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Establishment of a Mammary Carcinoma Cell Line from Syrian Hamsters Treated with N-Methyl-N-Nitrosourea

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Abstract

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CONFLICTS OF INTERESTS

The author(s) declare that they have no conflicts of interests.

AUTHORS' CONTRIBUTIONS

BC conducted the initial *in vivo* tumor growth analysis and the soft agar assay. BDL assisted with the experimental design and helped to draft the manuscript. EAO assisted with the animal follow-up, necropsies, and tumor identification. HEK-H designed the carcinogenesis study administered the MNU, assisted with the animal studies, and helped to draft the manuscript. JMM conceived of the study, participated in the experimental design, coordinated the experiment performed, and helped to draft the manuscript. MAC assisted with the hamster MNU studies, performed the animal follow-ups, and initiated the culture of the HMAM4 cells. MAS-K assisted with the experimental design, data analysis, and helped to draft the manuscript. NL performed the tamoxifen inhibition study. SBh assisted with the experimental design and helped to draft the manuscript. SBr performed the HMAM4 and HMAM5 cell line growth characterization and assisted with the detailed *in vivo* tumor growth analysis. SZ performed the histopathological analysis of the tumors. YYM conducted the detailed *in vivo* tumor growth analysis. X-LL performed the immunohistochemical analysis. All authors read and approved the final manuscript.

Conflict of Interest Statement

None Declared

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Clearly new breast cancer models are necessary in developing novel therapies. To address this challenge, we examined mammary tumor formation in the Syrian hamster using the chemical carcinogen N-methyl-N-nitrosourea (MNU). A single 50 mg/kg intraperitoneal dose of MNU resulted in a 60% incidence of premalignant mammary lesions, and a 20% incidence of mammary adenocarcinomas. Two cell lines, HMAM4A and HMAM4B, were derived from one of the primary mammary tumors induced by MNU. The morphology of the primary tumor was similar to a high-grade poorly differentiated adenocarcinoma in human breast cancer. The primary tumor stained positively for both HER-2/neu and pancytokeratin, and negatively for both cytokeratin 5/6 and p63. When the HMAM4B cell line was implanted subcutaneously into syngeneic female hamsters, tumors grew at a take rate of 50%. A tumor derived from HMAM4B cells implanted into a syngeneic hamster was further propagated *in vitro* as a stable cell line HMAM5. The HMAM5 cells grew in female syngeneic hamsters with a 70% take rate of tumor formation. These cells proliferate *in vitro*, form colonies in soft agar, and are aneuploid with a modal chromosomal number of 74 (the normal chromosome number for Syrian hamster is 44). To determine responsiveness to the estrogen receptor (ER), a cell proliferation assay was examined using increasing concentrations of tamoxifen. Both HMAM5 and human MCF-7 (ER positive) cells showed a similar decrease at 24 h. However, MDA-MB-231 (ER negative) cells were relatively insensitive to any decrease in proliferation from tamoxifen treatment. These results suggest that the HMAM5 cell line was likely derived from a luminal B subtype of mammary tumor. These results also represent characterization of the first mammary tumor cell line available from the Syrian hamster. The HMAM5 cell line is likely to be useful as an immunocompetent model for human breast cancer in developing novel therapies.

Keywords

adenocarcinoma; breast cancer; HMAM5 cell line; mammary tumor; MNU; N-methyl-N-nitrosourea; Syrian hamster

1. INTRODUCTION

Well characterized cancer cell lines are powerful research resources for studying cancer biology and new therapeutic strategies against this disease. Many of these cell lines represent the genetic alterations and features of breast cancer progression observed in human breast cancer. These cell lines allow for *in vivo* examination of complex multicellular interactions involved in the initiation and progression of breast cancer, using human xenograft cells implanted into immunocompromised animals and syngeneic rodent cells implanted into immunocompetent animals [1]. However, because breast cancer represents a heterogeneous molecular disease [2], no individual model system can adequately reflect its nature. Thus, additional breast cancer model systems are clearly necessary.

The Syrian hamster has been used for a number of different carcinogenesis and cancer chemoprevention models, but historically these have not included mammary carcinogenesis. These models have included the hamster cheek pouch model [3], in which polycyclic aromatic hydrocarbons such as 7,12-dimethylbenz[a]anthracene (DMBA) are painted on the cheek pouch either as a complete carcinogen or as the initiating agent followed by a tumor promoter such as benzoyl peroxide. The tumors that develop are typically squamous cell carcinomas that contain a mutation in codon 61 of the Ha-ras gene. In addition, Syrian hamsters are susceptible to diethyl-stilbestrol (DES) induced renal carcinomas, in which the kidneys are estrogen sensitive [4] and may behave more like breast cancer in this species; this was the basis for establishing a hamster renal carcinoma cell line [5]. Syrian hamsters also develop cholangiocarcinomas, bile duct hyperplasia, and pancreatic cancer when exposed to aromatic amines such as N-nitrosobis(2-oxopropyl)amine (BOP), which can be

partially prevented by certain phytochemicals [6, 7]. Inducing pancreatic cancer, however, requires augmentation pressure, which includes feeding the hamsters a choline-deficient diet and subjecting the animals to two to three rounds of ethionine-methionine-BOP injections [8].

The chief objective of this project was to establish a mammary cancer model in the Syrian hamster for investigating the potency of novel therapies alone and in combination with standard chemotherapy agents and radiotherapy. For example, the Syrian hamster has been identified as an animal model for oncolytic adenovirus vectors with promising results [9, 10]. The adenovirus is able to infect, replicate, and spread from cell-to-cell in cancer cell lines of this animal. Adenovirus replicates in the lungs, liver, and other organs [11]. However, hamsters are resistant to mammary cancer, and few hamster studies have been reported [12, 13]. Therefore, our initial effort was to establish mammary tumor models in the Syrian hamster using the chemical carcinogen N-methyl-N-nitrosourea (MNU). MNU-induced mammary carcinoma in rats is a well-established animal model for breast cancer, exhibiting similarities to human ER+ breast tumors [14]. This design was modeled after the rat mammary MNU model [14], in which rats were treated with a single dose of MNU (50 mg/kg body weight) at 50 days of age. In rats, this protocol leads to mammary tumor latency of approximately 4 weeks, with an incidence of 85 to 100% and a multiplicity of approximately 3.5 mammary tumors per animal. Most mammary tumors in rats resulting from this protocol were adenocarcinomas; other tumor types were rare [15].

In the current study, a 50 mg/kg dose of MNU resulted in a 60% incidence of premalignant mammary lesions, and a 20% incidence of mammary adenocarcinomas. One mammary tumor induced by MNU was used for the initiation and characterization of a new mammary carcinoma cell line HMAM4B. One tumor derived from HMAM4B cells implanted into a syngeneic hamster was further propagated as a stable cell line HMAM5; these cells grew in female syngeneic hamsters with a 70% take rate of tumor formation. These cell lines are likely to be useful as a model for human breast cancer in developing novel therapies.

2. MATERIALS AND METHODS

2.1 Syrian hamsters

Female inbred Syrian (Golden) hamsters (*Mesocricetus auratus*) at 4 weeks of age were obtained from Harlan (Prattville, AL animal colony) and housed in a temperature- and humidity-controlled AAALAC approved facilities with a 12 h light/dark cycle. All procedures were approved by the LSUHSC Institutional Animal Care and Use Committee (IACUC) or Southern Research Institute IACUC in accordance with NIH and USDA guidelines. The hamsters were housed one or two per cage, provided environmental enrichment, and allowed access to food and water *ad libitum*. The hamsters were acclimated to the laboratory conditions and handled at least 2 days/week to become accustomed to the human touch.

2.2 MNU experimental design

In the current experiment, female Syrian hamsters were acclimated for 2 weeks and at six weeks of age, were dosed once with MNU (50 or 100 mg/kg body weight) by intraperitoneal (i.p.) administration. MNU (Sigma; St Louis, MO) was dissolved in sterile acidified 0.9% saline immediately before use. Control hamsters received saline only. The hamsters were monitored by daily observation and palpation and weekly body weight measurement, and any tumors observed were recorded. Hamsters appearing to be moribund or in physical distress were sacrificed by CO₂ asphyxiation.

2.3 Histology

Necropsies were performed whenever possible: normal organ tissues and tumors were dissected, placed into cassettes, and fixed in 10% neutral buffered formalin overnight at 4° C, then changed to 50% ethanol. The cassettes were then rinsed and the tissues paraffin embedded using an automatic tissue processor. Afterwards, 5 μ tissue sections were cut and mounted on slides. All sections were deparaffinized, lightly counterstained with hematoxylin and eosin (H&E) and mounted with Permount and cover-slipped.

Immunohistochemical staining was performed using an automated processor (BioGenex I6000 Automated Staining System, San Ramon, CA) as described previously [16] under the following conditions: sections were incubated with either rabbit polyclonal anti-HER-2/neu (Abcam; Cambridge, MA) at 1:100 dilution, mouse monoclonal anti-pancytokeratin (Abcam) at 1:300 dilution, mouse monoclonal anti-cytokeratin 5 and 6 (Ventana Medical Systems; Tucson, AZ) at 1:100 dilution, mouse monoclonal anti-p63 (Ventana) at 1:100 dilution for 1 h. After incubation with secondary antibody (MultiLink-BioGenex Super Sensitive Link-Label IHC Detection System) for 30 min, sections were incubated with horseradish peroxidase label (BioGenex) for 15 min and exposed to diaminobenzidine (DAB) substrate for 3 min. Positive and negative (no primary antibody) controls were included in every experiment.

2.5 Initiation of cell lines

Hamsters were necropsied under sterile conditions. Briefly, after euthanasia, hamsters were immersed in betadine for 10 min, followed by immersion in 70% ethanol for 10 min. In a sterile laminar flow hood, using pre-sterilized equipment, the mammary tumors were dissected away from the underlying muscle using a scalpel and then finely minced into small pieces ($\leq 1 \text{ mm}^3$). The tumor fragments were soaked in penicillin (1 kU/ml), streptomycin (1 mg/ml) for 30 min, rinsed with PBS, then distributed into 75 cm^2 cell culture flasks, with Dulbecco's Modified Essential Medium (DMEM) containing 4,500 mg/L D-glucose, L-glutamine, 110 mg/L sodium pyruvate, and pyridoxine hydrochloride (Invitrogen; Carlsbad, CA), supplemented with 15% fetal bovine serum (FBS; Invitrogen), HEPES buffer (15 mM), penicillin (100 U/ml), streptomycin (100 $\mu\text{g}/\text{ml}$) and insulin (4 $\mu\text{g}/\text{ml}$). The culture flasks were incubated at 37° C in 95% air and 5% CO_2 . All tissue culture medium was removed and replaced with fresh medium every 7 days. Most of the tissues adhered after 24 hours accompanied by the migration of dividing cells outward, which were observed by microscopy.

2.6 Karyotyping

Karyotype analysis was performed by using a standard Giemsa banding method [17]. Metaphase HMAM4 cells were prepared and 5 G-banded karyotypes were analyzed. One hundred cells were analyzed for chromosome count distribution on Giemsa-stained chromosomes. Any cells with chromosome aberrations such as gaps, breaks, and acentric fragments were recorded.

2.7 Growth characterizations

Cells were plated at 1×10^4 cells/well in 6-well tissue culture plates. Every 24 h, three replicative plates were trypsinized and vital cells were counted with a hemocytometer. A growth curve was established, and the population doubling time was estimated from its exponential growth phase.

2.8 Determination of anchorage independence

Anchorage independence was assayed by growth in 0.5% agar in complete DMEM containing 10% FBS. Positive colonies greater than 50 cells were scored after 3 weeks in culture.

2.9 Proliferation assays

The human estrogen receptor (ER) positive breast cancer cell lines MCF-7 and the human ER negative breast cancer cell line MDA-MB-231 were obtained from the American Type Culture Collection (Manassas, VA). Cells were plated at 1×10^3 cells/well in 96-well tissue culture plates. After 24 h, the media was changed and cells were treated with tamoxifen (3.3 – 100 μ M) for 24 h. Subsequently, cell proliferation was quantified using a 5-bromo-deoxyuridine (BrdU) labeling kit (Roche Diagnostics Corp.; Indianapolis, IN) according to the manufacturer's instructions. Briefly, a BrdU labeling solution was added to each well for 16 h. Afterwards, the cells were fixed and incubated with an anti-BrdU antibody (conjugated with horseradish peroxidase), and the immune complexes were detected using a 3,3'-5,5'-tetramethylbenzidine (TMB) substrate reaction. The reaction products were quantified by measuring the absorbance using a multi-well ELISA plate reader. Results are expressed as a percentage of the results from tamoxifen treated cells relative to untreated cells, in which the proliferation of untreated cells equals 100%.

2.10 Establishment of an *in vivo* syngeneic tumor model

To determine the efficacy of tumor take in syngeneic female Syrian hamsters, tumors were established by subcutaneous (s.c.) injection of cultured cells each into the hind flanks of the animals. After shaving the animals at the site of injection, 1×10^7 cells were implanted and established tumors were monitored for growth using digital calipers. Tumor volume was determined by measuring the smallest (d) and largest (D) diameters with calipers, and the volumes were estimated using the formula: $V = D \times d^2 \times 0.52$.

2.11 Statistical analysis

Statistical analysis was performed using ANOVA with post hoc Newman-Keuls Student's t-tests, using GraphPad Prism 5.0 software (GraphPad Software, Inc.; La Jolla, CA). Data were considered statistically significant when $P < 0.05$.

3. RESULTS

3.1 MNU (50 mg/kg body weight) carcinogenesis study

Although the Syrian hamster is becoming a well-established model for studying novel therapies, there are no mammary cancer cell lines available for *in vitro* studies or for use *in vivo* in syngeneic tumor implantation models. Therefore, we sought to establish a mammary cancer cell line from primary Syrian hamster mammary tumors using the chemical carcinogen MNU.

Because of the high morbidity caused by an initial 100 mg/kg dose of MNU (Supplemental Data), a lower i.p. dose of MNU (50 mg/kg body weight) was used to treat a group of hamsters ($n = 10$). This group demonstrated an intermediate gain in body weight, between the control untreated and group receiving 100 mg/kg dose of MNU (data not shown). These hamsters were clearly healthier than animals treated with 100 mg/kg dose of MNU; they did not appear moribund until late in the study and their mammary glands were not atrophied upon histopathologic examination. Interestingly, this group presented with 6 of 10 animals having premalignant mammary lesions represented by papillary lesions, atypical ductal hyperplasia (ADH), and ductal carcinoma *in situ* (DCIS), as well as 2 of 10 animals having

mammary adenocarcinomas. Few other lesions were observed in the animals receiving the 50 mg/kg dose of MNU, although 3 animals also had sex cord stromal tumors of the ovary.

3.2 Establishment and characterization of a stable mammary carcinoma cell line

One of the mammary adenocarcinomas resulting from the MNU study using a 50 mg/kg dose was harvested and examined by histopathological analysis (Fig. 1). Examination of H&E stained tumor sections showed abnormal ducts containing secretory products separated by sheets of poorly differentiated epithelial cells. This pattern was similar to a high-grade poorly differentiated adenocarcinoma in human breast cancer. The tumor was established into primary culture as described in Materials and Methods. Interestingly, after initiating *in vitro* culture of the primary tumor, outgrowth of two different morphologic phenotypes of cells resulted: a uniformly round and a more elongated shaped. These lines were grown separately as HMAM4A (round morphology) and HMAM4B (elongated morphology) after this difference in shape was observed. These cell lines were passaged at least 15 times, and were capable of cryogenic storage and resuscitation. Isoenzyme analysis confirmed the HMAM4B cells were derived from Syrian hamster (*Mesocricetus auratus*) (data not shown).

3.3 Immunohistochemical analysis of the primary tumor

The mammary tumors and premalignant lesions from animals treated with a 50 mg/kg dose of MNU were analyzed by immunohistochemistry for the presence of pancytokeratin to confirm the carcinoma was epithelial cell origin and HER-2/neu to confirm mammary cell origin. Human T47D breast cancer cells were used as a positive control for HER-2/neu staining (data not shown). As controls, normal age-matched hamster mammary tissue stained positively for HER-2/neu and normal hamster uterus stained positively for pancytokeratin, indicating the primary antibodies cross-react with hamster (Fig. 2A). Both antibodies also demonstrated specificity, with staining patterns limited to cell membrane expression in epithelial cells with no staining in adjacent stroma. As negative controls, there was little staining using no primary antibodies, indicating the specificity of the secondary antibodies (data not shown). Interestingly, about half of the premalignant lesions (ADH) stained positively for HER-2/neu (Fig. 2B) and the other half were negative. As shown in Fig. 2C, the primary tumor from which HMAM4A and HMAM4B were derived, stained strongly for HER-2/neu. This result is not uncommon in human cancer; as a tumor progresses from DCIS to invasive ductal carcinoma (IDC), HER-2/neu expression often increases [18].

3.4 Injection of mammary cancer cell lines into syngeneic hamsters

HMAM4B cells were implanted by s.c. injection into the hind flanks of syngeneic untreated recipient female Syrian hamsters. The HMAM4B cells demonstrated a significant tumor growth rate after injection *in vivo*, although the take rate of tumors formed was 50% (Fig. 3). In addition, the lag time between tumor cell injection and appearance of measurable tumor (*i.e.*, tumor latency) was variable, ranging from 15 to 36 days. HMAM4A cells implanted into recipient female Syrian hamsters showed a similar take rate and variable lag time of tumor formation (data not shown). Histopathologic analysis of the HMAM4B tumors after necropsy demonstrated a phenotype consistent with high-grade mammary adenocarcinoma (data not shown). One of these tumors was harvested and passaged in cell culture as the HMAM5 cell line. We subsequently implanted the HMAM5 cell line by s.c. injection into the hind flanks of syngeneic untreated recipient female Syrian hamsters. Tumors derived from HMAM5 cells implanted Syrian hamsters showed similar histology as the HMAM4B tumors and the primary tumor from which these cell lines were derived; the HMAM5 tumors were composed of solid sheets and nests of poorly differentiated adenocarcinoma cells (data not shown). After HMAM5 cell implantation a greater take rate of tumor formation was observed *in vivo* (up to 70%) compared with HMAM4B cells. An analysis of

the mean body weight growth characteristics of the hamsters (Fig. 4A) and implanted HMAM5 tumor volumes *in vivo* (Fig. 4B), showed that after a consistent latency of approximately 21 days, the tumors grew at a similar rate in the animals until they were sacrificed at the upper limit allowed in our protocol (20% of total body weight).

3.5 Characterization of the HMAM5 mammary carcinoma cell line

The HMAM5 cell line has now been cultured for more than 40 passages, indicating that it is an immortal carcinoma cell line that does not senesce like primary cells (data not shown). The HMAM5 cells readily grew in culture with a doubling time of approximately 24 hours during exponential growth phase, similar to HMAM4B cells and the human MDA-MB-231 human breast cancer cell line (Fig. 5A). The HMAM5 cells also demonstrated anchorage-independent cell growth in soft agar (Fig. 5B), and the number of colonies formed was also similar to HMAM4B cells and the human MDA-MB-231 human breast cancer cell line.

Cytogenetic analysis was performed on HMAM5 cells; of 100 metaphases examined, the chromosome count ranged from 68 to 77 with a modal chromosome number of 74 (Fig. 6A). The modal chromosome number of 74 was also observed in HMAM4B cells. Although heterogeneous, 81% of the cells contained between 70 and 74 chromosomes. Further karyotype analysis of 5 metaphases demonstrated multiple copies of chromosomes A3, C11, C14, C15, E20, and F21, as well as numerous unknown chromosomes (UC, Fig. 6B). However, no chromosome aberrations were found.

To classify the molecular subtype of the hamster mammary tumors, additional markers were examined. Breast cancer can be divided into four distinct molecular classifications including: luminal A (ER+, PR+, HER2-), luminal B (ER+, PR+, HER2+), basal (ER-, PR-, HER2-), and basal-like HER-2/neu positive (ER-, PR-, and HER2+). The p63 and cytokeratin 5 molecular markers have also been used to distinguish the basal breast carcinomas from luminal. The majority of the luminal tumors have been shown to be negative for p63 and cytokeratin 5, and simultaneous of p63 and cytokeratin 5 is completely absent within the luminal B subtype [19, 20]. Thus, immunohistochemical analysis was performed using tumors established in syngeneic animals after subcutaneous injection of HMAM4B and HMAM5 cells. The results shown in Fig. 7 demonstrate no detectable staining of either for p63 or cytokeratin 5/6. As a control, samples of hamster skin were strongly positive for both p63 and cytokeratin 5/6.

To determine whether the HMAM5 cell line is estrogen dependent, responsiveness to tamoxifen, a nonsteroidal estrogen antagonist effective in arresting the growth of estrogen-responsive cells, was examined. This analysis was performed since no commercially available anti-ER antibodies tested were cross reactive to Syrian hamster. In this experiment, a human ER positive breast cancer cell line (MCF-7) and a human ER negative breast cancer cell line (MDA-MB-231) were used as positive and negative controls, respectively, for determining responsiveness to tamoxifen. As shown in Fig. 8, proliferation of both HMAM5 and MCF-7 cells showed a similar decrease at 24 h with increasing concentrations of tamoxifen. However, MDA-MB-231 cells were relatively insensitive to any decrease in proliferation from tamoxifen treatment. These results suggest that the HMAM5 cell line is derived from a luminal B subtype mammary carcinoma.

4. DISCUSSION

The results of the studies reported here have demonstrated that an i.p. injection of MNU will produce mammary carcinomas in the Syrian hamster, but many other tumors develop. High doses of MNU are associated with increased morbidity and a lower yield of mammary tumors because of toxicity of MNU and subsequent atrophy of the mammary glands. It is

unclear why hamsters are resistant to chemical-induced mammary carcinogenesis compared with rats. Differences in carcinogen metabolism might explain differences in susceptibility to polycyclic aromatic hydrocarbons, but since MNU is a direct-acting alkylating agent, metabolism is not a factor in this study. However, it has been reported that differences in DNA repair result in species-, age-, and tissue-specific susceptibility to alkylating agents [21]. The DNA repair enzyme, O⁶-methylguanine-DNA methyltransferase (MGMT) removes the alkyl group from the O⁶ position of guanine [21]. Pre-pubescent rats exposed to MNU were more susceptible to mammary carcinogenesis than adult rats, which correlated with lower activities of MGMT in the younger rats [22, 23]. Whether differences in tissue distribution of MGMT in hamsters affect their susceptibility to MNU-induced mammary carcinogenesis is currently unknown.

The primary models *in vivo* of breast cancer use transgenic mice as well as human and rodent cell lines implanted into recipient animals. The ability of new anticancer therapeutic agents to inhibit tumor growth *in vivo* has been used as a preclinical measure of activity prior to clinical trials. However, interspecies differences in gene expression as well as gene activity and function may alter toxicity and efficacy results. For example, in the case of therapeutic nucleoside analogs, differences in deoxynucleoside kinase and 5'-deoxynucleotidase activities between mice and rats may result in differences in metabolism and pharmacological responses [24]. In addition, recent studies demonstrated differences in conversion of nemorubicin, a doxorubicin derivative, to its active metabolite that could markedly affect its toxicity profile between species [25]. These results suggest that the new Syrian hamster mammary tumor model we generated could be useful in providing an additional species for evaluating preclinical anticancer drug efficacy, and provide additional support for the therapeutic potential in humans.

The new Syrian hamster mammary carcinoma cell line we generated may also have promising use as a syngeneic model in developing oncolytic adenovirus therapy strategies, because hamster models are permissive for adenoviral replication [10, 26, 27]. Oncolytic adenovirus vectors are most commonly evaluated for efficacy in immunodeficient mice using human xenograft tumor models. However, these models do not accurately reflect biodistribution and toxicity of oncolytic adenovirus vectors in humans because adenovirus replication is generally species specific, and human adenoviruses replicate poorly in normal mouse tissues such as liver [28]. Thus, a more realistic animal model that is permissive or at least semi-permissive for human adenovirus replication would be of great value to the field of adenovirus-based cancer therapy. In contrast to most other species examined, the Syrian hamster is permissive for human adenovirus replication [29–31]. The adenovirus is able to infect, replicate, and spread from cell to cell *in vitro* in cell lines derived from this animal, and replicates *in vivo* in the lungs, liver, and other organs [11]. Importantly, the Syrian hamster model has been used in toxicity studies required by the FDA for a phase I clinical trial [9].

In this study, we have established the first immortalized mammary carcinoma cell line currently available from Syrian hamsters. These cells proliferate *in vitro*, form colonies in soft agar, and contain 74 chromosomes (the normal chromosome number for Syrian hamster is 44). The cell line is clearly an adenocarcinoma derived from mammary. It could be transplanted into recipient hamsters, grown in primary culture again, and transplanted once again with a take rate of 70% in female recipients. Since initiation of primary mammary carcinomas in Syrian hamsters is so difficult, the availability of an immortalized cell line that can be re-implanted into syngeneic hosts should prove to be extremely valuable to the research community. Further analysis of tissues and cells generated by this model we describe may be useful in further understanding the mechanism for resistance to mammary cancer by hamsters. Furthermore, this model may serve as a new platform in addition to rats

and mice for the study of anticancer or chemopreventive agents, in which an intact immune system is required.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

MNU	N-methyl-N-nitrosourea
DMBA	7,12-dimethylbenz[a]anthracene
DES	diethyl-stilbestrol
BOP	N-nitrosobis(2-oxopropyl)amine
IACUC	Institutional Animal Care and Use Committee
i.p	intraperitoneal
PET	Positron Emission Tomography
¹⁸F-FDG	2-deoxy-2-[¹⁸ F]fluoro-D-glucose
LSO	lutetium oxyorthosilicate crystal
DMEM	Dulbecco's Modified Essential Medium
FBS	fetal bovine serum
s.c.	subcutaneous
ADH	atypical ductal hyperplasia
DCIS	ductal carcinoma <i>in situ</i>
MGMT	O ⁶ -methylguanine-DNA methyltransferase

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Figure 1. Histopathologic examination of a primary mammary tumor in MNU treated hamster Shown is a representative H&E stained section of a mammary adenocarcinoma from which the HMAM4 cell line was isolated.

Figure 2. Immunohistochemical analysis of normal mammary tissues and mammary tumors from Syrian hamsters

In the following histology images, (A) normal mammary tissue or uterus was compared to (B) mammary tissue displaying ADH and (C) a mammary tumor from a hamster treated with a 50 mg/kg dose of MNU. Shown are representative specimens stained by immunohistochemistry for pancytokeratin and HER-2/neu expression.

Figure 3. Growth characteristics of HMAM4B cells *in vivo* in syngeneic recipient Syrian hamsters

After s.c. injection of 1×10^7 HMAM4B cells into the left and right flanks, tumor growth at each injection site was monitored by caliper measurements. The take rate observed was 50% (i.e., 3/6 sites injected with HMAM4 cells developed a tumor).

Figure 4. Analysis of HMAM5 tumor growth *in vivo* in syngeneic recipient Syrian hamsters Shown are: (A) the mean body weight ($n = 10$) and (B) the mean tumor growth ($n = 7$) at each injection site after s.c. injection of 1×10^7 HMAM5 cells into the right flanks of recipient Syrian hamsters. The take rate observed was 70% (i.e., 7/10 animals injected with HMAM5 cells developed a tumor). Of the 7 animals followed, one hamster was euthanized at day 65 (E1) and another at day 79 (E2) due of excessive tumor burden. Each data point represents the mean $\pm$ standard error of the body weights or tumor volume measurements at each time point.

Figure 5. Growth characteristics of HMAM5 cells *in vitro*

Shown are: (A) representative growth curve of HMAM5 cells in culture and (B) soft agar assay of anchorage-independent cell growth assessed by colony formation of HMAM5 cells. Cell growth and colony formation characteristics of HMAM5 cells were compared with HMAM4B and MDA-MB-231 cells. Each data point represents the mean $\pm$ standard error of three independent experiments.

Figure 6. Cytogenic analysis of the HMAM5 cell line

(A) Summary of 100 metaphases analyzed showing the distribution of chromosome numbers. In Syrian hamsters, the normal chromosome number ($2n$) = 44. (B) Summary of 5 karyotypes examined showing the mean chromosome number. No chromosomal aberrations were observed. Each bar point represents the mean $\pm$ standard error of 5 karyotypes.

Figure 7. Immunohistochemical analysis of normal skin tissue and mammary tumors from Syrian hamsters

In the following histology images, sections of tumors from hamsters injected s.c. with HMAM4B or HMAM5 were stained with (A) cytokeratin 5/6 or (B) p63. Staining was compared to positive controls using sections from normal hamster skin. Shown are representative specimens.

Figure 8. The effect of tamoxifen treatment on cell proliferation

The effect of tamoxifen, an antagonist of the estrogen receptor, was used as a surrogate marker to determine the ER status of HMAM5 cells. MDA-MB-231 (an ER negative cell line), MCF-7 (an ER positive cell line), and HMAM5 were untreated or treated with 3.3, 10, 33, and 100 μM tamoxifen for 40 h. Cell proliferation was measured with a BrdU labeled ELISA kit. Each bar represents the mean $\pm$ standard error of values normalized to untreated cells. Indicated (*) by the Student's t-test, there was a statistically significant difference between the cell lines with $P < 0.05$.