Argonaute-2 protein (Ago2), a major component of RNA-induced silencing complex (RISC), has been viewed as a cytoplasmic protein. In this study, we demonstrated by immunofluorescence confocal microscopy that Ago2 is distributed mainly as a nuclear protein in primary human foreskin keratinocytes in monolayer cultures and their derived organotypic (raft) cultures, although it exhibits only a minimal level of nuclear distribution in continuous cell lines such as HeLa and HaCaT cells. Oncogenic human papillomavirus type 16 (HPV16) or type 18 (HPV18) infection of the keratinocytes does not affect the nuclear Ago2 distribution. Examination of human tissues reveals that Ago2 exhibits primarily as a nuclear protein in skin, normal cervix, and cervical cancer tissues, but not in larynx. Together, our data provide the first convincing evidence that the subcellular distribution of Ago2 occurs in a cell type- and tissue context-dependent manner and may correlate with its various functions in regulation of gene expression.

Experimental Procedures

**Cells and Tissues**—HeLa, HEK293, and HaCaT cells were cultured in DMEM supplemented with 10% FBS (HyClone, GE Healthcare). Primary human foreskin keratinocytes (HFKs) were cultured as described previously (13, 14) in EpiLife calcium-free medium (Life Technologies, Thermo Scientific) supplemented with 5% fetal bovine serum (HyClone) and human keratinocyte growth supplement (Life Technologies, Thermo Scientific) in the presence of mitomycin C-treated J2 feeder cells. Raft cultures derived from primary HFKs with or without human papillomavirus type 16 (HPV16) or type 18 (HPV18) infection were prepared as described (13, 14). Formaldehyde-fixed paraffin-embedded tissue sections of human normal cervix, cervical cancer, skin, and larynx were obtained from US Biomax, Inc. (Rockville, MD).

**Antibodies**—The following antibodies were used in the study: mouse monoclonal anti-Ago2/eIF2C2 (catalog number ab57113, Abcam, Cambridge, MA); hnRNP C1/C2 (catalog number ab10294, Abcam); polyclonal anti-PABPC-1 (catalog number sc-6279, Santa Cruz Biotechnology); rabbit polyclonal anti-Ago2/eIF2C2 (catalog number EPR10410, Abcam); polyclonal anti-PABPC-1 (catalog number sc-6279, Santa Cruz Biotechnology); and goat anti-TIA-1 (catalog number sc-1451, Santa Cruz Biotechnology) antibodies. All fluorophore-conjugated secondary antibodies, Alexa Fluor chicken anti-mouse 488, donkey anti-goat Alexa Fluor 546, and goat anti-rabbit Alexa Fluor 546, were purchased from Molecular Probes (Thermo Scientific).

**siRNA Knockdown of Endogenous Ago2**—HeLa cells or primary HFKs were transfected twice or thrice, respectively, at an interval of 48 h with 20 nm (HeLa) or 40 nm (HFKs) of SMARTpool human eIF2C2 siRNAs targeting Ago2 (L-004639-00,
FIGURE 1. Partial nuclear distribution of Ago2 in HeLa and HaCaT cells. A, specificity of anti-Ago2 antibody in Western blotting of Ago2 expressed from HeLa cells transfected with a nonspecific (—) or Ago2-specific (+ si-Ago2) siRNA. Total cell extract (5 μl) was used for the assay, and β-actin served as a loading control. Numbers on the left are protein molecular mass markers in kDa. B and C, subcellular distribution of Ago2 in HeLa (B) and HaCaT (C) cells. Immunofluorescence staining of endogenous Ago2 (green) was performed with an anti-Ago2-specific antibody tested by Western blotting (A). Nuclei were counterstained with Hoechst dye (blue). D, stress-induced translocation of cytoplasmic Ago2 into TIA-1-positive stress granules. E, colocalization of Ago2 with TIA-1 in stress granules upon arsenite treatment. HeLa cells with or without arsenite treatment were simultaneously stained with anti-Ago2 (green) and anti-TIA-1 (red) antibodies. The nuclei were stained with Hoechst stain. All images were captured by confocal microscopy. Scale bar = 10 μm. Dashed boxes represent selected zoomed area.
Dharmacon, GE Healthcare) or non-targeting siRNA negative control (D-001810-01, Dharmacon, GE Healthcare) using Lipofect transfection reagent (SignaGen Laboratories, Gaithersburg, MD). Total cell extract was collected 24 h (HeLa) or 48 h (HFKs) after the second siRNA transfection.

Subcellular Fractionation and Western Blotting Analysis—Nuclear and cytoplasmic fractionation of HeLa cells and primary keratinocytes were obtained using a nuclei isolation kit from Sigma (catalog number NUC101) according to the protocol provided by the company, with slight modification. Briefly, cells growing in a 35-mm dish were scraped in 1× PBS and lysed using 350 μl of Nuclei EZ lysis buffer. Nuclei were pelleted by centrifugation (500 × g) for 5 mins at 4 °C. The supernatant was collected as the cytoplasmic fraction, and the obtained nuclear pellet was resuspended/washed gently one time with 1× PBS and then resuspended in 350 μl of lysis buffer. Equal volumes of nuclear and cytoplasmic extracts or total protein samples were examined by a standard Western blotting protocol after solubilizing in Laemmli SDS buffer supplemented with 5% (v/v) 2-mercaptoethanol.

Pre-absorption of Anti-Ago2 Antibody with Recombinant HA-Ago2 Protein—The recombinant Ago2 protein was immunopurified from HEK293 cells transfected with an HA-tagged hAgo2 expression vector (Addgene). Briefly, the whole cell extract prepared at 48 h after transfection was incubated with anti-HA antibody-conjugated agarose beads. The bound HA-Ago2 on the beads was then eluted by incubation with HA peptides three times to obtain three elution fractions (E1, E2, and E3) (15), and its purity in each fraction was confirmed by a Silver Quest silver staining kit (Life Technologies, Thermo Fisher Scientific) after SDS-PAGE. To block the specific activity of Ago2 antibody, the purified HA-Ago2 (100 ng) from the mixed fractions 2–3 was incubated with 100 μl of anti-Ago2 antibody diluted (1:50) in blocking buffer (2% BSA in PBST (PBS with 0.02% Tween 20)) for 20 min at room temperature, and the immunocomplexes were removed by centrifugation at 13,000 rpm for 10 min at 4 °C. The supernatant was used as the Ago2-adsorbed antibody for immunostaining.

Immunofluorescence Analysis (IFA) and Confocal Microscopy—Indirect immunofluorescence antibody staining was performed as described previously (16, 17). Briefly HeLa and HaCaT cells grown on poly-D-lysine-treated glass coverslips were fixed with 4% paraformaldehyde in PBS for 20 min at room temperature, and the immunocomplexes were removed by centrifugation at 5% (v/v) 2-mercaptoethanol.

Results and Discussion

Minimal Nuclear Distribution of Ago2 in HeLa and HaCaT Cells, Two Continuous Keratinocyte Cell Lines—To detect cell compartmental distribution of endogenous Ago2 protein by confocal microscopy, we first confirmed the specificity of a commercially available anti-Ago2 antibody by Western blotting using the total cell extract from HeLa, a cervical cancer cell line infected by HPV18, with or without Ago2 siRNA (si-Ago2) treatment. The selected antibody was able to detect a single, prominent protein band with the expected size of ~100 kDa corresponding to endogenous Ago2 (Fig. 1A). The treatment of HeLa cells with Ago2-specific siRNA led to reduction of the Ago2-specific protein, confirming the antibody specificity toward Ago2 (Fig. 1A). The selected antibody was then used in Ago2 IFA on HeLa cells. In agreement with previous studies (3, 11), mouse anti-Ago2 antibody; 2nd, rabbit anti-mouse IgG antibody.
A small portion of Ago2 was found to be nuclear (Fig. 1B). To eliminate the possible effect of HPV viral oncogene expression on Ago2 distribution in HeLa cells, HaCaT, an immortal skin keratinocyte cell line lacking HPV infection, was further used for the study, and we found that a small portion of Ago2 in HaCaT cells is also nuclear, whereas the majority of Ago2 are cytoplasmic (Fig. 1C).

However, nuclear and cytoplasmic Ago2 are distinguishable for their responses to arsenite, a chemical inducer of cellular stress. Upon cellular stress induced by arsenite, both Ago2 and TIA-1, a stress granule (SG)-specific marker, are recruited to SGs involved in translation inhibition (Fig. 1D) (4, 18, 19). By IFA staining of Ago2 and TIA-1 in arsenite-treated HeLa cells, we found that both cytoplasmic Ago2 and TIA-1, but not nuclear Ago2 and TIA-1, are co-stained in numerous cytoplasmic SGs (Fig. 1E). In contrast, there is no SG formation in the cell nuclei where Ago2 and TIA-1 remain generally dispersed throughout the nucleus.

Ago2 Is Predominantly a Nuclear Protein in Primary Keratinocytes Both in Monolayer and in Organotypic (Raft) Culture Conditions—Considering that the physiological state of continuous cell lines growing in monolayer cultures differs significantly from cells in tissues, we performed IFA immunostaining of Ago2 in primary HFKs grown on coverslips. To our surprise, we found a predominant nuclear distribution of Ago2 in primary HFKs (Fig. 2A) in monolayer culture, which is in sharp contrast to what we saw in HeLa and HaCaT cell lines (Fig. 1, B and C). The nuclear Ago2 distribution became significantly
reduced in the HFKs when Ago2 was knocked down (Fig. 2B and supplemental Fig. S1A). Further nuclear and cytoplasmic fractionation and Western blotting analyses confirmed more Ago2 in the cytoplasm than in the nucleus in HeLa cells, but in contrast, more Ago2 in the nucleus than in the cytoplasm in HFKs (Fig. 2C). In addition, we found that, when compared with HeLa cells (supplemental Fig. 1B), HFKs did not form a visible amount of stress granules in response to arsenite (supplemental Fig. 1C).

Next we examined Ago2 distribution in HFK-derived organotypic cultures that mimic epithelial tissues with characteristic differentiation of keratinocytes from the undifferentiated

FIGURE 4. Tissue-context nuclear distribution of Ago2. A, immunostaining of endogenous Ago2 in normal cervix, skin, and larynx by anti-Ago2 antibody. B and C, distinguishable subcellular distribution of endogenous Ago2 and PABPC1. Normal or cancerous cervical tissues were double-stained with anti-Ago2 (green) and anti-PABPC1 (red) antibodies (B). Nuclei were counterstained with Hoechst dye (A and B). All images were captured by confocal microscopy. Scale bars = 20 μm. Dashed boxes represent zoomed areas. The levels of Ago2 and PABPC1 in the selected cells from normal cervix were measured by a line crossing over the stained cells. Nuclear DNA counterstained with Hoechst dye served to define the cell nuclear and cytoplasmic border (C). The intensity plot of individual measurements in arbitrary units (AU) was shown after normalization to maximum intensities.
basal layer to the terminally differentiated cornified layer. To exclude any possible nonspecific staining of the anti-Ago2 antibody, we applied the purified HA-tagged Ago2 (HA-Ago2, Fig. 2D) to specifically absorb anti-Ago2 antibody before its Ago2 staining. IFA staining of HFKs in the organotypic tissues revealed that Ago2 is predominantly localized in the nuclei of basal and parabasal keratinocytes (Fig. 2E). The infection of HFKs in the organotypic tissues with HPV16 or HPV18 does not affect the nuclear distribution of Ago2 (Fig. 2F and supplemental Fig. S2 A), although both HPVs promote cell proliferation of undifferentiated keratinocytes (20).

The nuclear specific IFA staining of Ago2 in the keratinocytes was further confirmed by using an Ago2-absorbed anti-Ago2 antibody. In this assay, the anti-Ago2 antibody at 1:50 dilution was pre-absorbed by the affinity-purified HA-Ago2 expressed from HEK293 cells (Fig. 2D). When compared with the anti-Ago2 antibody labeling without Ago2 pre-absorption, the pre-absorbed anti-Ago2 antibody exhibited a significant reduction in nuclear Ago2 staining, with the signal intensity nearly close to the background signal from the negative control (staining by the secondary antibody only) (Fig. 2F). These data rule out the possibility of any cross-reactivity of the anti-Ago2 antibody to other nuclear proteins. By counting ~150 HFKs in organotypic cultures with or without HPV16 (HKF16) or HPV18 (HF1K18) infection, we found that the nuclear Ago2 in all three conditions could be found in nearly ~95% of keratinocytes by anti-Ago2 antibody, but only in ~3% of the cells by the Ago2-2 absorbed anti-Ago2 antibody and in none in the cells by the secondary antibody only (Fig. 2G). Together, we conclude that Ago2 is expressed primarily as a nuclear protein in HFKs.

Intracellular Detection of Ago2 in HFK-derived Organotypic Tissues Defined by Lamin A/C Staining—Given that Ago2 is mainly a nuclear protein in HFKs, we wished to define whether Ago2 could also be distributed to the nuclear membrane as suggested in a previous study (21). To confirm Ago2 staining inside the nuclear membrane, we used an anti-lamin A/C antibody that recognizes both lamin A and lamin C (due to alternative RNA splicing) in the inner membrane of the nucleus. Double immunostaining of the cells revealed that Ago2 in the nucleus is clearly overlapped with DNA and surrounded by lamin A/C of the nuclear membrane (Fig. 3A). Nuclear distribution of Ago2 in HFKs was found to be independent of HPV 16 or HPV18 infection (Fig. 3A and supplemental Fig. S2B). We also acquired confocal z-stacks of these samples to examine Ago2 distribution in the different focal planes of the nucleus (Fig. 3B for line-guided orthogonal view of the nuclei and supplemental Fig. S3 for various focus planes up (+) or down (−) from the selected section). As shown in Fig. 3B and supplemental Fig. S3, a three-dimensional orthogonal view and the focus plane views up or down from the selected section indeed showed more Ago2 distribution inside of the nucleus, but separate from the nuclear membrane. This intranuclear distribution of Ago2, overlapping with the nuclear DNA but not with the nuclear membrane lamin A/C, is measurable by an arbitrary line drawn across three separate cells (Fig. 3B, arrows) and can be seen clearly in the videos generated from confocal z-stacks (supplemental Movies 1 and 2).

Nuclear Distribution of Ago2 Varies from Different Human Tissues—We next investigated Ago2 distribution in human tissues including cervix, skin, and larynx by immunostaining with the same mouse anti-Ago2 antibody used in Figs. 1–3. We showed that Ago2 protein is expressed primarily as a nuclear protein in normal cervix and skin, but expressed as a major cytoplasmic protein in larynx (Fig. 4A). Normal cervix staining with a rabbit monoclonal anti-Ago2 antibody also gave the same result, showing nuclear Ago2 distribution in the cervix (supplemental Fig. S4). To exclude any possibility of fewer Ago2 staining signals in the cytoplasm than in the nuclei in human cervix being an artifact of tissue fixation and processing, we performed double staining of Ago2 along with cytoplasmic PABPC1 protein both in the normal and in the cancerous cervical tissues. The double immunostaining of the cervical tissues showed an exclusive cytoplasmic distribution of PABPC1, confirming the good preservation of cytoplasmic antigens during tissue fixation and processing (Fig. 4B). Notably, Ago2 was highly enriched in the nucleus when compared with the cytoplasm in the fixed tissues (Fig. 4B, supplemental Movies 3 and 4). A signal intensity plot across a randomly selected cell showed the distinct distribution of nuclear Ago2 from cytoplasmic PABPC1 in the cell compartments (Fig. 4C). Altogether, these data indicate that Ago2 can be either a nuclear or a cytoplasmic protein in a tissue-context dependent manner.

In summary, immunostaining and Western blotting in this study have revealed the minimal presence of Ago2 in the nuclei of HeLa and HaCaT cells, but the predominant presence of Ago2 in the nuclei of primary HFKs and their derived organotypic cultures. Ago2 is also distributed primarily as a nuclear protein in skin, normal cervix, and cervical cancer tissues, but not in larynx. By measuring Ago2 signal intensity in the cells or tissues, the level of Ago2 in the nucleus was found to be much higher than that measured in the cytoplasm in HFKs and in the tissues of skin and cervix (supplemental Fig. S5). Therefore, the predominant cytoplasmic distribution of Ago2 observed in continuous cell lines HeLa or HaCaT cells does not reflect the accurate distribution of Ago2 in primary tissues such as cervix or skin.

Author Contributions—N. R. S., X. W., and V. M. performed and analyzed the experiments in this study. M. A. performed the experiments shown in Figs. S1A and 2D. M. K. provided technical assistance and contributed to the preparation of figures and supplemental movies. C. M. performed raft cultures and HPV infection of keratinocytes. Z. M. Z. designed the study and analyzed all experimental data. N. R. S. and Z. M. Z. wrote the paper. All authors reviewed the results and approved the final version of the manuscript.

References
1. Hutvagner, G., and Simard, M. J. (2008) Argonaute proteins: key players in RNA silencing. Nat. Rev. Mol. Cell Biol. 9, 22–32
2. Meister, G., Landthaler, M., Patkaniowska, A., Dorsett, Y., Teng, G., and Tuschl, T. (2004) Human Argonaute2 mediates RNA cleavage targeted by miRNAs and siRNAs. Mol. Cell 15, 185–197
3. Weinmann, L., Höck, J., Iavecic, T., Ohrt, T., Mütze, J., Schwille, P., Kremmer, E., Benes, V., Urlaub, H., and Meister, G. (2009) Importin 8 is a gene silencing factor that targets argonaute proteins to distinct miRNAs. Cell 136, 496–507
4. Leung, A. K., Calabrese, J. M., and Sharp, P. A. (2006) Quantitative analysis of Argonaute protein reveals microRNA-dependent localization to stress granules. Proc. Natl. Acad. Sci. U.S.A. 103, 18125–18130
5. Gagnon, K. T., Li, L., Chu, Y., Janowski, B. A., and Corey, D. R. (2014) RNAi factors are present and active in human cell nuclei. Cell Rep. 6, 211–221
6. Wei, Y., Li, L., Wang, D., Zhang, C. Y., and Zen, K. (2014) Importin 8 regulates the transport of mature microRNAs into the cell nucleus. J. Biol. Chem. 289, 10270–10275
7. Ando, Y., Tomaru, Y., Morinaga, A., Burroughs, A. M., Kawaji, H., Kubosaki, A., Kimura, R., Tagata, M., Ino, Y., Hirano, H., Chiba, J., Suzuki, H., Carninci, P., and Hayashizaki, Y. (2011) Nuclear pore complex protein mediated nuclear localization of dicer protein in human cells. PLoS ONE 6, e23385
8. Doyle, M., Badertscher, L., Jaskiewicz, L., Güttinger, S., Jurado, S., Hugen schmidt, T., Kutay, U., and Filipowicz, W. (2013) The double-stranded RNA binding domain of human Dicer functions as a nuclear localization signal. RNA 19, 1238–1252
9. Tang, S., Tao, M., McCoy, J. P., Jr., and Zheng, Z. M. (2006) The E7 oncoprotein is translated from spliced E6*I transcripts in high-risk human papillomavirus type 16- or type 18-positive cervical cancer cell lines via translation reinitiation. J. Virol. 80, 4249–4263
10. Gagnon, K. T., and Corey, D. R. (2012) Argonaute and the nuclear RNAs: new pathways for RNA-mediated control of gene expression. Nucleic Acid Ther. 22, 3–16
11. Chu, Y., Yue, X., Younger, S.T, Janowski, B. A., and Corey, D. R. (2010) Involvement of argonaute proteins in gene silencing and activation by RNAs complementary to a non-coding transcript at the progesterone receptor promoter. Nucleic Acids Res. 38, 7736–7748
12. Benhamed, M., Herbig, U., Ye, T., Dejean, A., and Bischof, O. (2012) Senescence is an endogenous trigger for microRNA-directed transcriptional gene silencing in human cells. Nat Cell Biol. 14, 266–275
13. Wang, X., Wang, H. K., Li, Y., Hafner, M., Banerjee, N. S., Tang, S., Briskin, D., Meyers, C., Chow, L. T., Xie, X., Tuschl, T., and Zheng, Z. M. (2014) MicroRNAs are biomarkers of oncogenic human papillomavirus infections. Proc. Natl. Acad. Sci. U.S.A. 111, 4262–4267
14. Wang, X., Wang, H. K., McCoy, J. P., Banerjee, N. S., Rader, J. S., Broker, T. R., Meyers, C., Chow, L. T., and Zheng, Z. M. (2009) Oncogenic HPV infection interrupts the expression of tumor-suppressive miR-34a through viral oncoprotein E6. RNA. 15, 637–647
15. Kang, J. G., Pripuzova, N., Majerciak, V., Kruhlak, M., Le, S. Y., and Zheng, Z. M. (2011) Kaposi’s Sarcoma-Associated herpesvirus ORF57 promotes escape of viral and human interleukin-6 from microRNA-mediated suppression. J. Virol. 85, 2620–2630
16. Majerciak, V., Kruhlak, M., Dagur, P. K., McCoy, J. P., Jr., and Zheng, Z. M. (2010) Caspase-7 cleavage of Kaposi sarcoma-associated herpesvirus ORF57 confers a cellular function against viral lytic gene expression. J. Biol. Chem. 285, 11297–11307
17. Massimelli, M. J., Majerciak, V., Kruhlak, M., and Zheng, Z. M. (2013) Interplay between polyadenylate-binding protein 1 and Kaposi’s sarcoma-associated herpesvirus ORF57 in accumulation of polyadenylated nuclear RNA, a viral long noncoding RNA. J. Virol. 87, 243–256
18. Detzer, A., Engel, C., Wünsche, W., and Szakal, G. (2011) Cell stress is related to re-localization of Argonaute 2 and to decreased RNA interference in human cells. Nucleic Acids Res. 39, 2727–2741
19. Karginov, F. V., and Hannon, G. J. (2013) Remodeling of Ago2-mRNA interactions upon cellular stress reflects miRNA complementarity and correlates with altered translation rates. Genes Dev. 27, 1624–1632
20. Longworth, M. S., and Laimins, L. A. (2004) Pathogenesis of human papillomaviruses in differentiating epithelia. Microbiol. Mol. Biol. Rev. 68, 362–372
21. Ahlenstiel, C. L., Lim, H. G., Cooper, D. A., Ishida, T., Kelleher, A. D., and Suzuki, K. (2012) Direct evidence of nuclear Argonaute distribution during transcriptional silencing links the actin cytoskeleton to nuclear RNAi machinery in human cells. Nucleic Acids Res. 40, 1579–1595