Mutations of the functional ARH1 allele in tumors from ARH1 heterozygous mice and cells affect ARH1 catalytic activity, cell proliferation and tumorigenesis

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ADP-ribosylation results from transfer of the ADP-ribose moiety of nicotinamide adenine dinucleotide (NAD) to an acceptor with ADP-ribose-acceptor content determined by the activities of ADP-riboyltransferases, which modify the acceptor, and ADP-ribose-acceptor hydrolase (ARH), which cleave the ADP-ribose-acceptor bond. ARH1 was discovered as an ADP-ribose(arginine)protein hydrolase. Previously, we showed that ARH1-knockout and ARH1 heterozygous mice spontaneously developed tumors. Further, ARH1-knockout and ARH1 heterozygous mouse embryonic fibroblasts (MEFs) produced tumors when injected into nude mice. In tumors arising in ARH1 heterozygous mice and MEFs, we found both loss of heterozygosity (LOH) of the ARH1 gene and ARH1 gene mutations. In the present report, we found that these mutant ARH1 genes encode proteins with reduced ARH1 enzymatic activity. Moreover, MEFs transformed with ARH1 mutant genes exhibiting different levels of ARH1 activity showed altered rates of proliferation, anchorage-independent colony growth in soft agar, and tumorigenesis in nude mice. MEFs transformed with the wild-type (WT) gene, but expressing low levels of hydrolase activity were also tumorigenic. However, transformation with the WT gene was less likely to yield tumors than transformation with a mutant gene exhibiting similar hydrolase activity. Thus, control of protein-ADP-ribosylation by ARH1 is critical for tumorigenesis. In the human cancer database, LOH and mutations of the ARH1 gene were observed. Further, ARH1 gene mutations were located in exons 3 and 4, comparable to exons 2 and 3 of the murine ARH1 gene, which comprise the catalytic site. Thus, human ARH1 gene mutations similar to their murine counterparts may be involved in human cancers.

INTRODUCTION

Lung cancer is the most common cause of global cancer-related mortality.1 Adenocarcinoma, and to a lesser extent, squamous cell carcinomas, major subtypes of non-small-cell lung carcinoma have been shown to have mutations in critical regulatory genes.1 Gene mutations may change protein structure and enzymatic activity and may be associated with novel activities, for example, interactions with other cellular proteins, resulting in aberrant regulation of transcriptional programs.2,3 Cellular kinases, for example, epidermal growth factor receptor and phosphatidylinositol 3 kinase, are frequently mutated and dysregulated in non-small-cell lung carcinoma.4,5 High rates of somatic mutations in human lung adenocarcinoma have been found in regulatory genes such as Ras and p53.1,4,5

Post-translational modifications (for example, phosphorylation, acetylation and methylation) of proteins regulate cell proliferation and tumor development in mice.6–8 Histone methylation has been shown to affect non-small-cell lung carcinoma tumorigenesis.3 Phosphorylation, acetylation and ubiquitination of p53 influence protein stability and transcriptional activity with affects on cell proliferation and tumorigenesis.5 Protein poly-ADP-ribosylation by a family of poly (ADP-ribose) polymerases (PARPs) contributes to a variety of biological functions including carcinogenesis, chromosomal stability, regulation of chromatin structure, transcription, DNA repair and telomere homeostasis9–11 and was induced by genotoxic stress.12

Mono-ADP-ribosylation is a post-translational modification of proteins, in which the ADP-ribose moiety of β-nicotinamide adenine dinucleotide (NAD) is transferred to specific amino-acid residues in target proteins.13 Mono-ADP-ribosylation is used by some bacterial toxins to alter the activity of critical proteins in mammalian cells, thereby disrupting regulatory, biosynthetic or metabolic pathways.14 Cholera toxin, for example, ADP-ribosylated arginine in the α-subunit of the stimulatory guanine nucleotide-binding (G) protein of the adenylyl cyclase system, resulting in its activation and an increase in intracellular cAMP.15 ADP-ribosylation of G-actin at Arg177 by Salmonella enterica toxin (SpWB) inhibits ATPase activity and thereby prevents actin polymerization.16,17 Other toxin ADP-ribosyltransferases (for example, Clostridium limosum exoenzyme C3, Pertussis toxin, diptheria toxin) use different proteins, and in some instances, different amino acids (for example, asparagine, cysteine and modified histidine) as substrates.18–20

In mammalian and avian systems, ADP-ribosylation of arginine appears to be a reversible modification of proteins.20 ADP-ribosyltransferases (ARTs) use β-NAD as the ADP-ribose donor and synthesize the α-anomeric ADP-ribo-arginine acceptor.13,19,20 Stereospecific ADP-riboarginine hydrolases (ARH) cleave α-ADP-ribose-arginine(protein), produced in the
transferase-catalyzed reaction, to regenerate the unmodified arginine, which is then available for ADP-ribosylation on its guanidino moiety.\textsuperscript{13,16,20–22} Thus, ARHs complete an ADP-ribosylation cycle that could reversibly regulate the function of substrate proteins.\textsuperscript{13,20,23}

Human neutrophil peptide 1 (HNP-1), a defensin found in human lungs is believed to modulate the innate immune response.\textsuperscript{24,25} ADP-ribosylated HNP-1 was isolated from the airways of patients with pulmonary disease (for example, asthma, pulmonary fibrosis).\textsuperscript{24,25} ADP-ribosylarginine was decreased reduced antimicrobial and cytotoxic activities compared with native HNP-1, but with less of an effect on its function as a T-cell chemoattractant.\textsuperscript{20,23} Other arginine ADP-ribosylations have been described. ADP-ribosylation of (arginine) P2X7 by ART2.2 leads to rapid apoptotic death of native murine T lymphocytes.\textsuperscript{20} Exposure of T cells to a low concentration of extracellular NAD lead to ADP-ribosylation of P2X7 by ART2.2, and resulted in cell shrinkage, fragmentation of DNA and exposure of phosphatidylserine.\textsuperscript{26,27}

ARH1 removes the regulatory ADP-ribose moiety from (arginine) proteins, regenerating unmodified acceptor proteins or molecules.\textsuperscript{23,28} ARH1 was identified as a 39-kDa protein in mammalian and avian tissues and cells, with human and mouse ARH1 sharing structural similarities.\textsuperscript{23,28} \textit{In vitro}, human and avian ARH1 enzymatic activities were stimulated by dithiothreitol and Mg\textsuperscript{2+}.\textsuperscript{20,30} Mg\textsuperscript{2+} is believed to be necessary for the correct orientation of the substrates at the catalytic site.\textsuperscript{30}

As noted, cholera toxin exerts its effects through the ADP-ribosylation of Gas. The effects of cholera toxin, as evidenced by both ADP-ribose-arginine content and Gas modification, were greater in ARH1-deficient mouse embryonic fibroblasts (MEFs) than in wild-type (WT) MEFs and were significantly reduced by overexpression of WT ARH1 in ARH1-deficient MEFs.\textsuperscript{31} Similarly, fluid accumulation induced by cholera toxin in intestinal loops was greater in ARH1-deficient (ARH1KO) mice than in their WT counterparts, as was ADP-ribosylation of Gas and ADP-ribosylarginine content of intestinal epithelial cells.\textsuperscript{31} These data support a role for ARH1 in the intoxication process.\textsuperscript{31}

Recently, we reported increased tumorigenesis associated with ARH1 deficiency.\textsuperscript{32} ARH1KO MEFs proliferated faster than their WT counterparts,\textsuperscript{32} and ARH1KO MEFs, but not their WT counterparts, formed colonies in soft agar and produced subcutaneous tumors in nude mice.\textsuperscript{32} Transfection of the WT ARH1 gene into ARH1KO MEFs (ARH1KO+wt) prevented colony formation in soft agar and tumors following their subcutaneous injection in nude mice, whereas transformation of the KO MEFs with an inactive double-mutant (D60, 61A) ARH1 gene had no effect.\textsuperscript{32} Consistent with these observations, ARH1KO mice spontaneously developed tumors more frequently than did their WT littermates. ARH1 heterozygous MEFs and mice shared tumorigenic properties similar to their ARH1-deficient counterparts, which appeared to result from mutation or loss of the functioning ARH1 allele.\textsuperscript{32}

In some tumors from ARH1 heterozygous mice or from nude mice after subcutaneous injection of ARH1 heterozygous MEFs, an ARH1 protein band was observed by immunoblotting. In all instances, mutations in the ARH1 gene were found in the tumor, but not in adjacent non-tumor tissue. Notably, no mutation was detected in complementary DNA (cDNA) from the ARH1 heterozygous MEFs that had been injected. In all likelihood, an ARH1 mutation in a small population of the heterozygous MEFs enabled them to proliferate more rapidly than did ARH1 heterozygous MEFs containing a normal allele, thus giving rise to colonies in soft agar and tumors in nude mice.\textsuperscript{32} Mechanisms observed for inactivation of the active ARH1 gene included loss of heterozygosity (LOH) and ARH1 gene mutations.\textsuperscript{32}

In this report, we characterize the functional effects of ARH1 mutations identified in lung adenocarcinomas in ARH1 heterozygous mice and in tumors in nude mice injected with ARH1 heterozygous MEFs. These mutations were observed in exons 2 and 3 of the ARH1 gene, which encode the region of the protein that forms the catalytic site.\textsuperscript{23,33} The mutations did not always inactivate the protein. Using ARH1-deficient MEFs transfected with the mutated ARH1 genes to measure of ARH1 function, different effects were seen with the different tumor-inducing gene mutations on proliferation, growth in soft agar and tumorigenesis in nude mice, suggesting that mutations in ARH1, in addition to effects on catalytic activity, could lead to conformational changes at the ARH1 active site, which alter other functions (for example, interaction with proteins). Given a possible role for the ARH1 gene in cancer, we next used the Catalogue of Somatic Mutations in Cancer (COSMIC) database from Trust Sanger Institute (England)\textsuperscript{24,25} to look for ARH1 gene mutations and LOH in human cancer. Mutations were found in exons similar in location to those seen in our murine model. These data are consistent with a potential role for ARH1 mutations in human, as well as murine cancer.

RESULTS
Mutations of the ARH1 gene in tumors

We reported that mutations in exons 2 and 3 of the ARH1 gene were detected by in lung adenocarcinoma isolated from ARH1 heterozygous mice and tumors in nude mice injected with ARH1 heterozygous MEFs.\textsuperscript{32} Six mutations were identified in lung adenocarcinoma of ARH1 heterozygous mice, and another eight mutations were found in tumors from nude mice injected with ARH1 heterozygous MEFs (Tables 1 and 2). Mutation types included missense mutations resulting from single-base substitutions (12 of 14, 85.7%), and deletion mutations with frame shifts (2 of 14, 14.3%) (Tables 1 and 2, Supplementary Table 1). The most frequent mutations of the coding strand were A>G (5 of 14, 35.7%) and T>C (4 of 14, 28.6%) (Tables 1 and 2, Supplementary Table 1).

Table 1. ARH1 mutations in lung adenocarcinoma from ARH1 heterozygous female mice

| Mouse ARH1 mutation | Amino-acid mutation | CDS\textsuperscript{c} mutation | Sample number\textsuperscript{a} | Mutation type | ARH1 activity (%) |
|---------------------|---------------------|-------------------------------|-----------------|-------------|------------------|
| mt1                 | p.I32V              | c.94A>G                       | 2               | Substitution – missense | 55.3 ± 1.2 |
| mt2                 | p.F31S              | c.92T>C                       | 2               | Substitution – missense | 4 ± 1.1 |
| mt3                 | p.F31S, p.I32V      | c.92T>C, c.94A>G              | 1               | Substitution – missense | 4.9 ± 1.3 |
| mt4                 | p.S78S, p.S79P stop codon | c.237G > del\textsuperscript{b} | 1               | Deletion – frameshift | None detected |
| mt5                 | p.S78S, p.S79P stop codon | c.233C > del\textsuperscript{b} | 1               | Deletion – frameshift | None detected |
| mt6                 | p.P117L             | c.350C>T                      | 3               | Substitution – missense | 42.9 ± 1.4 |

Abbreviations: ARH, ADP-ribose-acceptor hydrolase; MEFs, mouse embryonic fibroblasts. All mutations were in exon 2. \textsuperscript{a}Sample number: number of samples with a ARH1 gene mutation. \textsuperscript{b}Deletion mutant, frameshift: no recombinant protein, no stably ARH1-deficient MEFs. \textsuperscript{c}CDS: coding DNA sequence.
Effects of mutations on ARH1 activity
To determine the effects of mutations on ARH1 enzymatic activity, the ARH1 mutant genes were placed in a mammalian expression vector and expressed in ARH1KO MEFs. We generated stably transformed ARH1KO MEFs with ARH1 WT and mutant genes including mock (empty vector). Similar expression levels of protein were detected by western blots (Figure 1a). Surprisingly, when expressed in ARH1KO MEFs, the mutant proteins exhibited a wide variation of ARH1 catalytic activity (4–55% of WT activity) (Tables 1 and 2). However, ARH1 activity was not detected in ARH1KO MEFs transformed with an ARH1 frameshift or deletion mutant gene. Thus, some ARH1 proteins encoded by mutant genes isolated from lung adenocarcinoma, had 55% of WT activity when expressed in ARH1KO MEFs. Similarly, some tumors that developed from ARH1 heterozygous MEFs had ARH1 mutant alleles, which encoded proteins that when expressed in ARH1KO MEFs, had 42% of WT activity (Tables 1 and 2). These data suggested that effects on ARH1 enzymatic activity alone were not the sole basis for tumorigenesis. To examine this further, we used ARH1KO MEFs stably transformed with ARH1 mutant and WT genes and assessed the effects of ARH1 mutations on tumorigenesis.

### Table 2. ARH1 mutations in tumors of female nude mice injected subcutaneously with ARH1 heterozygous MEFs

| Mouse ARH1 mutation | Amino-acid mutation | CDS mutation | Exon<sup>a</sup> | Sample number<sup>b</sup> | ARH1 activity (%) |
|---------------------|---------------------|--------------|------------------|--------------------------|-------------------|
| mt7                 | p.E30G, p.F31S      | c.89A>G, c.92T>C | 2                | 1                        | 14.7 ± 0.5        |
| mt8                 | p.H130P             | c.389A>C      | 3                | 2                        | 29.5 ± 1.0        |
| mt9                 | p.H130L             | c.389A>T      | 3                | 2                        | 42.2 ± 0.6        |
| mt10                | p.A140T             | c.408G>A      | 3                | 2                        | 20.5 ± 0.4        |
| mt11                | p.L145R, p.R146G    | c.435G>C, c.436A>G | 3          | 1                        | 28.4 ± 0.6        |
| mt12                | p.I3717             | c.470T>C      | 3                | 3                        | 24.8 ± 0.5        |
| mt13                | p.S160C             | c.478A>T      | 3                | 2                        | 36.7 ± 1.2        |
| mt14                | p.F315              | c.92T>C, c.93G>G | 2                | 2                        | 14.8 ± 1.9        |

Abbreviations: ARH, ADP-ribose-acceptor hydrolase; cDNA, complementary DNA; MEFs, mouse embryonic fibroblasts. Histology: fibrosarcoma in all subcutaneous tumors of female nude mice. Mutation type: substitution – missense. <sup>a</sup>Exon number of ARH1 cDNA gene. <sup>b</sup>Sample number: number of samples with a ARH1 gene mutation. *CDS: coding DNA sequence.

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**Figure 1.** Proliferation of ARH1KO cells expressing a WT gene and mutant ARH1 genes from ARH1<sup>+/−</sup> mouse lung adenocarcinoma. (a) Immunoblotting with anti-ARH1 and anti-α-tubulin antibodies of proteins in cell lysates (50 μg) of ARH1KO MEFs transformed with ARH1 WT genes and mutant genes. ARH1KO MEFs that expressed ARH1 WT and mutant genes were used in the experiments (that is, ARH1 activity experiments, cell proliferation assays, soft agar colony forming assay, tumorigenesis studies in nude mice). (b) ARH1KO+WT (●), ARH1KO Mock (○), ARH1KO+mt1 (□), ARH1KO+mt2 (▲), ARH1KO+mt3 (●), and ARH1KO+mt6 (△) MEFs (5 × 10<sup>4</sup>) were seeded in 96-well plates and MTT assays were performed after growth for the indicated time. (c) ARH1KO+WT (●), ARH1KO mock (○), ARH1KO+mt1 (□), ARH1KO+mt2 (▲), ARH1KO+mt3 (●), and ARH1KO+mt6 (△) MEFs (1 × 10<sup>5</sup>) were seeded in 100 mm dishes and cell counting was performed after growth for the indicated time. Data are means ± s.e.m. of values from six assays performed in three experiments. Pairwise comparison showed that ARH1KO Mock, +mt2, +mt3 and +WT were significantly different from ARH1KO+mt1, and +mt6 (all at P < 0.001), and also all ARH1KO+mutations and ARH1KO mock were significantly different from ARH1KO+WT (all at P < 0.001).
Proliferation of ARH1KO cells expressing WT and mutant ARH1 genes from ARH1+/− mouse lung adenocarcinoma and ARH1+/− MEFs injected in nude mice

We reported previously that the proliferation of ARH1KO MEFs was faster than that of ARH1KO+wt and ARH1 WT MEFs. To characterize the ARH1 mutations, their effects on rates of proliferation of ARH1KO MEFs transformed with empty vector (ARH1KO+mock) were compared with ARH1KO MEFs transformed with an ARH1 WT gene that has 100% ARH1 activity (ARH1KO+WT1) and ARH1KO MEFs transformed with all ARH1 mutant genes (4–55% of WT activity, from ARH1KO+mt1 to ARH1KO+mt14) (Figures 1 and 2). Interestingly, the proliferation rate of ARH1KO MEFs transformed with all mutant genes (from ARH1KO+mt1 to ARH1KO+mt14, 4–55% of WT activity) was significantly (P < 0.0001) faster than that of ARH1KO+WT MEFs, and slower (P < 0.01 to 0.0001) than that of ARH1KO Mock MEFs (Figures 1 and 2). A comparison of ARH1KO MEFs transformed with all ARH1 mutant genes showed that the proliferation rate of MEFs transformed with a mutant gene expressing a protein having low ARH1 activity (4–5% of WT activity MEFs ARH1KO+mt2 or +mt3) was significantly faster (P < 0.01) than that of MEFs transformed with mutant genes expressing proteins having high ARH1 activity (42–55% of WT activity, ARH1KO+mt1 or +mt6) (Figure 1). Proliferation of ARH1KO MEFs containing ARH1 mutant genes expressing proteins with intermediate ARH1 activity (15–18% of WT activity, ARH1KO+mt7 or +mt14) was significantly (P < 0.01) faster than those containing high ARH1 activity (37–42% of WT activity, ARH1KO+mt9 or +mt13) (Figure 2). Thus, in the case of ARH1 mutations leading to tumor development, the rate of proliferation of transformed ARH1KO MEFs depended upon the levels of ARH1 activity. These data also suggested that the proliferation assay and enzymatic activity were not good surrogates for tumorigenesis.

Effects of ARH1 mutations of MEFs on growth and colony formation in soft agar

Previously, we found that ARH1KO MEFs, but not ARH1 WT and ARH1KO+WT MEFs formed colonies in soft agar. The soft agar colony formation assay is a common method to observe anchorage-independent growth, which correlates with tumorigenesis. All ARH1KO+mt1 to ARH1KO+mt14 MEFs transformed with ARH1 mutant genes and the DD mutant gene formed colonies in soft agar, whereas ARH1KO MEFs transformed with the ARH1 WT1 gene did not (Figure 3). Diameter of colonies with all mutant MEFs (ARH1KO+mt1 to +mt14) was significantly smaller than that of colonies formed by ARH1KO mock and DD mutant MEFs (Figures 3a and c). Diameters of colonies from MEFs transformed with the low catalytic activity group of ARH1 mutants (ARH1KO+mt3, +mt7, and +mt14, 5–15% of WT activity) were larger (P < 0.01) than that of colonies formed by the high activity group (ARH1KO+mt1, +mt6, +mt9, and +mt13, 37–55% of WT activity), but not larger than colonies formed by the intermediate ARH1 activity group (ARH1KO+mt8, +mt10, +mt11, and +mt12, 20–30% of WT activity) (Figures 3a and c).

Number of colonies formed by MEFs transformed with any of the mutant MEFs (ARH1KO+mt1 to +mt14) was greater (P < 0.01) than that of colonies seen with ARH1KO+WT1 MEFs, but was fewer (P < 0.01) than those formed by ARH1KO mock, and DD MEFs.

**Figure 2.** Proliferation of ARH1KO cells expressing mutant ARH1 genes isolated from ARH1+/− MEFs injected in nude mice. (a) ARH1KO+WT (□), ARH1KO Mock (●), ARH1KO+mt7 (×), ARH1KO+mt9 (■), ARH1KO+mt13 (○), ARH1KO+mt14 (▲) MEFs (1 × 10⁵) MEFs (5 × 10⁵) were seeded in 96-well plates and MTT assays were performed after growth for the indicated time. (b) ARH1KO+WT (□), ARH1KO Mock (●), ARH1KO+mt7 (×), ARH1KO+mt9 (■), ARH1KO+mt13 (○), ARH1KO+mt14 (+) MEFs (1 × 10⁵) were seeded in 100 mm dishes and cell counting was performed after growth for the indicated time. (c) ARH1KO+WT (□), ARH1KO Mock (●), ARH1KO+mt8 (▲), ARH1KO+mt10 (○), ARH1KO+mt11 (+), ARH1KO+mt12 (■) MEFs (5 × 10⁵) were treated and assayed as in a. (d) ARH1KO+WT (□), ARH1KO Mock (●), ARH1KO+mt8 (▲), ARH1KO+mt10 (○), ARH1KO+mt11 (+), ARH1KO+mt12 (■) MEFs (1 × 10⁵) were treated as in b before cell counting assays. Data are means ± s.e.m. of values from six assays performed in three experiments. (a,b) Pairwise comparison showed all mutations were significantly different from ARH1KO+WT, and ARH1KO Mock, +mt7 and +mt14 were significantly different from ARH1KO+mt9 and +mt13 (all at P < 0.001). (c,d) Pairwise comparison showed all mutations were significantly different from ARH1KO+WT and ARH1KO mock (all at P < 0.001).
Effects of ARH1 mutations on growth of MEFs in soft agar. (a) Diameters of ARH1KO MEFs and +DD (black bars, < 1% ARH1 activity of WT) MEFs, ARH1KO+WT MEFs (open bar), ARH1KO MEFs with mutants that have 36–55% activity of WT (dark-gray bars), ARH1KO with mutants that have 5–15% activity of WT (light-gray bars), and ARH1KO with mutants that have 20–30% activity of WT (open bars) formed colonies in soft agar after incubation for 42 days (37°C, with 5% CO2). In all instances, ARH1 WT genes were isolated to confirm the absence of mutations. Data are means ± s.e.m. of values from three experiments with duplicate assays per experiment with each cell line for colony formation in soft agar. Pairwise comparison showed all MEFs containing mutant genes were significantly different from ARH1KO+WT, ARH1KO mock and +DD MEFs (P < 0.001). Also, ARH1KO+mt1, +mt6, +mt9 and +mt13 (36–55% activity of WT) MEFs were significantly different from ARH1KO+mt3, +mt7 and +mt14 MEFs (5–15% activity of WT) (P < 0.05). Numbers in parentheses indicate the % ARH1 activity of ARH1KO+WT MEFs. (AC): ARH1 mutant gene from lung adenocarcinoma in ARH1WT mice. (B) Number of ARH1KO MEFs and ARH1KO+DD MEFs (black bars, < 1% ARH1 activity of WT), ARH1KO+WT MEFs (open bar), ARH1KO MEFs with mutants that have 36–55% activity of WT (dark-gray bars), ARH1KO MEFs with mutants that have 5–15% activity of WT (light-gray bars), and ARH1KO MEFs with mutants that have 20–30% activity of WT (open bars) that formed colonies in soft agar after incubation for 42 days (37°C, with 5% CO2). Data are means ± s.e.m. of values from three experiments with duplicate assays per experiment with each cell line for colony formation in soft agar. Pairwise comparison showed all MEFs containing mutant genes were significantly different from ARH1KO+WT, Mock and +DD MEFs (P < 0.01). Also, ARH1KO+mt1, +mt6, +mt9 and +mt13 MEFs (36–55% activity of WT) were significantly different from ARH1KO+mt3, +mt7 and +mt14 MEFs (5–15% activity of WT) (P < 0.05). (C) Appearance (x 100) of colonies in soft agar after 32 days of growth of ARH1KO MEFs transformed with ARH1 WT and mutant genes.

(Figures 3b and c). Data regarding diameter of colonies in soft agar with the different groups of MEFs were similar to data related to the number of colonies. The numbers of colonies seen with the low ARH1 activity MEF group (ARH1KO+mt3, mt7, and mt14, 5–15% of WT activity) were greater (P < 0.05) than those seen with the intermediate ARH1 activity group (ARH1KO+mt8, mt10, mt11, and mt12, 20–30% of WT activity) and the high activity group (ARH1KO+mt1, +mt6, +mt9, and +mt13, 37–55% of WT activity) (Figures 3b and c). These data indicate that MEFs transformed with ARH1 mutant genes encoding proteins with residual ARH1 activity display anchorage-independent growth in soft agar with the number of colonies and diameters dependent on hydrolase activity.

Effects of ARH1 mutant gene on growth of MEFs in nude mice. Growth of cells in athymic nude mice was used as a measure of tumorigenecity. It was observed previously that ARH1 genotype affected tumorigenesis; ARH1KO and ARH1 heterozygous MEFs, but not ARH1 WT and ARH1KO+WT MEFs developed tumors in nude mice. Using ARH1KO MEFs transformed with ARH1 mutant genes, the effects of ARH1 mutant genes and activities of encoded proteins on subcutaneous tumor mass in athymic nude mice were determined. ARH1KO mock MEFs formed tumors in nude mice, whereas ARH1KO MEFs transformed with ARH1 WT gene did not (Figure 4). Interestingly, all ARH1KO MEFs transformed with ARH1 mutant genes formed tumors in nude mice (Figure 4). The growth rates of tumors formed in ARH1KO MEFs transformed with mutant genes were significantly (P < 0.001–0.0001) different from ARH1KO mock MEFs (Figure 4a). Also, the growth rates of tumors resulting from ARH1KO MEFs transformed with ARH1 mutant genes encoding proteins of the high catalytic activity group (ARH1KO +mt1, +mt6, +mt9, and +mt13, 37–55% of WT activity) were significantly (P < 0.001) slower than those transformed with the low ARH1 activity group (ARH1KO+mt3, +mt7 and +mt14, 5–15% of WT activity) (Figure 4a). Growth rates of tumors observed with the intermediate ARH1 activity group (ARH1KO+mt8, +mt10, +mt11, and +mt12, 20–30% of WT activity) placed between the low ARH1 activity group (ARH1KO+mt3, +mt7 and +mt14, 5–15% of WT activity) and high ARH1 activity group (ARH1KO+mt1, +mt6, +mt9, and +mt13, 37–55% of WT activity) (Figure 4b). In addition, ARH1KO MEFs transformed with a WT
gene but expressing low levels of ARH1 protein and activity (KO/W2, 9% of WT activity) developed tumors, but they grew at a slower rate (P < 0.01) than ARH1 KO MEFs transformed with ARH1 mutant genes having similar ARH1 activity (Figure 4b). Interestingly, growth of tumors seen with ARH1 KO MEFs transformed with ARH1 WT gene but expressing intermediate level of ARH1 protein and activity (KOWT6, 43% of WT activity) did not develop tumors in nude mice and were thus similar to ARH1KO+WT1 that was designated as 100% ARH1 activity. Thus, all ARH1 KO MEFs transformed with ARH1 mutant genes developed tumors, and were thus similar to ARH1KO mock MEFs rather than ARH1KO+WT MEFs. Further, the levels of expression of the WT gene and mutant gene were critical to tumor potential.

**ARH1 gene mutations in human cancer**

Based on our tumorigenesis data, it appears that ARH1 deficiency and mutations were associated with development of lung adenocarcinoma and other cancers. Next, we asked whether human tumors may have ARH1 mutations and whether the mutations would preferentially occur in exons encoding the catalytic site, as was the case in the murine model. The human cancer database used to search for ARH1 mutation data was the COSMIC database from Trust Sanger Institute, Genome Research Limited (England). Thirty-two ARH1 mutations in human cancers (for example, lung, breast and colon) were found in the COSMIC database (Table 3). ARH1 mutations were observed in human ARH1 exons 3 and 4, which are equivalent to mouse ARH1 exons 2 and 3 (Figure 5). The ARH1 mutations in human cancer were mainly missense mutations with single-base substitution (71.2%, 23 out of 32) similar to the data seen with ARH1 heterozygous mice (Supplementary Tables 1 and 2). The most frequent mutations of the coding strand were G > T (9 out of 30, 30%), G > A (6 out of 30, 20%) and C > T (6 out of 30, 20%) (Supplementary Table 2). In particular, human ARH1 gene mutations were more frequent in lung cancer (1.6%) than in cancers of other tissues (Supplementary Table 3). All ARH1 mutations in human and mouse were seen in parts of the coding region that comprise the active site (Figure 5). Some of the ARH1 sites mutated in the human gene were similar in location to those found in the mouse ARH1 gene. Also, the human ARH1 equivalent amino acid to mouse ARH1 D61, which was shown previously to be critical for ARH1 activity, is D56. It was found to be mutated in human cancer.

As tumorigenesis was observed in both ARH1-deficient and heterozygous mice, ARH1 has properties of a tumor-suppressor gene, and cancers follow a two-hit model. In agreement, we reported that 6 of 16 lung adenocarcinomas found in ARH1 heterozygous mice had LOH. We, therefore, also looked for LOH involving the human ARH1 gene as a potential mechanism for inactivation of ARH1 in human cancers. ARH1 LOH in human cancers was found in various types of tumors and tissues (Supplementary Table 4). In the human cancer database, percentage of LOH in lung (15.1%) and kidney (18.0%) cancers was greater than that observed in other tissues. Based on these data, it appears that ARH1 may participate in the pathogenesis of both human, as well as murine cancer.

**DISCUSSION**

Previously, we reported that ARH1 MEF genotype affected cell proliferation, anchorage-independent growth and tumorigenesis in nude mice. As expected, the transformed ARH1 WT gene rescued the phenotypes of the ARH1-deficient MEF mice in vitro and in vivo. ARH1 heterozygous mice and MEFs injected into nude mice developed tumors, which appeared to be the result of loss of the functioning ARH1 alleles, either by LOH or mutation.

In this report, we looked at the effects of ARH1 gene mutations on ARH1 activity in vitro, and on their effects on the cell biological properties of ARH1-deficient MEFs. We found 12 missense mutations and 2 deletion mutations in the murine ARH1 gene. Surprisingly, a wide range of ARH1 activities were observed (4–55% of WT activity) in ARH1 KO MEFs transformed with ARH1 mutant genes. As ARH1 activity was measured with an ADP-ribosyl (arginine) substrate, the differential ARH1 activities of the WT and mutated ARH1 proteins are relative to catalytic activity with ADP-ribosyl(arginine). When the WT and mutated ARH1 proteins use ADP-ribosylated proteins as substrates as would occur in vivo, the relative activities would reflect the context of the ADP-ribosylarginine in the protein quaternary structure. ADP-ribosylated protein substrates formed under physiological conditions by ARTs may prove better or worse than the model substrate for WT ARH1. Similarly, ARH1 mutations may have more or less activity toward the in vivo substrates than WT ARH1.

Furthermore, even levels of WT hydrolase were critical to tumorigenesis. ARH1 KO MEFs transformed with WT2 (9% of WT activity) formed tumors in nude mice. However, ARH1 KO MEFs transformed with W6 (43% of WT activity) and ARH1 KO MEFs transformed WT1 (100% of WT activity) did not form tumors in nude mice, whereas ARH1 KO MEFs transformed with an ARH1 mutant with activity similar to KO+WT6 resulted in tumors in nude mice. The data suggest that the mutated ARH1 proteins may also be unable to hydrolyze in vivo ADP-ribosylated protein found in the tumor, thus resulting in...
Figure 5. ARH1 mutation sites in human and murine cancer with ARH1 protein pairwise alignments. Eighty-three percent identities and 91% similarities (middle line) were seen between human ARH1 (upper line) and murine ARH1 (lower line) in ARH1 protein alignments. Highlight shows the ARH1 mutation sites in human and murine cancer.

Table 3. ARH1 gene mutations reported in human cancer

| No. | CDS mutation | AA mutation | Primary tissue | Histology | Count | Zygosity | Gender | Type |
|-----|-------------|-------------|----------------|-----------|-------|----------|--------|------|
| 1   | c.4G>T      | p.E2        | Lung           | SCC       | 1     | Unknown  | Male   | Substitution – nonsense |
| 2   | c.30G>T     | p.L10L      | Lung           | SCC       | 1     | Unknown  | Female | Substitution – coding silent |
| 3   | c.31A>C     | p.S11R      | Lung           | SCC       | 1     | Unknown  | Female | Substitution – missense |
| 4   | c.78C>T     | p.F26F      | Lung           | SCC       | 1     | Unknown  | Male   | Substitution – coding silent |
| 5   | c.113C>T    | p.A38V      | Kidney         | SCC       | 1     | Unknown  | Male   | Substitution – missense |
| 6   | c.140A>T    | p.D47V      | Breast         | Carcinoma | 1     | Heterozygous | Female | Substitution – missense |
| 7   | c.166G>A    | p.D56N      | Stomach, intestinal | AC | 1     | Unknown  | Female | Substitution – missense |
| 8   | c.218C>A    | p.A73D      | Breast         | Carcinoma | 1     | Heterozygous | Female | Substitution – missense |
| 9   | c.289C>T    | p.R97W      | Colon          | AC        | 1     | Heterozygous | Female | Substitution – missense |
| 10  | c.362C>T    | p.P121L     | Lung           | SCC       | 1     | Unknown  | Female | Substitution – missense |
| 11  | c.400C>T    | p.R134W     | Lung           | SCC       | 1     | Unknown  | Female | Substitution – missense |
| 12  | c.401C>G    | p.R134Q     | Prostate       | AC        | 1     | Unknown  | Female | Substitution – missense |
| 13  | c.438C>T    | p.S146S     | Cecum          | SCC       | 1     | Heterozygous | Female | Substitution – coding silent |
| 14  | c.481A>G    | p.M161V     | Urinary tract, bladder | Carcinoma | 1     | Unknown  | Male   | Substitution – missense |
| 15  | c.513G>A    | p.G171G     | Lung           | AC        | 1     | Unknown  | Male   | Substitution – coding silent |
| 16  | c.510delG   | p.A172fs    | Colon          | AC        | 1     | Heterozygous | Female | Deletion – frameshift |
| 17  | c.526G>T    | p.A176S     | Lung           | AC        | 1     | Unknown  | Female | Substitution – missense |
| 18  | c.577A>T    | p.K193S     | Endometrium    | Carcinoma | 1     | Heterozygous | Female | Substitution – missense |
| 19  | c.625T>G    | p.S209A     | Lung           | AC        | 1     | Heterozygous | Female | Substitution – missense |
| 20  | c.649C>G    | p.L217V     | Lung           | AC        | 1     | Heterozygous | Female | Substitution – missense |
| 21  | c.653A>C    | p.Q218P     | Ovary          | Carcinoma | 1     | Heterozygous | Female | Substitution – missense |
| 22  | c.688_699nsACCTA | p.K232fs    | Esophagus      | AC        | 1     | Unknown  | Female | Insertion – frameshift |
| 23  | c.716G>T    | p.G239V     | Lung           | AC        | 1     | Unknown  | Female | Substitution – missense |
| 24  | c.757G>T    | p.E253      | Lung           | SCC       | 1     | Unknown  | Male   | Substitution – non-sense |
| 25  | c.771C>A    | p.F257L     | Endometrium    | Carcinoma | 1     | Heterozygous | Female | Substitution – missense |
| 26  | c.844G>A    | p.R295Q     | Endometrium    | Carcinoma | 1     | Heterozygous | Female | Substitution – missense |
| 27  | c.884G>T    | p.R295L     | Lung           | AC        | 2     | Unknown  | Male   | Substitution – missense |
| 28  | c.916A>G    | p.T306A     | Lung           | SCC       | 1     | Unknown  | Female | Substitution – missense |
| 29  | c.936C>A    | p.C312      | Breast         | Carcinoma | 1     | Heterozygous | Female | Substitution – missense |
| 30  | c.944G>T    | p.G315V     | Colon          | AC        | 1     | Unknown  | Female | Substitution – missense |
| 31  | c.1017G>A   | p.E339E     | Lung           | AC        | 1     | Unknown  | Female | Substitution – coding silent |
| 32  | c.1043G>A   | p.G348E     | Breast         | AC        | 1     | Heterozygous | Male  | Substitution – missense |

Abbreviations: AC²a, adenocarcinoma; SCC³a, squamous cell carcinoma. These data were obtained from the COSMIC database (COSMIC v67, v68 and v69 release) http://cancer.sanger.ac.uk/cancergenome/projects/cosmic/.
cancers, despite the presence of ARH1 enzymatic activity. Thus, ARH1 mutation or level of ARH1 activity may affect rate of cell proliferation and tumorigenesis in nude mice.

Mono-ADP-ribosylation of proteins (that is, Gas, Ras, ExoS, P2X7 and HNP-1) on arginine residues by mammalian ARTs or bacterial ADP-ribosylating toxins affected function by different mechanisms.\(^{15,24,25,37-40}\) The resulting effects on acceptor proteins can be activating or inactivating.\(^{41}\) ART1, an arginine-specific ART expressed on the surface of airway epithelial cells and neutrophils,\(^{42,43}\) catalyzes the transfer of the ADP-ribose moiety of NAD to arginines (R14, R24) of HNP-1, inhibiting its antibacterial and cytotoxic activities.\(^{24,46}\) Cholera toxin ADP-ribosylation inhibits Ras intrinsic GTPase activity, which converts the active GTP-bound Gas to the inactive, GDP-bound Gas. ADP-ribosylation of arginine (R187) by inhibiting GTPase activity, prolongs the lifetime of the active, GTP-bound Gas. The resulting persistent activation of adenyl cyclase by Gas-GTP increases cyclic AMP content.\(^{15}\) Moreover, Pseudomonas aeruginosa exoenzyme S (ExoS) catalyzes the ADP-ribosylation of Ras at Arg41 and Arg128.\(^{37}\) ADP-ribosylated of Arg41 inhibits the binding of the guanine nucleotide exchange factor, which inhibits downstream effector (Raf) signaling.\(^{44}\)

Our finding with murine tumors may be applicable to human cancers. Some human tumors have ARH1 mutations encoding amino acids similar in position to those seen with ARH1 genes isolated from murine tumors. Based on the human somatic tumor mutation database and our results of murine ARH1 gene mutations, ARH1 appears to be tumor-suppressor gene.

**MATERIALS AND METHODS**

Materials and methods for cell culture, quantification of viable cells, isolation of proteins from cultured cells and assessment colony formation in soft agar, tumorigenicity in nude mice and cholera toxin activity are described previously.\(^{23,44,45}\)

**COSMIC database search for ARH1 gene**

Data in Table 3, Supplementary Table 1, 3 and 4 were based on the COSMIC database search for ARH1 gene mutations. Maximum tumor volume was used to compare outcome variables. All statistical tests were considered significant at the 0.05 level.

**CONFLICT OF INTEREST**

The authors declare no conflict of interest.

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ARH1 gene mutations affects tumorigenesis
J Kato et al

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