RhoC GTPase, a Novel Transforming Oncogene for Human Mammary Epithelial Cells That Partially Recapitulates the Inflammatory Breast Cancer Phenotype

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ABSTRACT

Inflammatory breast cancer (IBC) is the most aggressive form of breast cancer and is phenotypically distinct from other forms of locally advanced breast cancer. In a previous study, we identified specific genetic alterations of IBC that could account for a highly invasive phenotype. RhoC GTPase was overexpressed in 90% of IBC archival tumor samples, but not in stage-matched, non-IBC tumors. To study the role of RhoC GTPase in contributing to an IBC-like phenotype, we generated stable transfectants of human mammary epithelial cells overexpressing the RhoC gene. The HME-RhoC transfectants formed large colonies under anchorage-independent growth conditions, were more motile, and were invasive. In conjunction with an increase in motility, overexpression of RhoC led to an increase in actin stress fiber and focal adhesion contact formation. Furthermore, orthotopic injection into immunocompromised mice led to tumor formation. Taken together, these data indicate that RhoC GTPase is a transforming oncogene in human mammary epithelial cells and can lead to a highly invasive phenotype, akin to that seen in IBC.

INTRODUCTION

Primary IBC accounts for approximately 6% of new breast cancer cases annually in the United States (1). IBC is clinically well characterized by skin changes such as erythema, skin nodules, peau d’aurance, and nipple retraction (2, 3). IBC is also distinguished by a very rapid onset of characteristics of locally advanced breast cancer, typically within 6 months. Almost all tumors have lymph node metastases at the time of diagnosis, and approximately 36% have gross distant metastasis (2, 3). This number greatly increases 1 year after diagnosis, presumably due to the presence of occult metastases, suggesting that IBC cells acquire metastatic capabilities early in tumor formation. Not surprisingly, even with multimodality treatment, the 5-year disease-free survival is less than 45%, thus making IBC the most deadly form of locally advanced breast cancer (3).

Until recently, relatively little was known about the genetic mechanisms underlying the development and progression of IBC. In a previous study, our laboratory identified genes that strongly correlated with the aggressive and invasive IBC phenotype (4). In this study, we describe the potential role of one of those genes, RhoC GTPase, in modulating the invasive IBC phenotype.

RhoC GTPase is a member of the Ras superfamily of small GTPases. Activation of Rho proteins by soluble factors, such as serum or modulating the invasive IBC phenotype. In this study, we describe the potential role of one of those genes, RhoC GTPase, in modulating the invasive IBC phenotype.

RhoC GTPase was overexpressed in 90% of IBC archival tumor samples, but not in stage-matched, non-IBC tumors. To study the role of RhoC GTPase in contributing to an IBC-like phenotype, we generated stable transfectants of human mammary epithelial cells overexpressing the RhoC gene. The HME-RhoC transfectants formed large colonies under anchorage-independent growth conditions, were more motile, and were invasive. In conjunction with an increase in motility, overexpression of RhoC led to an increase in actin stress fiber and focal adhesion contact formation. Furthermore, orthotopic injection into immunocompromised mice led to tumor formation. Taken together, these data indicate that RhoC GTPase is a transforming oncogene in human mammary epithelial cells and can lead to a highly invasive phenotype, akin to that seen in IBC.

MATERIALS AND METHODS

Cell Lines. As described previously, cell lines were maintained under defined culture conditions for optimal growth in each case (8–10). Briefly, HME cells were immortalized with human papilloma virus E6/E7 (11) and grown in 5% FBS (Sigma Chemical Co., St. Louis, MO)-supplemented Ham’s F-12 medium (JRH BioSciences, Lenexa, KS) containing insulin, hydrocortisone, epidermal growth factor, and cholera toxin (Sigma Chemical Co.). The SUM149 cell line was developed from a primary IBC tumor and grown in 5% FBS-supplemented Ham’s F-12 medium containing insulin and hydrocortisone. The HME cells were characterized as being keratin 19 positive, ensuring that they are from the same differentiation lineage as the SUM149 IBC tumor cell line.

Transfection Experiments. Low passage (passage 10) immortalized HME cells were cotransfected with the PTP-200-hydro vector and either pFLAG-RhoC GTPase, pFLAG-β-gal, or pFLAG-CMV-4 (Sigma Chemical Co.) alone using FuGene 6 transfection reagent (Roche-Boehringer Mannheim, Mannheim, Germany). Transfection efficiency was 22–85% as determined by β-gal staining of the pFLAG-β-gal-transfected cells using X-Gal reagent (1 mg/ml X-Gal, 4 mM potassium ferricyanide, 4 mM potassium ferrocyanide, 2 mM magnesium chloride-hexahydrate in PBS (pH 7.4); Life Technologies, Inc., Gaithersburg, MD) and 2% formaldehyde and 0.2% glutaraldehyde in cation-free PBS (pH 7.4), Sigma Chemical Co.) for 16 h at 37°C. Stable transfectants were established by culturing the cells in the described medium supplemented with 100 μg/ml hygromycin (Life Technologies, Inc.) for 14–20 days. Expression of the transgene was determined by RT-PCR and immunoprecipitation followed by Western blot analysis.

Constructs. RhoC GTPase from the SUM149 cell line was amplified by RT-PCR and cloned using the pGEM-T Easy kit (Promega, Madison, WI). The full-length RhoC GTPase cDNA was sequenced and checked against the published sequence of any mutations. Using EcoRI (Roche-Boehringer Mannheim), the RhoC cDNA was excised and ligated into pFLAG-CMV-4 (Sigma Chemical Co.) and resequenced.

Expression Analysis of RhoC GTPase Transfectants. Total RNA was isolated from cells using Trizol reagent (Life Technologies, Inc.) according to the manufacturer’s recommendations. One μg of total RNA was converted to cDNA using an avian myeloblastosis virus reverse transcription system (Promega). A 100-μg aliquot of the resulting cDNA was amplified in a double PCR with 25 ng each of pFLAG/GAPDH- or RhoC GTPase/GAPDH-specific primers. PCR products were separated on a 1.2% agarose gel and imaged on an Alpha Image 950 documentation system (Alpha Innotech, San Leandro, CA). Densitometry of images was performed using NIH Image version 1.62.
Proteins were harvested from cell cultures using radioimmunoprecipitation assay buffer (1× PBS, 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS, 0.1 mg/ml phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate, and 0.3 mg/ml aprotonin; Sigma Chemical Co.). Whole cell lysates (100 μg) were precleared using normal mouse IgG and protein A/G-agarose (Santa Cruz Biotechnology Inc., Santa Cruz, CA). The cleared supernatants were incubated overnight with antibodies specific for the FLAG epitope (Eastman Kodak, Rochester, NY). Antibody-bound proteins were precipitated after the addition of protein A/G-agarose and washed four times with 1× PBS. Immunoprecipitates were resuspended in 20 μl of 2× Laemmli electrophoresis buffer, boiled for 3 min, and centrifuged briefly to pellet the free protein A/G-agarose. Samples were separated by SDS-PAGE on an 8% gel under nondenaturing conditions and transferred to a nitrocellulose membrane. Nonspecific binding was blocked by overnight incubation with 2% powdered milk (Kroger, Cincinnati, OH) in Tris-buffered saline with 0.05% Tween 20 (Sigma Chemical Co.). Anti-FLAG immunoprecipitate blots were incubated with the FLAG antibody. Protein bands were visualized by ECL (Amersham-Pharmacia Biotech, Piscataway, NJ).

Monolayer Growth Rate. Monolayer culture growth rate was determined as described previously (12) by conversion of MTT (Sigma Chemical Co.) to a water-insoluble formazan by viable cells. Three thousand cells in 200 μl of medium were plated in 96-well plates and grown under normal conditions. Cultures were assayed at 0, 1, 2, 3, 5, and 7 days by the addition of 40 μl of 5 mg/ml MTT and incubation for 1 h at 37°C. The MTT-containing medium was aspirated, and 100 μl of DMSO (Sigma Chemical Co.) were added to lyse the cells and solubilize the formazon. Absorbance values of the lysates were determined on a Dynatech MR 5000 microplate reader at 540 nm.

Anchorage-independent Growth in Soft Agar. A 2% stock of sterile, low melting point agarose was diluted 1:1 with 2× MEM. Further dilution to 0.6% agarose was made using 10% FBS-supplemented Ham’s F-12 medium complete with growth factors, and 1 ml was added to each well of a 6-well plate as a base layer. The cell layer was then prepared by diluting agarose to 0.3% and 0.6% with 10³ cells in 2.5% FBS-supplemented Ham’s F-12 medium/1.5 ml/well. Colonies greater than or equal to 100 μm in diameter were counted after a 3-week incubation at 37°C in a 10% CO2 incubator.

Invasion through a Matrigel-coated Filter. The invasion assay was performed as described previously, with minor modifications (12). A 10-μl aliquot of Matrigel (10 mg/ml) was spread onto a 6.5-mm Transwell filter with 8 μm pores (Costar, Corning, NY) and air dried in a laminar flow hood. Once dried, the filters were reconstituted with a few drops of serum-free medium. The lower chamber of the Transwell was filled with either serum-free or serum-containing media. Cells were harvested and resuspended in serum-free medium with 0.1% BSA at a concentration of 3.75 × 10⁵ cells/ml, and 0.5 ml of cell suspension was added to the top chamber. The chambers were incubated for 24 h at 37°C in a 10% CO2 incubator. The cell suspension was aspirated, and excess Matrigel was removed from the filter using a cotton swab. The filters were then cut away from the Transwell assembly and fixed with methanol, gel side down, to a glass microscope slide. The fixed filters were stained with H&E, and the cells in 20 random ×40 magnification fields counted. These cells were assumed to have invaded the Matrigel/filter. The number of cells that had invaded the serum-free containing lower chambers was considered background, and this number was subtracted from the number of cells that had invaded in response to the serum-containing medium.

Random Motility Assay. Random motility was determined using a gold-colloid assay (13). Gold-colloid was layered onto glass coverslips and placed into 6-well plates. Cells were plated onto the coverslips and allowed to adhere for 1 h at 37°C in a CO2 incubator (12,500 cells/3 ml). To stimulate the cells, the serum-free medium was replaced with 5% FBS-containing Ham’s F-12 medium supplemented with growth factors and allowed to incubate for 3 h at 37°C. The medium was aspirated, and the cells were fixed using 2% glutaraldehyde. The coverslips were then mounted onto glass microscope slides, and areas of clearing in the gold-colloid corresponding to phagokinetic cell tracks were counted.

Rhodamine-Phalloidin Staining of Actin Filaments. Visualization of actin filaments was accomplished by staining with a conjugated phalloidin. Cells were grown on glass coverslips for 48 h and washed with PBS followed by fixation with 1:1 ice-cold acetone and methanol. After a 30-min incubation in PBS containing 1% BSA, 5 μl of methanolic rhodamine-phalloidin stock (Molecular Probes, Eugene, OR) were added to each coverslip and allowed to stain for 20 min at room temperature. After repeated washing with PBS, the coverslips were mounted onto glass microscope slides using Gel/Mount (Bio-media Co., Foster City, CA). Cells were visualized under an Olympus fluorescence microscope equipped with a 573 nm filter.

Orthotopic Injection into Nude Mice. Ten-week-old female NCR athymic nude mice were orthotopically injected with HME-, HME-β-gal-, or HME-RhoC GTPase-transfected cells. Injection into the MFP of 20 mice/group was performed as described previously (14). Briefly, cells were harvested by trypsinization, washed, and resuspended in HBSS at a concentration of 1.5 × 10⁵ cells/0.2 ml. Mice were anesthetized using 10 mg/ml ketamine, 1 mg/ml xylazine, and 0.01 mg/ml glycopyrrolate, and an incision below the thoracic MFP was made. Using a 27-gauge needle, the cell suspension was injected directly into the expected MFP, and the wound was closed with a single wound clip. Tumor growth measurements were taken weekly until tumors reached 2.5 cm in diameter. Mice were necropsied, and tumor tissues were dissected for histological analysis.

RESULTS

Expression of RhoC GTPase in Immortalized HME Cells Induces Anchorage-independent Growth but Does Not Alter the Monolayer Growth Rates. To study the effect of RhoC GTPase overexpression on the phenotype of HPV-immortalized HME cells, stable HME-RhoC GTPase-overexpressing transfectants were established. Quantitative RT-PCR using primers specific for the pFLAG-CMV vector and for RhoC GTPase verified expression levels of the RhoC transgene (Fig. 1A). Levels of RhoC GTPase expression were found to be 6–20-fold higher in RhoC GTPase transfectants when compared with nontransfected HME cells.

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compared with the untransfected or HME-β-gal control-transfected HME cells. This level of expression is comparable with that of the SUM149 IBC cell line, which was found to have an 8-fold higher expression of RhoC as compared with normal HME cell lines and non-IBC breast cancer cell lines as determined by Northern blot analysis (4). Also, this level of expression was comparable to archival IBC tumor specimens, which were previously found to have 5–12-fold higher levels of RhoC expression compared with the surrounding normal tissue as determined by in situ hybridization (4). Expression of the FLAG-tagged RhoC GTPase protein was confirmed by immunoprecipitation with an anti-FLAG antibody followed by immunoblotting with the same antibody (Fig. 1B).

Because the Rho proteins can affect activation of transcription (15), we studied the monolayer growth rate of eight clones of the HME-RhoC transfectants. As shown in Fig. 2A, no significant changes in the monolayer growth rates were observed for any of the HME-RhoC transfectant clones or the mixed polyclonal population, as compared with the control cell lines using the MTT assay. Furthermore, regardless of quantitative differences in the level of RhoC transgene expression, no difference in growth rate was observed between the various clones and the polyclonal population.

When tested for their ability to grow under anchorage-independent conditions, which is a strong indicator of malignant transformation, the HME-RhoC transfectants formed 6–176-fold more colonies than the control cell lines. As shown in Fig. 2B, the number of colonies greater than 100 μm in size formed by individual HME-RhoC clones ranged from 6–176 colonies/well in comparison to the HME-β-gal cells, which had a range of 0–6 colonies/well. This increase in anchorage-independent growth directly correlated with levels of expression of the RhoC transgene. The polyclonal population had the highest level of colony formation, and this recapitulated the behavior of the SUM149 IBC cell line in soft agar. The SUM149 cell line expressed RhoC GTPase at 8-fold higher levels than the HMEs. In the same assay, using the less stringent condition of 0.3% soft agar, the average HME-RhoC transfectant colony size was greater than 600 μm.

Overexpression of RhoC GTPase Produces a Motile and Invasive Phenotype in Immortalized HME Cells through Formation of Actin Stress Fibers and Focal Adhesion Points. The term IBC was coined in 1924 to describe the appearance of the skin overlaying the affected breast (16). This term is somewhat of a misnomer because IBC is not characterized by significant infiltration of inflammatory cells. The inflamed appearance of the skin is due to edema caused by...
invasion into and obstruction of the dermal lymphatics by tumor emboli (3).

Rho proteins are most noted for their ability to control polarity, protrusion, and adhesion during cell motility by rearranging the actin cytoskeleton (6). Given the clinical observations, coupled with the finding that RhoC GTPase is overexpressed in patient tumor samples (4), we sought to ascertain whether RhoC GTPase overexpression could generate highly invasive and motile HME cells.

Using a modified blind-well chamber model, we measured the amount of migration of cells across a porous membrane coated with the extracellular matrix conglomerate Matrigel in response to a chemoattractant. In this assay, all cell lines tested were invasive to varying degrees after 24 h. The data in Fig. 3 are expressed as the fold increase in invasion observed over the invasion level of the untransfected HME cell line. The HME-β-gal control-transfected cell line was 1.5-fold more invasive than the untransfected cells, whereas the HME-RhoC transfectants were, on average, 5-fold more invasive than either the untransfected or HME-β-gal cell lines. In comparison, the SUM149 IBC cell line was 4-fold more invasive than the control (untransfected and β-gal) HME cell lines. As was observed previously in the anchorage-independent growth assays, the ability to invade directly correlated with the level of RhoC GTPase expression of the HME-RhoC transfectant clones. Invasion rates from 1–19-fold over the control cell lines were observed, depending on the RhoC transgene expression levels. These data demonstrate that expression of RhoC GTPase directly correlates with the ability of the cells to invade, with a clear threshold effect dependent on RhoC expression level.

Similar results were obtained when the cells were assessed for their ability to move in response to serum stimulation during a random motility assay. Fig. 4A depicts representative photomicrographs of a gold-collloid random motility assay comparing HME-β-gal and HME-RhoC transfectants. In this assay, cells are layered onto a gold-collloid, on which motile cells produce tracks that can then be quantitated. The data depicted in Fig. 4B are represented as a percentage of the total number of cells of each clone that moved in response to serum activation over a 3-h period. In this assay, we found that more than 90% of the HME-RhoC cells were highly motile in response to serum stimulation. This was true for the different HME-RhoC GTPase clones. In comparison, only 10% of the HME-β-gal control-transfected HME cells were motile under the same conditions. Furthermore, in this assay, none of the untransfected HME cells were motile. Rho proteins are reported to form actin stress fibers and focal adhesion points, whereas rac and Cdc42 are involved in lamellipodia and filopodia formation (17). The dynamic balance of formation and reformation of these structures leads to cellular motility. Recent evidence has implicated Rho in the control of lamellipodia through activation of Rho effector proteins (18). To test whether RhoC GTPase was effecting the motility of the HME transfectants through the formation of stress fibers and focal adhesions, we stained the cells with a rhodamine-tagged phalloxin that targets actin structures and compared the patterns with those of the HME controls (untransfected and β-gal HME cells). As seen in Fig. 5, B and C, focal adhesion points and actin stress fibers are clearly visible and abundant in the HME-RhoC transfectants. The same was true for the SUM149 IBC cell line (data not shown). These prominent structures were not seen in the untransfected HME cells (data not shown) or in HME-β-gal control-transfected cells (Fig. 5A). The HME-RhoC clone 3 cell highlighted in Fig. 5B clearly shows actin stress fiber formation emanating from a focal adhesion point and leading to a discernible lamellipodial extension. Taken together, these features imply much greater motility and ability to effect cytoskeletal reorganization in the RhoC transfectants than control HMEs.

Malignant Transformation of HME Cells by RhoC GTPase. The HME-RhoC GTPase transfectants were assessed for their ability to form tumors in athymic nude mice. The SUM149 IBC cell line, untransfected HME, HME-β-gal, and the polyclonal population of the HME-RhoC GTPase transfectants were each injected into the MFP of female nude mice. As shown in Table 1, none of the untransfected HME cells or HME-β-gal cells formed tumors in nude mice. Untransfected HME cells have never been known to form tumors in nude mice. In contrast, both the SUM149 IBC cell line and the Rhoc GTPase HME cells grew tumors in nude mice, in 86% (17 of 20) and 25% (5 of 20) of cases, respectively. The incidence of tumor growth by the SUM149 cell line is comparable with the SUM190 cell line, the only other known available IBC cell line (data not shown). These data indicate that overexpression of RhoC GTPase is weakly sufficient for malignant transformation of immortalized HME cells and that other genetic abnormalities in the IBC cell lines derived from established tumors contribute to the full-blown malignant phenotype.
DISCUSSION

Differential display and more recently, gene arrays, are leading to the isolation of many genes with altered patterns of expression in cancer. The task of sorting out their specific role and relative importance in the cancer phenotype is, in general, exceedingly complex. In the case of IBC, however, our previous work indicated that RhoC GTPase overexpression is specifically correlated with this phenotype in contrast to slow-growing locally advanced breast cancers. By exploiting insight from the clinical behavior of IBC, we focused on sorting out the specific contributions of RhoC GTPase to the IBC phenotype. In deciding how to go about defining the role of RhoC GTPase in IBC, we also considered the known functions of the Rho family members.

The mammalian Rho GTPase family can be divided into six different groups with the following members: (a) Rho (RhoA, RhoB, and RhoC); (b) Rac (RhoG and Rac1–3); (c) Cdc42 (Cdc42Hs, G25K, and TC10); (d) Rnd (Rho6, Rho7, and RhoE); (e) RhoD; and (f) TTF (19). RhoA, RhoB, and RhoC share a high degree of homology with one another, with RhoA and RhoC being the most homologous (20). Studies performed on Swiss 3T3 cells have helped to define the role that each of the Rho GTPase family members plays in controlling cytoskeletal reorganization (21). Rho controls actin stress fiber and focal adhesion contact formation, whereas rac and Cdc42 are responsible for the formation of lamellipodia and filopodia, respectively (17). Several upstream pathways that activate Rho as well as the downstream targets of activated Rho have been identified (15, 22–25). The ability of Rho to form stress fibers and focal adhesions is due to the phosphorylation and activation of two of these targets, Rho-kinase/ROK/ROCK (24, 26, 27) and the myosin-binding subunit of myosin phosphatase (18, 28). Phosphorylation of myosin-binding subunit by Rho inhibits myosin phosphatase, whereas activated Rho-kinase directly phosphorylates the myosin light chain. The phosphorylation of myosin light chain regulates the formation of stress fibers and focal adhesion contacts. Rho-kinase can also phosphorylate adducin, which leads to cell membrane ruffling and cell motility (29). It is also becoming clear that the various members of the Rho family (Rho, rac, and Cdc42) are able to undergo molecular “cross-talk,” creating dynamic molecular interactions leading to cell motility (30, 31).

There is increasing evidence that the effects of Rho family mem-
even at lower (but still increased) expression levels, stress fiber and expected, there appears to be a threshold effect at the cellular level; expression of RhoC GTPase influenced the level of bulk motility. As seen with anchorage-independent growth, the level of expression of activated Rho GTPase can affect the expression of molecules, such as cadherins, that are integral in signal transduction pathways, thereby influencing transcription of other downstream genes (34, 35). Transcription of other genes, including those influencing motility, can also be regulated directly through activation of the mitogen-activated protein kinase subfamilies c-Jun NH2-terminal kinase (35), and p38 kinase (36).

Our initial finding that Rhoc was a key molecule in an invasive cancer such as IBC was consistent with observations of other groups in different tumor types (37). Suwa et al. (37) established a correlation between Rhoc GTPase overexpression and the progression and prognosis of aggressive ductal adenocarcinoma of the pancreas. Other studies have demonstrated that activated Rho is an integral component for mediating cell motility required for producing intrahepatic metastasis of hepatocellular carcinoma (39).

Overexpression of Rhoc GTPase in immortalized HME cells produced a striking phenotypic change that, for the most part, recapitulates that of the SUM149 IBC cell line. The extent of the phenotypic change appears to correlate with the level of Rhoc expression in the various HME-Rhoc clones. However, the highest expressers do not completely recapitulate the SUM149 cell line, suggesting that other genetic alterations in the IBC cell line contribute to the full-blown malignant phenotype of the SUM149 cell line, other genes, such as Ras, p53, or antiapoptotic genes may need to be altered as well. Therefore, overexpression of Rhoc GTPase represents a single but important component of HME transformation.

The set of experiments that test the potential oncogenicity of a gene vary according to cell type and study. However, in general, they comprise a battery of assays that test in vitro- and in vivo-specific qualities of the phenotypic behavior (40, 41). The experiments we chose for this work were guided by the specific IBC phenotypic characteristics of the human tumors and the IBC parent cell line (SUM149) as well as by the putative function of the Rhoc proteins. For this particular system, we conclude that all of our experiments credential Rhoc GTPase as an oncogene in breast cancer, which, to a significant extent, recapitulates the invasive behavior of our IBC model system. In addition, given the specific role that Rhoc plays in the invasive phenotype of IBC, interfering with its function suggests a new therapeutic target in a particularly challenging form of breast cancer. As the upstream and downstream signaling pathways are explored, these too may present new targets for therapeutic intervention.

### Table 1 Tumor take for various cell lines

<table>
<thead>
<tr>
<th>Cell line</th>
<th>No. of mice with tumor</th>
<th>Tumor take (%)</th>
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<tbody>
<tr>
<td>HME</td>
<td>0/20</td>
<td>0</td>
</tr>
<tr>
<td>HME-β-gal</td>
<td>0/20</td>
<td>0</td>
</tr>
<tr>
<td>HME-RhoC</td>
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<td>25</td>
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<td>SUM149</td>
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References:


Unpublished observations.
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