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ORIGINAL ARTICLE

AMPH-1 is a tumor suppressor of lung cancer by inhibiting Ras-Raf-MEK-ERK signal pathway

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Abstract

Amphiphysin 1 (AMPH-1) is a nerve terminal-enriched protein and it is a 128-kD protein with three identified functional domains. Some studies found that AMPH-1 was a dominant autoantigen associated with breast cancer and melanoma. However, its function in lung cancer is unknown. Here, we showed that AMPH-1 knockdown dramatically increased cell proliferation, attenuated cell apoptosis, and promoted cell cycle progression in human lung cancer cells. In vivo xenograft studies confirmed that the AMPH-1-knockdown cells were more tumorigenic than the controls. Moreover, we demonstrated that silencing AMPH-1 markedly activated Ras-Raf-MEK-ERK signal pathway. In summary, our results identified the anti-oncogenic function of AMPH-1 in lung cancer in vitro and in vivo. It is proposed that AMPH-1 may have potential as a new therapeutic target in human lung cancer treatment.

Keywords *Amphiphysin* 1 · Apoptosis · Tumorigenic · Ras-Raf-MEK-ERK signal pathway

Introduction

Lung cancer is the leading cause of cancer deaths worldwide, accounting for one third of all deaths from cancers. It is broadly divided into small-cell lung cancer (SCLC, ~20%), and non-small-cell lung cancer (NSCLC, ~80%) [1]. NSCLC comprises adenocarcinoma, bronchioloalveolar, squamous, anaplastic, and large-cell carcinomas [2]. Activating mutations of the Kras gene, found in 30 to 50% of NSCLC samples, are one of the most common genetic alterations in human lung cancer, particularly for smokers [3–5]. Mouse models for lung cancer that express mutant Kras conditionally, somatically, or inducibly have been established [6–10]. Mutant Kras alone is sufficient to initiate lung cancer in mice; however, the tumors never metastasize [11]. Whereas about 70% of patients with lung cancer present with locally advanced or metastatic disease at the time of diagnosis, 90% die of metastasis in clinic [12, 13]. The development and progression of lung cancer is a multistep process characterized by the accumulation of numerous alterations of tumor suppressor genes and oncogenes, leading to perturbations of key cell regulatory and growth control pathways [14–16]. Therefore, the simple mouse models could not mimic all the developmental stages of lung cancer.

Amphiphysin 1 (AMPH-1) is a nerve terminal-enriched protein [17]. It is a 128-kD protein with three identified functional domains, including an N-terminal Bin/amphiphysin/Rvs (BAR) domain, a middle clathrin- and adaptor-binding domain (CLAP), and a C-terminal SH3 domain [18–20]. Amphiphysin 1 is a phosphoprotein expressed at high levels in neurons, where it participates in synaptic vesicle endocytosis and neurite outgrowth [21–23], and it involves the recruitment of dynamin to sites of clathrin-mediated endocytosis [24, 25]. Until now, a few studies found that AMPH-1 was a dominant autoantigen in a small minority of stiff-person syndrome

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(SPS) patients associated with breast cancer [26–28] and melanoma [17]. Amphiphysin Ab is frequently coexpressed with other paraneoplastic antibodies and amphiphysin Ab-associated SPS is a recognized clinical entity [17]. Other studies also showed that the yeast homologs of amphiphysin, Rvs161 and Rvs167 [29, 30], were involved in the transition from exponential cell growth to stationary phase upon exposure to nutrient starvation, implicating a possible role in the biology of cancer.

In this study, we detected the function of AMPH-1 in lung cancer. We knocked down *AMPH-1* in two lung cancer cells and found that *AMPH-1* silencing attenuated cell apoptosis and promoted cell cycle progression. We further evaluated the in vivo growth of the *AMPH-1*-knockdown cells in a mouse xenograft model. Moreover, we demonstrated that silencing *AMPH-1* markedly activated Ras-Raf-MEK-ERK signal pathway. Our study demonstrated a novel function for *AMPH-1* in the suppression of lung cancer.

Materials and methods

Cell culture, RNA interference

The A549 and H441 cells were purchased from ATCC. The stable cell line was generated by integration of retroviral shRNA vectors specific for AMPH-1 or a control gene from OriGene (Rockville, MD). The siRNA against AMPH-1 (1#) shown as follows: 5′- GCGAGAACUCCGAGGAUAUTT-3′. The shRNA against AMPH-1 (2#) was 5′- GCAGGAAG CUAGUGGACUATT-3′.

Colony formation

Cells seeded in triplicate into 96 well dishes density of 5×10^4 cells per well. Individual colonies were fixed and stained with a solution containing 0.2% crystal violet in 10% ethanol for 30 min. The amount of dye was quantitated in a spectrophotometer (Thermo Scientific™) or plate reader at 570 nm.

Apoptosis assay and cell cycle analysis

Cell apoptosis was detected by annexin V-FITC/PI Apoptosis Detection Kit (Abcam, Cambridge, MA, USA) according to the manufacturer's protocols. Briefly, cells were resuspended by cold PBS at a density of 1×10^6 cells/ml. The cells were washed in ice-cold PBS, resuspended in 200 μl of binding buffer, and incubated with 5 μl annexin V-FITC solution with 5 μl of propidium iodide (PI) for 15 min at 4 °C in the dark. The results were analyzed by flow cytometry (Beckman Coulter) with wavelength emission filters of 488–530 nm for the fluorescence of annexin V and of 488–630 nm for the fluorescence of PI.

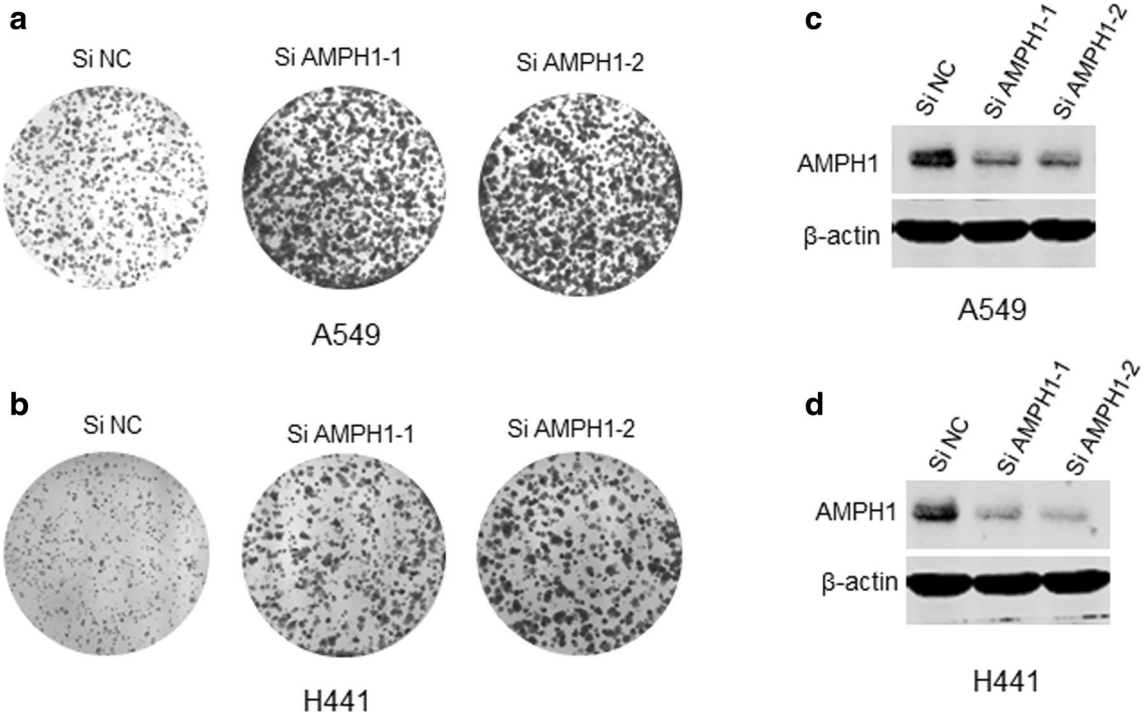

Fig. 1 Knockdown of *AMPH-1* promotes lung cancer cell growth. **a**–**b**. Soft agar colony formation and statistical analysis for A549 cells (**a**) and H441 cells (**b**) transfected with scrambled siRNA (negative control, NC) and AMPH-siRNA, respectively. **c**–**d** Western blot analysis of *AMPH-1* protein expression in A549 cells and H441 cells treated with scrambled siRNA (control) or *AMPH-1* siRNA

Western blot

For Western blot, proteins were separated on 10% SDS polyacrylamide gels and transferred to NC membranes. Total protein from each sample was loaded and immune blots were analyzed using primary antibodies. After incubation with fluorescent labeled secondary antibody, specific signals for proteins were visualized by LI-COR Odyssey Infrared Imaging System.

Antibodies were purchased from Cell Signaling (RAS, P-C-RAF, P-MEK, P-ERK, and caspase 3 cleaved), Proteintech (AMPH-1), sigma (β-Actin).

Immunohistochemistry

Immunohistochemistry (IHC) was performed for continuous sections from paraffin-embedded blocks. Antigen retrieval was performed by microwaving for 3 min in citrate-buffered solution (pH 6.0). Blocking was done by incubation with 10% goat serum at room temperature for 30 min. Sections were incubated with AMPH-1 (1:500), p-ERK (1:300), Ki67(1:200), and E-cadherin (1:500) antibodies that are indicated in this study for overnight at 4 °C. Staining with secondary antibody (conjugated with horseradish peroxidase) was performed for 1 h at room temperature. Sections were finally stained with 3, 3-diaminobenzidine tetrahydrochloride (DAB) and counterstained with hematoxylin. The images were captured with software (Leica Q-win V2.0, Germany) accompanying with Leica DMLA microscope (Leica Inc., Germany).

Xenograft experiments

The five female BALB/c nude mice at the age of 5 weeks were anesthetized. Sh*AMPH-1* and control cells were suspended in cold PBS (1×10^6 cells/100 μl) and injected

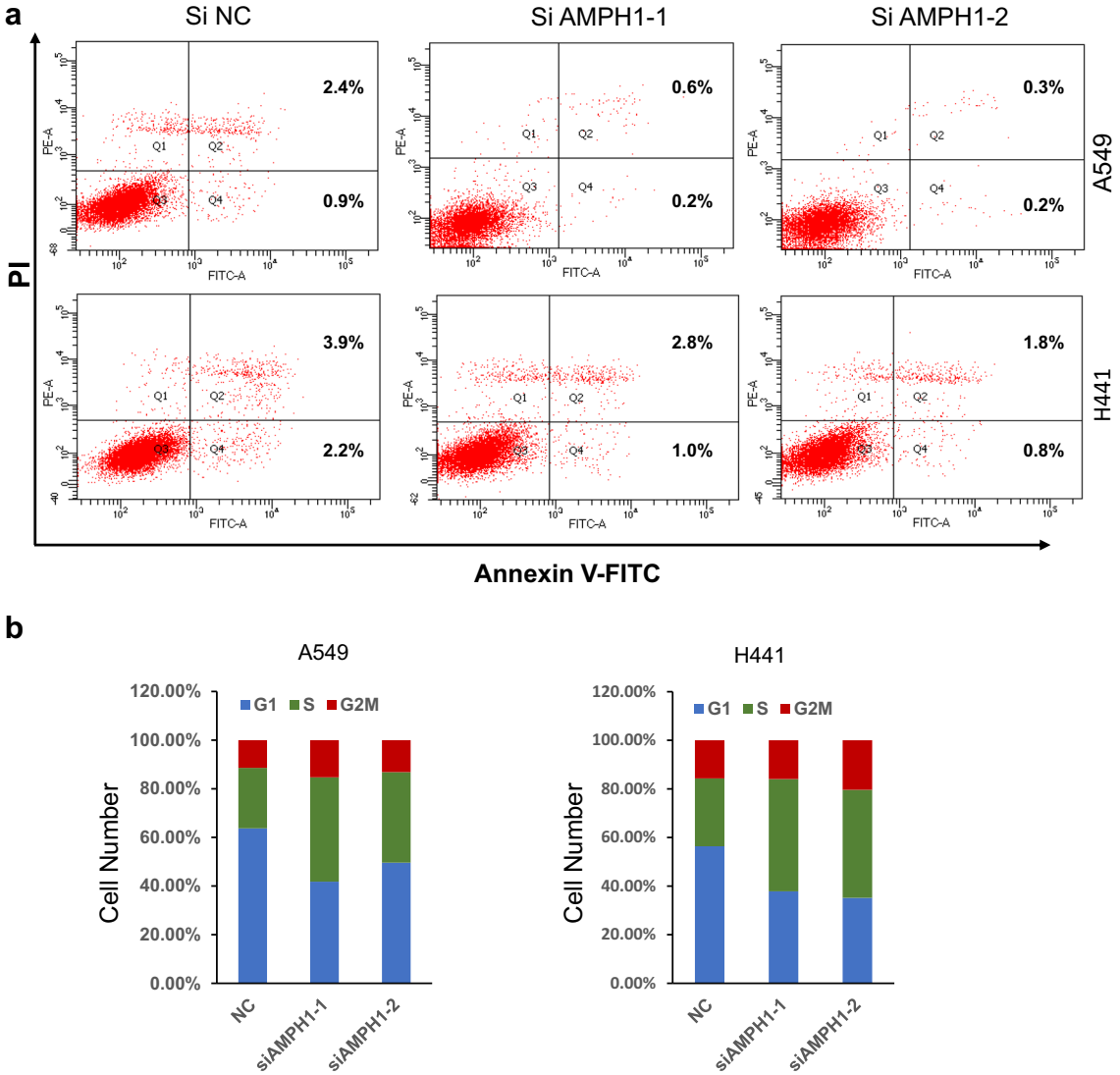

Fig. 2 *AMPH-1* silencing attenuates cell apoptosis and promotes cell cycle progression. **a** Cell apoptosis for A549 and H441 cells was evaluated by flow cytometry. FL1-H is annexin V and FL2-H is PI. **b** Cell cycle distribution analysis for *AMPH-1* knockdown (shAMPH-1) and control (shNC) A549 and H441 cells

into the mice subcutaneously on flanks. Mice were bred in the Animal Core Facility by following procedures approved by the Jiangsu Agri-Animal Husbandry Vocational College.

Results

Knockdown of *AMPH-1* promotes lung cancer cell growth

In order to determine the function of *AMPH-1* in lung cancer, we examined its role in regulation of lung cancer cell proliferation. Knockdown of *AMPH-1* by two different shRNAs dramatically increased the cell growth in A549 and H441 cells (Fig. 1a, b) using soft agar colony formation assay. In addition, Western blot showed that AMPH-1 protein levels were markedly reduced after transfection of siRNA (Fig. 1c, d).

AMPH-1 silencing attenuates cell apoptosis and promotes cell cycle progression

To determine whether tumor proliferation was affected due to cell apoptosis, A549 and H441 cells transfected with siAMPH-1 and siControl were stained with annexin V and propidium iodide (PI) and analyzed by flow cytometry. The cell ratio undergoing late apoptosis and early apoptotic cell population was reduced (Fig. 2a).

For cell cycle analysis, knockdown of *AMPH-1* in cells caused a concomitant lower G1 population, as demonstrated in Fig. 2b. Meanwhile, the S phase cell population was elevated in both cell lines with *AMPH-1* knockdown compared to control groups. Therefore, these data suggested that *AMPH-1* silencing attenuated cell apoptosis and inhibited cell cycle progression.

Knockdown of *AMPH-1* activated Ras-Raf-MEK-ERK signal pathway

The Ras-Raf-MEK-ERK pathway is primarily responsible for mitogenesis in metazoans, and mutational activation of this pathway is common in cancer. Next, we evaluated the effects of *AMPH-1* knockdown on the activity of Ras-Raf-MEK-ERK signal pathway. Western blot analyses showed that *AMPH-1* knockdown increased the levels of RAS, P-C-RAF, P-MEK, and P-ERK in A549 and H441 cells (Fig. 3a, b).

Since caspase activation is one of the characteristic symbols of cell apoptotic process, we also detected caspase 3 activity in human lung cancer cells. As expected, the cleaved caspase 3 was markedly decreased in A549 and H441 cells (Fig. 3a, b) with AMPH-1 silencing compared with their control groups.

Knockdown of *AMPH-1* promotes breast cancer growth in xenograft mouse model

To further investigate the effects of *AMPH-1* signaling on lung carcinogenesis in vivo, AMPH-1-knockdown A549 stable cells (shN and shAMPH-1) were injected into the flanks of nude mice subcutaneously. As expected, *AMPH-1*-knockdown A549 cells showed more tumorigenicity compared with the control groups (Fig. 4a). In addition, the tumor weights in AMPH-1 silencing group were significantly increased compared with these of control group (Fig. 4b). Immunohistochemical staining of RAS and P-ERK was remarkably reduced in knockdown group compared to controls (Fig. 4c). Thus, these in vivo results showed silencing *AMPH-1* promoted lung cancer growth dependent on the Ras-Raf-MEK-ERK pathway.

Discussion

The main finding of the present study showed the oncogene role of *AMPH-1* for lung cancer formation. *AMPH-1* knockdown dramatically increased human lung cancer cell proliferation in vitro and in vivo, attenuated cell apoptosis, and inhibited cell cycle progression. To gain additional insight into the mechanism, we demonstrated that silencing *AMPH-1* markedly activated Ras-Raf-MEK-ERK signal pathway.

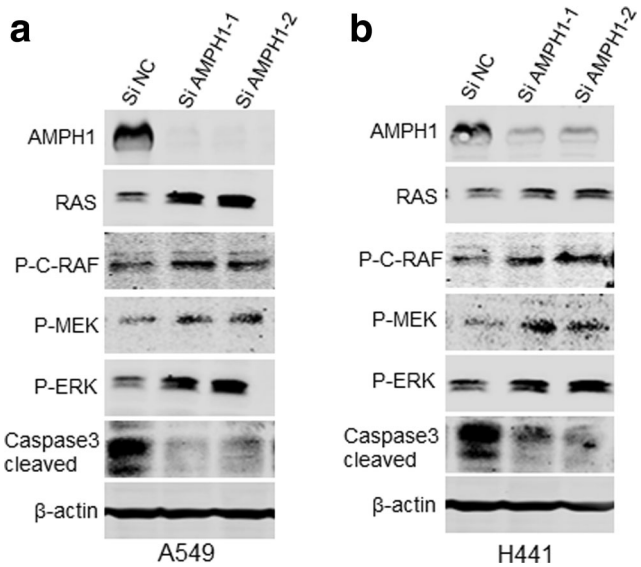

Fig. 3 Knockdown of *AMPH-1* activated Ras-Raf-MEK-ERK signal pathway. **a**–**b** A549 and H441 cells were treated with scrambled siRNA (control) or *AMPH-1* siRNA for 72 h and then collected for Western blot analysis. Total protein was isolated from A549 (**a**) and H441 cells (**b**) and analyzed by immunoblotting with the antibodies against Ras, P-C-RAF, p-MEK, caspase 3 cleaved, and P-ERK

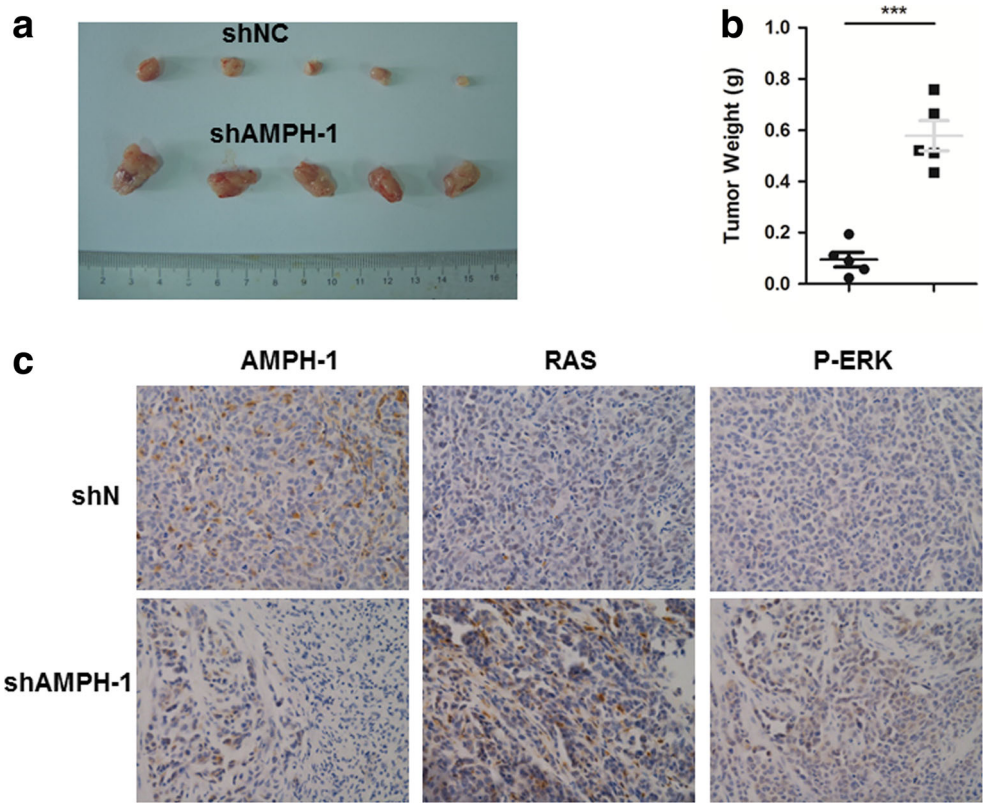

Fig. 4 Knockdown of *AMPH-1* promotes lung cancer growth in xenograft mouse model. **a**–**b** Nude mice were injected with 10^6 cells and tumors were removed at 3 weeks post injection; each experimental group contained five tumors. The AMPH-1 silencing significantly increased the final tumor size (**a**) and tumor weights (**b**). **c** Immunohistochemical staining for AMPH-1, RAS, and P-ERK

Thus, we concluded that *AMPH-1* serves as one tumor suppressor of human lung cancer formation by regulating Ras-Raf-MEK-ERK signal pathway.

Human amphiphysin 1 is a protein highly concentrated in nerve terminals [25] and it is a nerve terminal protein with a putative role in endocytosis, recognized by autoantibodies present in the serum and CSF samples from female patients with paraneoplastic stiV man syndrome (SMS) and breast cancer [18, 26, 27]. Previous studies identified anti-amphiphysin I antibodies in the serum of three patients with small-cell lung carcinoma (SCLC) [31, 32]. Similarly, in lung cancer, we found that *AMPH-1* served as one tumor suppressor. Furthermore, it was reported that amphiphysin1 had the capacity to inhibit vitronectin-mediated cell adhesion, spreading, and migration in vitro [33]. Therefore, it would be interesting to pursue some signal pathway involved by *AMPH-1*.

Activating mutations in Ras genes are observed in approximately 30% of human cancers and oncogenic K-Ras^{G12D} is one of the most common cancer-associated genetic lesions [34]. A number of previous studies have demonstrated that mutations in the kinases of the RAS-RAF-MEK-ERK transduction pathway were frequently observed in human cancer [35]. Our data indicated that Ras-Raf-MEK-ERK signaling pathway may be one of the important effectors of *AMPH-1* downstreams. However, it is still unclear if inhibition of *AMPH-1* is able to induce lung tumorigenesis. Furthermore, the clinical relevance of *AMPH-1* is also important for further study.

In conclusion, *AMPH-1* knockdown results in lung cancer cell proliferation and tumor formation and decreased cell apoptosis. Our study demonstrates a novel function of *AMPH-1* in suppression of lung cancer, indicating a tumor suppressor role in lung cancer development. Meanwhile, *AMPH-1* knockdown activated Ras-Raf-MEK-ERK signaling pathway. Therefore, therapeutic approaches aiming at activating *AMPH-1* might be an essential factor in protecting against human lung cancer formation, whose effects are mediated by Ras-Raf-MEK-ERK signaling pathway.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All experimental protocols and methods were approved by the Jiangsu Agri-Animal Husbandry Vocational College. We also confirmed that all methods were performed in accordance with the relevant guidelines and regulations. Mice were bred in the Animal Core Facility by following procedures approved by the Jiangsu Agri-Animal Husbandry Vocational College of Institutional Animal Care and Use Committee.

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