby enhancing the translation.

clude the possibility that $p16^{Ink4a}$ is also involved, it is conceivable that the $p19^{ARF}$-p53 pathway is a major target of HuR to control the life span of mouse cells.

In human cells, HuR directly associates with ARE in the 3′UTR of *Ink4a* mRNA (25). This region is shared by *ARF* mRNA; therefore, it is possible that HuR also regulates $p14^{ARF}$ expression in human cells. Unlike in human cells, in MEFs, the loss of HuR has no influence on $p16^{Ink4a}$ levels, while $p19^{ARF}$ is increased. Consistently with these results, HuR associates with the 5′UTR of *ARF* mRNA, which is not shared by *Ink4a*. However, the interaction of HuR with *ARF* mRNA is weak and observed only after UV-mediated cross-linking. Therefore, it is likely that HuR forms a much more fragile complex with *ARF* mRNA than with other mRNAs. Alternatively, the effect of HuR may be indirect; HuR may target another factor(s) that regulates $p19^{ARF}$ expression. In this regard, it is worthy of note that HuR regulates the translation of β-*catenin* and *Jun-B* mRNAs by modifying the stability of linc-p21 RNA (51). This could be clarified by identifying *ARF* mRNA-interacting molecules. Additionally, HuR has been proposed to recruit RISC to human *Ink4a* mRNA independently of miRNA, thereby destabilizing it (25). Although the involvement of miRNA needs to be further clarified, it is possible that RISC-mediated regulation may also be involved in mice, since deletion of *dicer-1* causes $p19^{ARF}$-p53-dependent cellular senescence (52). Furthermore, we detected *ARF* mRNA in the Ago2 complex. Nonetheless, the interaction of Ago2 and *ARF* mRNA was not decreased upon HuR depletion, implying that RISC is not involved in HuR-mediated *ARF* regulation.

HuR exclusively affects translation in $p19^{ARF}$ expression. Loss of HuR enhances ribosome association with *ARF* mRNA. This translational activation is associated with the nucleolar accumulation of *ARF* mRNA. We found that *ARF* mRNA associates with nucleolin, which is required for $p19^{ARF}$ induction in HuR-depleted cells. Nucleolin associates with numerous mRNAs and shuttles between the nucleolus and the cytoplasm. The influences of nucleolin on target mRNA differ depending on the target transcript. A recent report by Abdelmohsen and colleagues demonstrated that nucleolin is required for ribosome binding and subsequent translation of its target mRNA (45). Consistently with our results, they also observed *CDKN2A*, as well as both *ARF* and *Ink4a*, among the nucleolin-associated mRNAs. Together with these observations, our data suggest that HuR-associated *ARF* mRNA is retained in the nucleoplasm and is not efficiently translated upon nuclear export (Fig. 14). However, in the absence of HuR, *ARF* mRNA localizes to the nucleolus, where it associates with nucleolin. As nucleolin enhances ribosome recruitment to its target mRNA (45), $p19^{ARF}$ synthesis is increased under these conditions. Interestingly, *p53* mRNA also accumulates in the nucleoli upon DNA damage, when *p53* mRNA translation is increased (44). Hence, nucleolar localization of mRNA may reflect general aspects of stress-dependent mRNA translation. It has recently been shown by Miceli and colleagues that oncogenic Ras activates the transcription of the *ARF* gene, as well as the translation of *ARF* mRNA through mTORC1 (53). In this context, it is noteworthy that mTORC1 activity can affect the binding of HuR to *ornithine decarboxylase* mRNA (54). Therefore, it would be interesting to see if mTORC1 and HuR cooperate in *ARF* mRNA regulation.

Cellular senescence is known to be involved in metabolic disorders as well as cancers. Among these, senescence in adipose tissue is associated with insulin resistance (46). HuR has also been shown to function in adipocytes by regulating C/EBPβ expression (55, 56). Our results reveal that, although C/EBPβ may be affected by HuR status, it has little effect on adipogenesis, which is consistent with a previous report that *C/EBPβ*-null MEFs are capable of undergoing adipogenesis (57). Instead, the function of HuR in adipogenesis depends largely on *ARF*, as HuR knockdown had virtually no effect on adipogenesis in *ARF*-null MEF or 3T3-L1 cells, in which the p53 pathway was inactivated by *mdm2* amplification (Fig. 11 and data not shown) (58). Impaired adipogenesis is observed with concomitant reductions in clonal expansion during the initial stage of adipogenesis, which is alleviated in an *ARF*-null background. Hence, it is likely that adipogenic failure in HuR-depleted MEFs is attributed largely to $p19^{ARF}$. There were no ab-

normalities in the shapes and sizes of adipose tissues in adipose-specific *HuR* knockout mice. However, these mice revealed progressive insulin resistance with age. The reason why this phenotype was not observed in young animals can be explained by differences in the levels of *ARF* mRNA among these animals. In young mice, increased translation by HuR loss does not lead to expression of sufficient amounts of p19^{ARF} because of low *ARF* mRNA levels. However, in older animals, larger amounts of *ARF* mRNA and an increased rate of translation synergistically induced p19^{ARF} in adipose tissue. SA-β-Gal activity was not as strong as in cultured cells, and we failed to quantitatively detect an increase in enzyme activity. Nonetheless, the senescence program is likely activated in those cells, since *PAI-1* was significantly induced. It should be further clarified whether the phenotype is completely dependent on p19^{ARF} or whether other adipocyte-related factors are involved. Also, it would be interesting to see if HuR is linked to metabolic disorders, such as type 2 diabetes mellitus. In this regard, it is noteworthy that there was strong linkage between the human *ARF* and *Ink4a* loci and the disease (59–61).

HuR is deregulated in many types of cancers (40), and there is no doubt that cellular senescence is a central tumor-suppressive mechanism in mammals. Hence, it is plausible that deregulated HuR activity and expression leads to an uncontrolled senescence program, thereby allowing cells to bypass senescence. This can be achieved by suppressing the activity of the p16^{Ink4a}-Rb pathway and the p19^{ARF}-p53 pathway in humans and mice, respectively. Moreover, HuR is downregulated in aged human tissues, which may contribute to an age-associated phenotype, such as decreased insulin sensitivity. Our data demonstrate a novel function of HuR in the maintenance of the cellular replicative life span and will lead to further understanding of the mechanism and biological roles of cellular senescence.

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We declare that we have no conflict of interest.

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Brief report

Somatic mosaicism for oncogenic *NRAS* mutations in juvenile myelomonocytic leukemia

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Juvenile myelomonocytic leukemia (JMML) is a rare pediatric myeloid neoplasm characterized by excessive proliferation of myelomonocytic cells. Somatic mutations in genes involved in GM-CSF signal transduction, such as *NRAS*, *KRAS*, *PTPN11*, *NF1*, and *CBL*, have been identified in more than 70% of children with JMML. In the present study, we report 2 patients with somatic mosaicism for oncogenic *NRAS* mutations (G12D and G12S) associated with the development of JMML. The mutated allele frequencies quantified by pyrosequencing were various and ranged from 3%-50% in BM and other somatic cells (ie, buccal smear cells, hair bulbs, or nails). Both patients experienced spontaneous improvement of clinical symptoms and leukocytosis due to JMML without hematopoietic stem cell transplantation. These patients are the first reported to have somatic mosaicism for oncogenic *NRAS* mutations. The clinical course of these patients suggests that *NRAS* mosaicism may be associated with a mild disease phenotype in JMML. (*Blood*. 2012;120(7):1485-1488)

Introduction

Juvenile myelomonocytic leukemia (JMML) is a rare myeloid neoplasm characterized by excessive proliferation of myelomonocytic cells. Somatic mutations in genes involved in GM-CSF signal transduction, such as *NRAS*, *KRAS*, *PTPN11*, *NF1*, and *CBL*, have been identified in more than 70% of children with JMML.[1-3] The term "somatic mosaicism" is defined as the presence of multiple populations of cells with distinct genotypes in one person whose developmental lineages trace back to a single fertilized egg.[4] Somatic mosaicism of various genes, including some oncogenes, has been implicated in many diseases. For example, somatic mosaicism for *HRAS* mutations is found in patients with Costello syndrome.[5-7] Whereas germline mutations in causative genes (ie, *PTPN11*, *NRAS*, *NF1*, and *CBL*) are found in JMML patients,[3,8-11] the presence of somatic mosaicism for these genes has never been reported. In the present study, we describe 2 cases of JMML in which the patients display somatic mosaicism for oncogenic *NRAS* mutations (G12D and G12S).

Study design

Written informed consent for sample collection was obtained from the patients' parents in accordance with the Declaration of Helsinki, and molecular analysis of the mutational status was approved by the ethics committee of the Nagoya University Graduate School of Medicine (Nagoya, Japan).

Patient 1. A 10-month-old boy had hepatosplenomegaly and leukocytosis (72.1×10^9/L) with monocytosis (13.3×10^9/L; Table 1). The patient's BM contained 7% blasts with myeloid hyperplasia. Cytogenetic analysis revealed a normal karyotype and colony assay of BM mononuclear cells (BM-MNCs) showed spontaneous colony formation but GM-CSF hypersensitivity assay was not tested. The diagnostic criteria for JMML, as developed by the European Working Group on Myelodysplastic Syndrome in Childhood, was fulfilled,[12] and the patient was treated with IFN-α and 6-mercaptopurine. His clinical and laboratory findings gradually resolved without hematopoietic stem cell transplantation. However, 11 years after the diagnosis of JMML, the patient developed thrombocytopenia (7.6×10^9/L) and BM findings showed trilineage dysplasia with low blast count compatible with refractory anemia. The patient did not have any physiologic abnormalities, such as facial deformity, and there was no family history of malignancy or congenital abnormalities.

Patient 2. A 10-month-old boy had anemia, hepatosplenomegaly, and leukocytosis (31.8×10^9/L) with monocytosis (6.4×10^9/L; Table 1). The patient's BM exhibited myeloid hyperplasia and granulocytic dysplasia with 5% blasts. Cytogenetic

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Table 1. Patient characteristics

	Patient 1	Patient 2
Age, mo	10	10
Sex	Male	Male
Liver, cm	12	5
Spleen, cm	8	10
WBCs, $\times 10^9$/L	72.1	31.8
Monocytes, %	18.5	20
Blasts, %	4	2
Hb, g/dL	8.9	5.4
Platelets, $\times 10^9$/L	59	100
HbF, %	2.1	1.7
BM blasts, %	7	5
Karyotype	46,XY [20/20]	46,XY [20/20]
Monosomy 7 (FISH)	Negative	Negative
Spontaneous colony formation	Positive	Positive
Gene mutation	*NRAS*, G12D 35G > A	*NRAS*, G12S 34G > A
Treatment	IFN-α-2b, 6-MP	None
Observation period, mo	231	103
Outcome	Alive	Alive
Fraction of mutant alleles, % (pyrosequencing)		
Nail (whole)	24	12.5 (average)
Nail (left hand)	ND	26
Nail (right hand)	ND	13
Nail (left foot)	ND	8
Nail (right foot)	ND	3
Buccal smear cells	43	21
Hair bulbs	5	ND
Family studies		
Father	Wild-type	Wild-type
Mother	Wild-type	Wild-type
Sibling	ND	Wild-type

Hb indicates hemoglobin; 6-MP, 6-mercaptopurine; and ND, not done.

analysis revealed a normal karyotype. Colony assay of BM-MNCs showed spontaneous colony formation and GM-CSF hypersensitivity. Although the diagnostic criteria for JMML were fulfilled,[12] the patient's clinical symptoms and leukocytosis improved spontaneously within a few months without cytotoxic therapy or hematopoietic stem cell transplantation. The patient has remained healthy and has experienced no hematologic or physiologic abnormalities. The most recent follow-up examination was conducted when the patient was 8 years of age.

Detailed methods for experiments are described in supplemental Methods (available on the *Blood* Web site; see the Supplemental Materials link at the top of the online article).

Results and discussion

DNA sequencing for JMML-associated genes (ie, *NRAS*, *KRAS*, *PTPN11*, and *CBL*) was performed (Figure 1 and Table 1). In Patient 1, the *NRAS* G12D mutation was identified in BM-MNCs at the time of diagnosis of both JMML and MDS. We identified the same G12D mutation in DNA derived from buccal smear cells and nails of both hands; however, the sequence profile of the nails showed a low signal for the mutant allele compared with signal of blood cells. In Patient 2, the *NRAS* G12S mutation was identified in DNA from BM-MNCs, buccal smear cells, and nails of the left hand. However, the sequence profiles of buccal smear cells and nails of the left hand showed a low signal for the mutant variant. No mutation was detected in DNA from the PB-MNCs of the patient's parents or sibling.

We used pyrosequencing to quantify the fraction of mutated alleles in DNA samples from different somatic tissues (Figure 1 and Table 1). The frequency of mutated alleles varied by tissue type as follows. For Patient 1: BM-MNCs, 50%; nails, 24%; buccal smear cells, 43%; and hair bulbs, 5%. For Patient 2: buccal smear cells, 21%; nails of left hand, 26%; nails of right hand, 13%; nails of left foot, 8%; and nails of right foot, 3%. We cloned the PCR product of *NRAS* exon 2 from the nails of Patient 1 and picked up 15 clones. The clones were sequenced. Four of the 15 clones (27%) contained the mutant allele, which is consistent with the results of pyrosequencing analysis (24% mutant allele). Because the confirmed detection level by pyrosequencing technique was above 5%, results with a low percentage (< 5%) of mutant allele (ie, hair bulbs in Patient 1) should be interpreted with caution.[13,14]

We diagnosed 2 JMML patients as having somatic mosaicism of *NRAS* mutations: G12D for Patient 1 and G12S for Patient 2. The diagnoses were based on negative familial studies and mutational allele quantification analyses that showed diversity in the chimeric mutational status of different somatic tissues. Although DNA from buccal smear cells might be contaminated with WBCs, we also identified mutations in DNA from the nail tissue, which is known to be a good biologic material without contamination from hematopoietic cells, in both patients. These data suggest that a portion of the *NRAS*-mutated somatic cells were derived from one cell that acquired the mutation at a very early developmental stage. Although both somatic and germline mutations of RAS pathway genes (ie, *PTPN11*, *NRAS*, *NF1*, and *CBL*) are found in some JMML patients,[3,8-11] somatic mosaicism for these genes has never been reported. To the best of our knowledge, the present study is

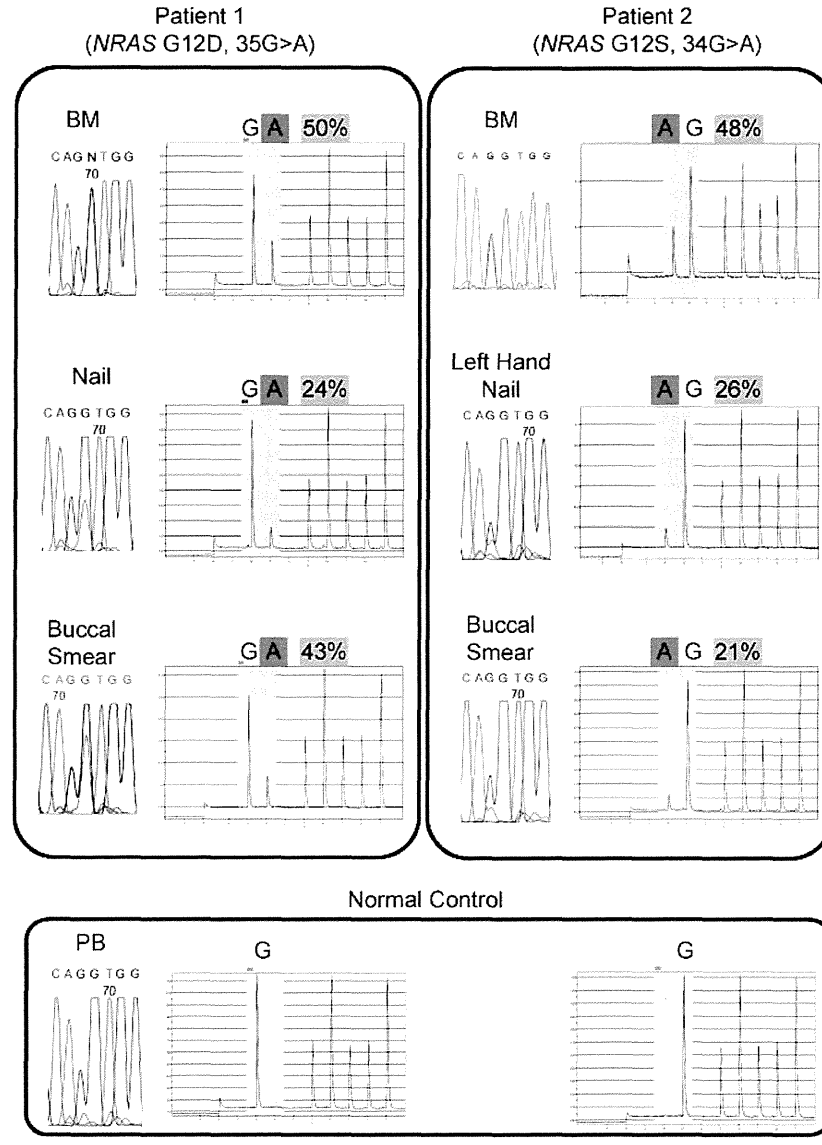

Figure 1. Direct sequencing and quantitative mutational analysis of NRAS in JMML patients. *NRAS* mutations are detected by direct sequencing and quantified by pyrosequencing. Direct sequencing identified oncogenic *NRAS* mutations: for Patient 1, G12D, 35G > A; for Patient 2, G12S, 34G > A) in BM-MNCs at diagnosis of JMML and in the nails and buccal smear cells. Quantification by pyrosequencing revealed that the fractions of mutated allele varied among different tissue types. For Patient 1: BM, 50%; nail, 24%; and buccal smear, 43%. For Patient 2: BM, 48%; left-hand nail, 26%; and buccal smear, 21%.

the first report of JMML patients with somatic mosaicism of mutations in RAS pathway genes.

Germline RAS pathway mutations are often associated with dysmorphic features similar to Noonan syndrome or its associated diseases. Correspondingly, JMML patients with germline *NRAS* or *CBL* mutations exhibit characteristic dysmorphic features.[3,10] Although our patients did not show any dysmorphic or developmental abnormalities, they should receive careful medical follow-up, especially for the occurrence of other cancers, because of the oncogenic nature of the mutations.

In general, JMML is a rapidly fatal disorder if left untreated.[8] However, recent clinical genotype-phenotype analyses have revealed heterogeneity in their clinical course. We and other researchers have reported that patients with *PTPN11* mutations have a worse prognosis than patients with other gene mutations, including *NRAS* and *KRAS*.[15,16] Both of the JMML patients in the present study with somatic mosaicism of oncogenic *NRAS* mutations have had a mild and self-limiting clinical course. We analyzed nails of other 3 JMML patients with RAS mutations who experienced aggressive clinical course and none showed somatic mosaicism (data not shown). In analogy to the mild phenotype of JMML patients with germline mutations in *PTPN11*, we speculate that JMML patients with somatic mosaicism of RAS genes might have a mild clinical course. We are planning to confirm these observations in larger cohort.

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Authorship

Contribution: S.D. and H.M. designed and conducted the research, analyzed the data, and wrote the manuscript; A.S., M.M.-E., M. Sato, H.K., A.K., M. Sotomatsu, and Y.H. treated the patients; Y.T., Y.F.-H., K.Y., H.H., H.K., N.Y., H.S., A.N., X.W., O.I., Y.X.,

N.N., M.T., A.H., and K.K. conducted the research; and S.K. designed the research, analyzed the data, and wrote the manuscript.

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LETTERS TO THE EDITOR

Novel splicing-factor mutations in juvenile myelomonocytic leukemia

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Myelodysplastic syndromes (MDS) and myelodysplastic/myeloproliferative neoplasms (MDS/MPN) are heterogeneous groups of chronic myeloid neoplasms characterized by clonal hematopoiesis, varying degrees of cytopenia or myeloproliferative features with evidence of myelodysplasia and a propensity to acute myeloid leukemia (AML).[1] In recent years, a number of novel gene mutations, involving *TET2*, *ASXL1*, *DNMT3A*, *EZH2*, *IDH1/2*, and *c-CBL*, have been identified in adult cases of chronic myeloid neoplasms, which have contributed to our understanding of disease pathogenesis.[2–7] However, these mutations are rare in pediatric cases, with the exception of germline or somatic *c-CBL* mutations found in 10–15% of chronic myelomonocytic leukemia (CMML) and juvenile myelomonocytic leukemia (JMML),[8] highlighting the distinct pathogenesis of adult and pediatric neoplasms.[9]

Recently, we reported high frequencies of mutations, involving the RNA splicing machinery, that are largely specific to myeloid neoplasms, showing evidence of myeloid dysplasia in adult.[10] Affecting a total of eight components of the RNA splicing machinery (U2AF35, U2AF65, SF3A1, SF3B1, SRSF2, ZRSR2, SF1 and PRPF40B) commonly involved in the 3′ splice-site (3′SS) recognition, these pathway mutations are now implicated in the pathogenesis of myelodysplasia.[10] To investigate the role of the splicing-pathway mutations in the pathogenesis of pediatric myeloid malignancies, we have examined 165 pediatric cases with AML, MDS, chronic myeloid leukemia (CML) and JMML for mutations in the four major splicing factors, *U2AF35*, *ZRSR2*, *SRSF2*, and *SF3B1*, commonly mutated in adult cases.

Bone marrow or peripheral blood tumor specimens were obtained from 165 pediatric patients with various myeloid malignancies, including *de novo* AML ($n=93$), MDS ($n=28$), CML ($n=17$) and JMML ($n=27$), and the genomic DNA (gDNA) was subjected to mutation analysis (Supplementary Table 1). The status of the RAS pathway mutations for the current JMML series has been reported previously (Supplementary Table 2).[11,12] Nineteen leukemia cell lines derived from AML (YNH-1, ML-1, KASUMI-3, KG-1, HL60, inv-3, SN-1, NB4 and HEL), acute monocytic leukemia (THP-1, SCC-3, J-111, CTS, P31/FUJ, MOLM-13, IMS/MI and KOCL-48) and acute megakaryoblastic leukemia (CMS and CMY) were also analyzed for mutations. Peripheral blood gDNA from 60 healthy adult volunteers was used as controls. Informed consent was obtained from the patients and/or their parents and from the healthy volunteers. We previously showed that for *U2AF35*, *SRSF2* and *SF3B1*, most of the mutations in adult cases were observed in exons 2 and 7, exon 1, and exons 14 and 15, respectively.[10] Therefore, we confirmed mutation screening to these 'hot-spot' exons. In contrast, all the coding exons were examined for *ZRSR2*, because no mutational hot spots have been detected. Briefly, the relevant exons were amplified using PCR and mutations were examined by Sanger sequencing, as previously described.[10] The Fisher's exact test was used to evaluate the statistical significance of frequencies of mutations for *U2AF35*, *SF3B1*, *ZRSR2* or *SRSF2* in adult cases and pediatric cases. This study was approved by the Ethics Committee of the University of Tokyo (Approval number 948-7).

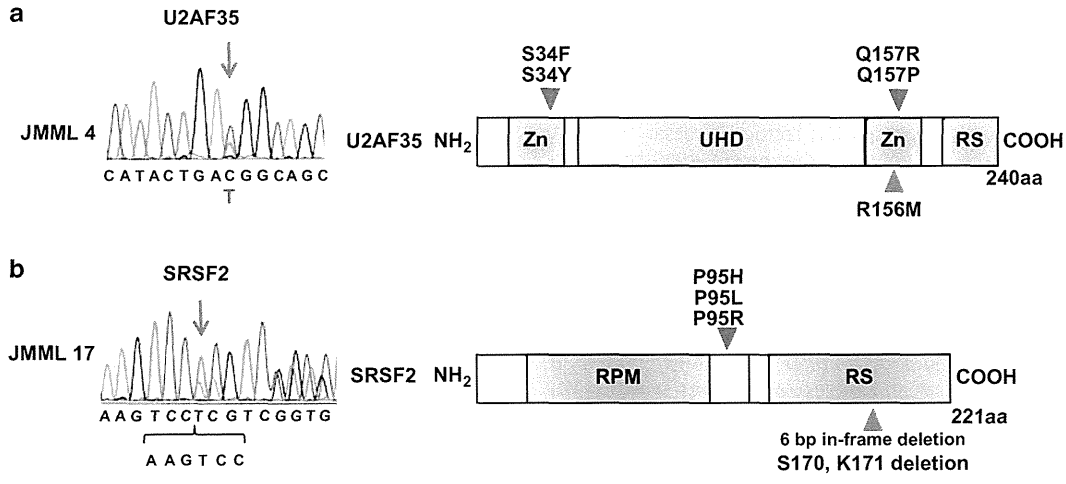

Figure 1. Novel *U2AF35*and *SRSF2* mutations detected in JMML cases. (**a**) Left panel: sequence chromatogram of a heterozygous mutation at R156 in N-terminal zinc-finger motifs of *U2AF35* detected in a JMML case (JMML 4) is shown. Mutated nucleotides are indicated by arrows. Right panel: illustration of functional domains and mutations of U2AF35. Red arrow heads indicate hot-spot mutations at S34 and Q157 detected in the adult cases.[10] Blue arrow head indicates the missense mutation at R156. (**b**) Left panel: sequence chromatogram of a 6-bp in-frame deletion (c.518-523delAAGTCC) in *SRSF2* detected in JMML 17 is shown. Mutated nucleotides are indicated by arrows. Right panel: illustration of functional domains and mutations of SRSF2. Red arrow head indicates hot-spot mutation at P95 frequently detected in the adult cases.[10] Blue arrow head indicates a 6-bp in-frame deletion leading to deletion of S170 and K171.

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No mutations were identified in the 28 cases with pediatric MDS, which included 13 cases with refractory anemia with excess blasts, 5 with refractory cytopenia of childhood, 2 with Down syndrome-related MDS, 2 with Fanconi anemia-related MDS, 2 with secondary MDS and 4 with unclassified MDS. Similarly, no mutations were detected in 93 cases with *de novo* AML or in 17 with CML, as well as 19 leukemia-derived cell lines. Our previous study in adult patients showed the frequency of mutations in *U2AF35*, *SF3B1*, *ZRSR2* or *SRSF2* to be 60/155 cases with MDS without increased ring sideroblasts and 8/151 *de novo* AML patients, emphasizing the rarity of these mutations in pediatric MDS ($P<5.0\times10^{-6}$) and AML ($P<0.02$) compared with adult cases. We found mutations in two JMML cases, JMML 4 and JMML 17. JMML 4 carried a heterozygous *U2AF35* mutation (R156M), whereas JMML 17 had a 6-bp in-frame deletion (c.518-523delAAGTCC) in *SRSF2* that resulted in deletion of amino acids S170 and K171 (Figure 1). Both nucleotide changes found in *U2AF35* and *SRSF2* were neither identified in the 60 healthy volunteers nor registered in the dbSNP database (http://www.ncbi.nlm.nih.gov/projects/SNP/) or in the 1000 genomes project, indicating that they represent novel spliceosome mutations in pediatric cases.

U2AF35 is the small subunit of the U2 auxiliary factor (U2AF), which binds an AG dinucleotide at the 3′SS, and has an essential role in RNA splicing.[13] With the exception of a single A26V mutation found in a case of refractory cytopenia with multilinage dysplasia, all the U2AF35 mutations reported in adult myeloid malignancies involved one of the two hot spots within the two zinc-finger domains, S34 and Q157, which are highly conserved across species, suggesting the gain-of-function mutations.[10] In JMML 4, the R156M U2AF35 mutation affects a conserved amino acid adjacent to Q157, suggesting it may also be a gain-of-function mutation, leading to aberrant pre-mRNA splicing possibly in a dominant fashion.

SRSF2, better known as SC35, is a member of the serine/arginine-rich (SR) family of proteins.[14] SRSF2 binds to a splicing-enhancer element in pre-mRNA and has a crucial role not only in constitutive and alternative pre-mRNA splicing but also in transcription elongation and genomic stability.[14] All mutations thus far identified in adult cases exclusively involved P95 within the intervening sequence between the N-terminal RNA-binding domain and the C-terminal RS domain.[10] This region interacts with other SR proteins, again suggesting that the P95 mutation may result in gain-of-function.[10] This proline residue is thought to determine the relative orientation of the two flanking domains of SRSF2, and a substitution at this position could compromise critical interactions with other splicing factors necessary for RNA splicing to take place. In contrast, the newly identified 6-bp in-frame deletion in JMLL 17 results in two conserved amino acids, S170 and K171, within the RS domain. Although it may affect protein–protein interactions, the functional significance of this deletion remains elusive.

JMML is a unique form of pediatric MDS/MPN characterized by activation of the RAS/mitogen-activated protein kinase signaling pathway; in 90% of cases, there are germ line and/or somatic mutations of *NF1*, *NRAS*, *KRAS*, *PTPN11* and *CBL*.[8] Although JMML shares some clinical and molecular features with CMML, its spectrum of gene mutations suggests that it is a neoplasm distinct from CMML.[15] This was also confirmed by the current results that the splicing-pathway mutations are rare in JMML, whereas they are extremely frequent (~60%) in CMML.[10] Although the two JMML cases carrying the splicing-pathway mutations had no known RAS-pathway mutations, both the pathway mutations frequently coexisted in CMML.[8]

To summarize, no mutations of *SF3B1*, *U2AF35*, *ZRSR2* or *SRSF2* are found in pediatric MDS and AML. In our study, except for *ZRSR2*, mutations were examined focusing on the reported hot spots in adult studies, raising a possibility that we may have missed some mutations occurring in other regions. However, these hot spots represent evolutionally conserved amino acids and have functional relevance, it is unlikely that the distribution of hot spots in children significantly differs from adult cases and as such, we could safely conclude that mutations of *SF3B1*, *U2AF35*, *ZRSR2* and *SRSF2* are rare in myeloid neoplasms in children. Finally, mutations of *U2AF35* and *SRSF2* may have some role in the pathogenesis of JMML, although further evaluations are required.

CONFLICT OF INTEREST

The authors declare no conflict interest.

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Supplementary Information accompanies the paper on the Leukemia website (http://www.nature.com/leu)