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TITLE:

T24 HRAS Transformed NIH/3T3 Mouse Cells (GhrasT-NIH/3T3) in Serial Tumorigenic Passages Give Rise to Increasingly Aggressive Tumorigenic Cell Lines T1-A and T2-A and Metastatic Cell Lines T3-HA and T4-PA.

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Abstract 9-11-14 (360 words)

Cancer cells often arise progressively from “normal” to “pre-cancer” to “transformed” to ‘local metastasis’ to “metastatic disease” to “aggressive metastatic disease”. A series of mouse cell lines have been developed sequentially to mimic this type of progression starting with the NIH/3T3 cell line transformed by transfection with HRAS oncogene DNA from the T24 human bladder carcinoma to produce the Ghrast-NIH/Swiss cell line. To start, these cells were injected s.c. into a NIH/Swiss mouse to produce a primary tumor from which the T1-A cell line was established. T1-A cells injected i.v. in the tail vein produced a local metastatic tumor from which the T2-A cell line was established. T2-A cells injected i.v. into a nude NIH/Swiss mouse produced a metastasis in the liver from which the T3-HA (H=hepatic) cells line were developed. T3-HA cells injected i.v. into a nude mouse produced a metastasis in the lung from which the T4-PA (P=pulmonary) cell line was established. PCR analysis indicated the human T24 HRAS oncogene was carried along with each in vitro/in vivo step and found in the T2-A and T4-PA cell lines. Light photomicrographs indicated the five transformed cells are morphologically similar. Ghrast-NIH/Swiss cells injected s.c produced tumors in 4% of NIH/Swiss mice in 6-10 weeks; T1-A cells injected s.c produced tumors in 100% of NIH/Swiss mice in 7-10 days. T1-A, T-2A, T3-HA and T4-PA cells when injected i.v. produced local metastasis in non-nude or nude NIH/Swiss mice. Evaluation of the mice injected indicated a general increase in metastatic potential of each cell in the progression as compared to the Ghrast-NIH/3T3 transformed cells. A new photomicrographic technique to follow growth rates within six preselected 2x2mm grids per plate is described. Average doubling times of the transformed cells Ghrast-NIH/3T3 (17 hrs), T1A (17.5 hrs), T2A (15.5 hrs), T3-HA (17.5 hrs) and T4-PA (18.5 hrs) (average 17.2 hrs) were significantly faster (P=0.006) than NIH Swiss primary embryonic cells and NIH/3T3 cells (22 hrs each). This cell series is being used in studies of cancer cell inhibitors, mitochondrial biogenesis and gene expression and is available for further study by other investigators for intra- and inter-laboratory comparisons of whole genome and transcriptome sequencing.

Key Words: NIH/3T3, HRAS, transformed, tumorigenic progression, metastasis

Highlights:

- A Tumor Progression Model derived from a new T24 HRAS transformed NIH/3T3 cell line
- T1-A, T2-A, T3-HA, T4PA lines derived from Ghrast-NIH/3T3, T1-A, T2-A, T3-HA tumors
- The human HRAS oncogene remained in both primary and metastatic tumors
- These five new cell lines showed a progressive increase in tumorigenic potential
- 18 hour doubling times were observed from daily photomicrographs on gridded plates
INTRODUCTION

It is well established that human cancers develop in a multistep sequence with environmental influences including chemical, physical, and viral agents being major etiological contributors (Meeker & J, 1985; Weinberg, 2014). Numerous transitions occur in this “tumorigenic progression” as the cells change from “normal” to “immortalized” (Bischoff et al., 1991; Stevens et al., 2014) to “transformed” and finally go on to the “metastatic state” (Bernstein & Weinberg, 1985; Liotta, 1988). Recent work using next-generation sequencing has also demonstrated that one rapid catastrophic “chromothripsis” event can shatter DNA into pieces leading to hundreds of genomic rearrangements, a phenomenon shown to have occurred in two to three percent of all cancers (Stephens et al., 2011). These cells may become dominant in a heterogeneous tumor population allowing cancer to form relatively quickly in these cases as they then continue to progress through a multistage process. Chromothripsis has been implicated in some of the genomic rearrangements in the sequenced genome of the universally employed human cancer model HeLa cell line (Adey et al., 2013; Landry et al., 2013). The predominant hyper-triploid profile of eight HeLa cell strains is consistent with published karyotypes of various HeLa strains, suggesting that their becoming mostly hyper-triploid occurred either during tumorigenesis or early in the establishment of the HeLa cell line (Adey et al., 2013). This finding illustrates how transformed cells in culture for shorter periods than HeLa’s 68 years should remain relatively stable once established from tumors with inherent malignant and metastatic characteristics. This affirms the usefulness of model cancer cell lines in evaluating cancer tumor cell treatments between various laboratories

More recent results from studies of tumor cells in vivo have clearly demonstrated the importance of micro-environmental cell-to-cell interactions as well as paracrine and endocrine influences on the ways tumor cells and their progeny change over time and space (Espina & Liotta, 2011; Shibue & Weinberg, 2011)(Liu et al., 2014). Cancer stem cells and their role in the initiation events leading to cancer cell transformation and their self renewing characteristics have shed light on the tumorigenic process and highlighted problems associated with effective killing of both non-cancer stem cells and cancer stem cells (Korkaya & Wicha, 2013; Liu et al., 2014; Scheel & Weinberg, 2012; Wang et al., 2014).

The use of established cell lines combined with cutting edge technology has recently revealed how the insertion of human papillomavirus HPV18 is affecting the tumorigenic capacity of HeLa cells (Adey et al., 2013; Landry et al., 2013). Studies of two sub-clones of the human neuroblastoma cell line STA-NB-10 have shown how MYCN gene amplification occurred in double minutes or homogeneously staining regions (HSRs) (Storlazzi et al., 2010).

Forty one years ago the human HRAS gene transfection was first reported by Robert Weinberg’s lab to transform the immortalized NIH/3T3 cell line (C. Shih, Padhy, Murray, & Weinberg, 1981; C Shih, Shilo, Goldfarb, Dannenberg, & Weinberg, 1979). Subsequent work by Weinberg and Michael Wigler and Mariano Barbacid cloned the human transforming ras gene from the T24 and EJ bladder carcinomas cell lines, reviewed by Malumbres and Barbacid (Malumbres & Barbacid, 2003). The transforming DNA from the EJ bladder human carcinoma cell line (EJ-Ha-ras) was identified as the activated HRAS oncogene. The EJ-6-2-Bam-6a cell line (ATTC CRL-1888) was established by transfection of the activated HRAS oncogene into immortalized NIH/3T3 cells. The ras oncogenes (HRAS, KRAS and NRAS) are known to exploit their signaling pathways to help drive tumorogenesis in a large number of cancer types (Pylayeva-Gupta, Grabocka, & Bar-Sagi, 2011). This EJ-6-2-Bam-6a cell line is a tertiary transfectant created from a BamHI digest of the DNA from the human EJ-Ha-ras cells transfected into the NIH/3T3 cell line. This transfected cell line paired with the untransfected NIH/3T3 cell is being used globally to evaluate the effects of HRAS transformation in the tumorigenic process. They are being used for many recent approaches to understand the control of cell death in the malignant cell (Koziel et al., 2013), evaluating the mechanisms cancer cells utilize to alter their metabolism (Leprivier et al., 2013) and devising ways to target cancer cells with specific decorated nanoparticles to image and ultimately kill cancer cells when attached to tumor specific toxins (Pan & Feng, 2009).

To better understand the importance of transformations and tumor progression changes on disease status, prognosis, and treatment, universally available tumor model systems will continue to help establish a testable dataset in the application of modern molecular techniques. These can be
combined with bioinformatic analysis and applied to large datasets of clinical genomics that are being assembled around the world. Model systems may even be useful to help address quality-control issues relevant to development of universal standards for intra- and inter-laboratory comparisons of reproducibility and sample tracking within large world wide efforts to standardize clinical genomic data (Miliaras, 2014).

The aim of the present study was to establish cell lines from a new HRAS ras transfection system using the T24 human bladder carcinoma and establish cells derived from it that represent various stages in one tumor progression that includes several additional metastatic cell types with the advantage that all transformed cells in the series are derived from a common transfected parent population, namely, the GhrasT-NIH/3T3 cell line used in this study. This cell line was generated by transfection with DNA from the T24 human bladder carcinoma. The design to intentionally expose different metastatic cells to in-vivo micro-environmental effects as they adapt to multiple targets was accomplished by alternating between in vitro and in vivo growth in the development of this series. The first cell line in the series (T1-A) was cultured from a primary tumor in a NIH/Swiss mouse injected s.c. with the GhrasT-NIH/3T3 cells. The second cell line (T2A) was cultured from a subsequent secondary local metastasis in a NIH/Swiss mouse injected i.v. with T1-A cells. The third cell line (T3HA) was cultured from a tertiary liver metastatic tumor in a nude NIH/Swiss mouse injected i.v. with T2-A cells. The fourth cell line (T4PA) was cultured from a quaternary metastatic tumor in a nude NIH/Swiss mouse injected i.v. with T3-HA cells. The tumorigenic properties of these T24 derived cell lines are reported along with their growth and morphological properties that appear to be unique from other reports on a different EJ-6-2-Bam-6a cell line transfection system. A new photomicrographic method was utilized here to measure the in vitro growth rates of these cell lines as they replicate in specific 2x2 mm areas on gridded plates. This new series of cells has proven useful in studies of growth inhibition testing of various compounds, as well as mitochondrial biogenesis, and gene expression studies in this lab (D.B. Ray, pers. comm.) and should be useful for further study in other laboratories. These lines offer the opportunity to evaluate single cell variations within and among a variety of cell lines derived from a common tumorigenic event(s).

**METHODS**

**Animals:** A breeding pair of NIH Swiss mice was obtained from L. Watson at NIH or the Jackson Labs and bred as an outbred strain as described by the Jackson Lab protocols. All mice at the Oral Roberts University Medical School were housed in the animal facility of the Biomedical Research Center, fully accredited by the American Association for the Accreditation of Laboratory Animal Care, and cared for in accordance with the guidelines of the National Research Council for the care and use of laboratory animals under the supervision of a licensed veterinarian. All mice at Grove City College were cared for with the same animal studies guidelines that had been approved by the Grove City College IRB. Nude mice (Nu/-/Nu-) were obtained from Jackson Labs, Farmington, Connecticut (Common name: nu/nu; Strain Name: NUI; Stock Number: 002019) or obtained from a breeding pair (Nu+/Nu-) offspring. These are from a spontaneous mutation athymic (nude) inbred mice on an N:NIH(s) background homozygous for nude marker, Nu-/N- and immuno-compromised. The two main defects of mice homozygous for the nude spontaneous mutation (Foxn1nu, formerly Hfh11nu) are abnormal hair growth and defective development of the thymic epithelium. The Jackson Laboratory imported the nude mutation from the NIH on an outbred stock. As of 2008, the strain has been inbred for at least 100 generations. The studies reported here with these mice were completed between 1995 and 2000. NFS/NCr inbred mice were obtained from the Jackson Lab and maintained as an inbred colony. They have a very low incidence of spontaneous lymphoma.

**Source, derivation and growth characteristics of each cell or cell line:**

All cells and cell lines were grown at 37° C in 5% CO2 / 95% air in Dulbecco’s modified Eagle’s medium (DMEM) (Catalog No. 30- 2002) supplemented with extra added glucose (4.5 g/L), 10% fetal calf serum, and 20 Units/ml penicillin, and 20 ug/ml streptomycin.

Extra glucose was included to insure a rich glucose source for rapidly growing tumor cells that are known to utilize aerobic glycolysis as a major source of energy and glycolytic intermediates (Warburg, 1956) (Wallace, 2010).
1) Normal primary NIH Swiss embryonic cell cultures (mortal): These cells were prepared from 17 to 19 day old NIH Swiss mouse embryos by fine mincing of whole embryos with scissors (Todaro & Green, 1963). These minces were used for growing primary normal cell cultures and for DNA extractions as needed. The tissue was disaggregated with 0.25% trypsin, cells were collected by centrifugation, resuspended in phosphate buffered saline and viable cells, identified by Trypan blue exclusion, were counted in a hemocytometer. Cells were plated at 4.3 x 10^6 cells/60 mm diameter dishes and grown in Dulbecco's modified medium (D-MEM) supplemented as described above. Cultures were expanded and subcultures were frozen and stored in liquid nitrogen tanks as stocks.

2) NIH/3T3 cells (immortal): The "immortal" NIH/3T3 cells (ATCC® CRL-1658™) cells were purchased from the American Type Culture Collection at passage # 126. These cells were originally developed (Jainchil, Aaronson, & Todaro, 1969) by growing primary embryonic cells, prepared as described above, using a rigid transfer schedule. These cells were non-tumorigenic when 1 x 10^6 cells were injected s.c. into 23 NFS/Ncr inbred mice or 105 outbred NIH Swiss mice (Table 1). They produce tumors in BALB/c nude mice (Greig et al., 1985). These cells grow rapidly and when transferred at 4.3 x 10^5 cells/50 mm diameter culture dish they form confluent monolayers within 2 to 3 days. Cultures were expanded and subcultures were frozen and stored in liquid nitrogen as stocks.

3) Tumorigenic ras oncogene-transfected NIH/3T3 cells (transformed): The HRAS oncogene-transfected NIH/3T3 cells were obtained from Dr. David A. Goldthwait at the Department of Biochemistry, Case Western Reserve University in Cleveland, Ohio and designated as G-hrasT-NIH/3T3 cells. (G denotes their source from the Goldthwait lab, T denotes their transformed cell type.) They were isolated from a focus of transformed cells following transfection with DNA from the T24 Human bladder carcinoma. This cell line contains the Harvey-ras oncogene isolated from the T24 human bladder carcinoma that has been shown to have a glycine to valine substitution at the twelfth amino acid residue of the T24 oncogene encoded p21 protein (E. Premkumar Reddy, 1983). The pT24-C3 plasmid (ATCC 41000™) used for the transfection of the NIH/3T3 cell line by Dr. Goldthwait is a pBR322 derived plasmid containing the 6.6 Kilo base pair Bam HI fragment of the T24-Ha-ras1 oncogene and is carried in E. coli C-600 strain (Goldthwait, D. A., unpublished results). G-hrasT-NIH/3T3 cultures were expanded and subcultures were frozen and stored in liquid nitrogen as stocks.

4) Highly tumorigenic ras T-1A cells: Primary tumors that formed at the G-hrasT-NIH/3T3 cells’ injection sites in two of the NIH Swiss mice were excised, minced and cultured as described above giving rise to the T1-A and T1-C cell lines. The T1-A and T1-C cells were sub-cultured several times and stocks were saved in frozen in liquid nitrogen.

5) T2-A cell line from local metastasis in NIH Swiss mouse: To seek a metastatic lesion derived from these T1A cells, 1 x 10^6 cells were injected into the tail veins of several NIH Swiss mice. A tumor was located in the rump near the base of the tail of one mouse within 13 days of tail vein injection. This tumor was excised, minced and cultured and named T2-A cells. These cells were expanded and subcultures were frozen and stored in liquid nitrogen. Thus this T2-A cell line was derived from a fast growing local metastasis close to but not at the site of injection in the tail.

6) T3-HA metastatic cells from distant liver metastasis in nude NIH Swiss mouse: In attempts to acquire a highly metastatic cell line in this series, 1 x 10^6 T2-A cells were injected into the tail veins of four immune compromised nude NIH/Swiss mice. After 3.5 weeks a mouse was sacrificed and contained a metastatic lesion in the liver that was excised, minced, cultured and designated T3-HA (H = hepatic) cells. These cells were expanded in culture and stored in liquid nitrogen.

7) T4-PA metastatic cells from distant lung metastasis in nude NIH Swiss mouse: To determine if the T3-HA cells derived from the first distant metastatic lesion would generate second metastatic lesions in the liver, 0.7 x 10^6 T3-HA cells were injected into the tail veins of four NIH/Swiss nude mice. After 4 weeks, a metastatic lesion was found in the lung of the one mouse. It was excised, minced, cultured and cells designated T4-PA (P = pulmonary) cells. These cells were expanded and stored in liquid nitrogen.
**DNA Extraction**

Total DNA was isolated from tissue or cell pellets by a modification of the methods of Davis, et. al. (Davis, Kuehl, & Battey, 1986).

**Human ras oncogene Polymerase Chain Reaction (PCR) analysis:** The presence of this human oncogene in the ras transfected G-hrasT-NIH/Swiss and its descendent cell lines was confirmed by polymerase Chain Reaction amplification with the use of human specific H-ras primers. Mouse specific primers were used for a control PCR. Primers were designed by methods similar to those of Lowe et al (Lowe, Sharefkin, Yang, & Dieffenbach, 1990) as published previously (Ray et al., 2005, page 422).

Primers designed:

HN, MN = human or mouse primer that becomes the non-template strand of the DNA
HT, MT = human or mouse primer that becomes the template strand of the DNA

Human ras primers: designed and named from GenBank: J00277; formerly V00574 (E. P. Reddy, Reynolds, Santos, & Barbacid, 1982; Tabin et al., 1982). These human specific primers will amplify the same region in either the normal human proto-oncogene or the human T24-Ha-ras1 oncogene

HN-3630 5’-CTG-TCT-TCA-ACA-TCC-CAA-ATG-CC-3’
(note: although this C should be a G to match the published sequence, V00574, the PCR was successful)

HT-4781 5’-AGT-GTG-GTA-TTC-CCT-GGA-CAA-AAG-G-3’

Mouse ras primers: designed and named from GenBank: Z50013.1 (Przybojewska & Plucienniczak, 1996). These mouse specific primers will amplify a region the normal mouse proto-oncogene.

MN-1121 5’-GGC-CTT-AGT-TCT-TCT-TGT-CC-3’

MT-1336 5’-AAC-CAA-CAC-AAA-TAG-GGA-GC-3’

PCR was performed as described previously (Ray et al., 2005). DNA was purified as described above and purity and concentration were analyzed via UV/VIS spectrophotometry. Each 50 ul reaction contained 10 mM Tis-HCl, pH 8.3, 50 mM KCl, 1.5 mM MgCl2, 200 uM each of dATP, dCTP, dGTP, dTTP, 0.2 uM primer one (forward) and 0.2 uM primer two (reverse), ~0.5 ug DNA, 1.25 Units of Taq-Gold polymerase (Applied Biosystems). The thermocycler program that included 10 min at 95º C, a 40 cycle repeat of: 1 min at 94º C, 1 min at 55º C, and 1 min at 72º C, followed by a polishing time of 6 min at 72º C. Agarose slab gel electrophoresis was performed and EtBr stained gels were photographed by a Gel Doc XR from BIO-RAD, and Quantity One® software was used to analyze the gels and assign a specific base pair value to each DNA fragment based on the sizing standards.

**Determination of cell growth rates:** Cells were plated in the DMEM described above in 60mm diameter dishes with 2x2mm square grids (Corning cat# 430196). Six predetermined squares were pre-marked with marking pen on each dish prior to use. The squares were scattered in such a way that each 2x2mm square area would be representative of the entire plate. Photographs were taken daily when cells reached 20-40% confluency (day 0) with an Olympus DP12 inverted Microscope with a Digital Camera System at 100X in the center of each selected 2x2 mm square. Cell counts for each square were determined from the photomicrographs and the average for the 6 squares was determined for each day. Results for each day were normalized to fold increase relative to day 0. Statistical significance was determined via T-test.

**RESULTS and DISCUSSION**

**Normal NIH Swiss Embryonic Cells:** The normal primary NIH Swiss embryonic cells used to establish the other cell lines in this tumorigenic progression model were obtained from 17 to 19 day old NIH Swiss mouse embryos (Todaro & Green, 1963). They are anchorage dependent, contact inhibited, and mortal,
surviving about 30 cell divisions before they become senescent like all diploid cell cultures (Littlefield, 1982; Todaro & Green, 1963).

**NIH/3T3 cell line**: The NIH/3T3 cell line was derived from NIH Swiss embryonic cells that have escaped this senescent “crisis” to become, immortal, contact inhibited and although not transformed they already have obtained properties associated with transformed cells (Littlefield, 1982). They were not tumorigenic (Table 1) in the inbred NFS/NCr mice (0 tumors in 23 mice injected) or in the outbred NIH Swiss strain of mice (0 tumors in 105 mice injected). Each mouse was injected with $1 \times 10^6$ cells s.c.. This negative result with NFS/NCr mice was the same as the results of Bernstein and Weinberg (Bernstein & Weinberg, 1985). Variable NIH/3T3 tumorigenic and metastatic capabilities of NIH/3T3 cells have been reported. NIH/3T3 cells have been shown to produce tumors in BALB-c nude mice (Greig et al., 1985). Differences in NIH/3T3 cell stocks, passage numbers, site of tumor cell inoculation and cell number inoculations may be responsible for different outcomes. In addition, in some transfection experiments, untransfected control NIH/3T3 cells have been shown to undergo “spontaneous transformation”, thought to be more frequent when these cells are maintained at confluency for extended times prior to subculturing (Greig et al., 1985; Rubin, 1981).

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Recipient Mouse</th>
<th>Injection Site</th>
<th># of Tumors per Mouse</th>
<th>% Tumors</th>
<th>Time Period</th>
</tr>
</thead>
<tbody>
<tr>
<td>NIH/3T3</td>
<td>NFS/NCr (inbred)</td>
<td>hind quarter</td>
<td>0/23</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>NIH/3T3</td>
<td>NIH Swiss (outbred)</td>
<td>hind quarter</td>
<td>0/105</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>G-hrasT-NIH/3T3</td>
<td>NFS/NCr (inbred)</td>
<td>hind quarter</td>
<td>10/41</td>
<td>21</td>
<td>8-10 weeks</td>
</tr>
<tr>
<td>G-hrasT-NIH/3T3</td>
<td>NIH Swiss (outbred)</td>
<td>hind quarter</td>
<td>4/104</td>
<td>4</td>
<td>6-10 weeks</td>
</tr>
<tr>
<td>G-hrasT-NIH/3T3</td>
<td>nude NIH/Swiss</td>
<td>hind quarter</td>
<td>3/3</td>
<td>100</td>
<td>7 N.A.</td>
</tr>
<tr>
<td>T1-A</td>
<td>NFS/NCr (inbred)</td>
<td>hind quarter</td>
<td>3 N.D.</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>T1-A</td>
<td>NIH Swiss (outbred)</td>
<td>hind quarter</td>
<td>20/20</td>
<td>100</td>
<td>7-10 days</td>
</tr>
<tr>
<td>T1-A</td>
<td>NIH Swiss (outbred)</td>
<td>tail vein, i.v.</td>
<td>1/1</td>
<td>100</td>
<td>8-25 days</td>
</tr>
<tr>
<td>T1-C</td>
<td>NFS/NCr (inbred)</td>
<td>hind quarter</td>
<td>8 N.D.</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>T1-C</td>
<td>NIH Swiss (outbred)</td>
<td>hind quarter</td>
<td>10/14</td>
<td>94</td>
<td>7-10 days</td>
</tr>
</tbody>
</table>

1. $1 \times 10^6$ cells injected per mouse
2. not available, 3. not determined
3. This tumor was a local metastasis in the base of the tail from which the T-3A cell line was established.

**G-hrasT-NIH/3T3**: Transfecting the Harvey-ras oncogene into the NIH/3T3 cell line is well known to transform it into a tumorigenic cell type (Bernstein & Weinberg, 1985). To confirm this, the tumorigenic capability of the ras oncogene transfected NIH/3T3 cells (GhrasT-NIH/3T3) was tested for tumor formation in normal mice. Injection of $1 \times 10^6$ cells s.c. into the rear flank of mice produced (20-30 mm
diameter) primary tumors in 14 of 45 (31%) of inbred NFS/N mice and in 4 of 104 (4%) of outbred NIH/Swiss mice all within 6 to 10 weeks (Table 1). This was different than the findings of Bernstein and Weinberg who reported 100% tumor formation when injecting equivalent amounts of the EJ-6-2-Bam-6a cells (ATCC-1888TM) into the NFS/NCr mouse strain.

Consistent with the results of Bernstein and Weinstein’s EJ-6-2-Bam-6a cells results (Bernstein & Weinberg, 1985) injection of these GhrasT-NIH/3T3 cells s.c. into the rear flank of nude NIH Swiss mice produced tumors at the site of injection in each mouse (Table 1, footnote). Other studies (Greig et al., 1985) with a third ras oncogene transfected NIH/3T3 cell line, A51 (Goldfarb, Shimizu, Perucho, & Wigler, 1982), injected s.c. into BALB/c nude mice generated primary tumors in 15-25 days. Some of the differences between the findings with the GhrasT-NIH/3T3 cells and the EJ-6-2-Bam-6a cells could be related to the number of transfections to produce these two cell lines or the specific site where the ras oncogene BamHI fragment inserted into the two different NIH/3T3 mouse cell genomes during each transfection process. In summary, GhrasT-NIH/3T3 cells are transformed, immortal and tumorigenic (Table I).

**Highly tumorigenic ras T1-A cell line:** Primary tumors that formed in two of the NIH Swiss mice at the ras-NIH/3T3 cells’ injection sites were excised, minced and cultured as described above giving rise to the T1-A and T1-C cell lines. Their tumorigenic capability was tested by s. c. injection into the hindquarters of NIH Swiss mice. T1-A cells produced 20 tumors in 20 mice (100%) and T1-C produced 13 tumors in 14 mice (93%) all within 7-10 days (Table 1). This indicated the T1-A and T1-C cell lines were significantly more aggressive at producing tumors than the GhrasT-NIH/3T3 cells that required 6 to 10 weeks for tumor growth. All but one of the 34 mice injected developed a primary tumor and the tumors developed 4 to 10 times faster than the ras-transformed GhrasT-NIH/3T3 cells did in this outbred NIH/Swiss mouse (Table I). Thus it appears that the inherent tumorigenic capabilities of the T1-A and T1-C cells has increased in two separate NIH/Swiss mice as this tumor line moves from cultured cells to growth in vivo.

**T2-A cell line from local metastasis in NIH Swiss mouse:** To seek a metastatic lesion derived from these T1A cells, 1 x 10⁶ cells were injected i.v. into the tail veins of NIH Swiss mice (Table 2). A tumor developed in the rump near the base of the tail of one mouse within 13 days, again illustrating a more rapid tumorigenic process than the original GhrasT-NIH/3T3 cells. No metastatic lesions were found in the mice injected. Cells were cultured from this tumor and designated T2-A. So this T2-A cell line was derived from a fast growing local metastasis close to but not at the site of injection in the tail.

<table>
<thead>
<tr>
<th>Cell Line (passage #)</th>
<th>Recipient</th>
<th># of mice injected</th>
<th>Outcomes</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>T1-A (n.a.)</strong></td>
<td>NIH Swiss (outbred)</td>
<td>4</td>
<td>Four had a local metastatic tumor in rump near tail after 13 days, none with obvious internal metastatic lesions. The T2A cell line was established from one of these local mets.</td>
</tr>
<tr>
<td><strong>T2-A (7, 9)</strong></td>
<td>Nude NIH Swiss (inbred)</td>
<td>10</td>
<td>Four died within 21-27 days; most of the other 6 all had local mets in Rump near tail, two others developed distant metastatic tumors in the liver, lung, pericardium, or throat. The T3-HA cell line was established from one met in the liver.</td>
</tr>
<tr>
<td><strong>T3-HA (2, 3)</strong></td>
<td>Nude NIH Swiss (inbred)</td>
<td>3</td>
<td>One died after 58 days, one had no mets after 54 days and one had metastatic tumors in the liver and lung after 29 days. T4-PA cell line was established from this lung.</td>
</tr>
<tr>
<td><strong>T4-PA (2, 3)</strong></td>
<td>Nude NIH Swiss (inbred)</td>
<td>4</td>
<td>One died after 29 days, one had no mets after 34 days and one had metastatic tumors in the liver and lung after 29 days. Cells cultured from these lesions failed to thrive.</td>
</tr>
</tbody>
</table>

1n.a., not available
**T3-HA metastatic cells from distant liver metastasis in nude NIH Swiss mouse:** In attempts to acquire a highly metastatic cell line in this series, $1 \times 10^6$ T2-A cells were injected into the tail veins of four immune compromised nude NIH/Swiss mice. Each week one mouse was dissected to determine the existence of metastatic lesions. At 3.5 weeks a mouse was sacrificed and observed to contain a metastatic lesion in both the liver and a lung. They were each excised, minced, cultured and designated T3-HA (H = hepatic) and T3-PA (P = pulmonary). These cells were expanded in culture and stored in liquid nitrogen. So these cell lines were derived from tumors that had formed from cells migrating from the tail vein to the liver (T3-HA) or to the lung (T3-PA) to form metastatic lesions, thereby showing that the T2-A cells were capable of metastasizing to distant locations in the nude NIH/Swiss mice. This illustrated the first distant metastatic property by the T2-A cells and the establishment of the T3-HA and T3-PA metastasized cell lines in this tumor progression model.

**T4-PA metastatic cells from distant lung metastasis in nude NIH Swiss mouse:** To determine if the T3-HA cells derived from the first distant metastatic liver lesion would go to the liver and generate second metastatic lesions in the liver, $0.7 \times 10^6$ T3-HA cells were injected into the tail veins of four NIH/Swiss nude mice. Each week one mouse was dissected to determine metastatic lesions. After 4 weeks a metastatic lesion occurred in the lung of one mouse. It was excised, minced, cultured and designated T4-PA (P = pulmonary) cell line. These cells were expanded and stored in liquid nitrogen. This T4-PA cell line was derived from a tumor that had formed from cells migrating from the tail vein to the lung leaving the circulation to form a metastatic lesion thereby showing that the T3-HA cells were capable of metastasizing to a distant location other than the liver in the nude NIH/Swiss mice. This illustrated the distant metastatic property of the T3-HA cells and the establishment of the T4-PA metastasized cell lines in this tumor progression model.

**Tumorigenicity and Metastasis comparisons:** Further studies were undertaken to determine if the T3-HA cells and T4-PA cells metastasize to specific sites. After the T1-A cells were discovered to rapidly produce primary tumors when injected s.c. in immune-competent NIH Swiss outbred mice (Table 1), they were then injected in the tail vein (i.v.) of these NIH/Swiss mice to search for metastatic lesions. These produced only local metastatic lesions near the tail (Table 2). The T2-A cell line was derived from one of these local metastatic lesions. Then to try to enhance the opportunity to produce a metastatic lesion, T2-A cells were injected in the tail vein of a group of partially immune-compromised nude NIH Swiss mice. If successful, this approach would provide a metastatic lesion derived from one local metastatic lesion into another group of nude mice to acquire a serially related distant metastatic cell line. The outcomes (Table 2) indicated that the lung and liver are the most common sites for distant metastasis to occur in nude mice injected with either the T2-A or T3-HA cells. The T4-PA cells were more metastatic since the few nude mice injected created lesions in a broader variety of target tissues (Table 2). Although only a few mice were injected with either cell line, the T4-PA cell appears to more likely have undergone a cellular change allowing it to escape the circulation more easily and invade a broader variety of distant tissues (Table 2). Representative photomicrographs of all the cell types are shown in Figure 1. Further studies are needed to confirm these initial apparent tumorigenic differences among these new cell lines that have progressed in series from a common origin, the GhrasT-NIH/3T3 cells.
Location of the Ras Oncogene in the cell lines by PCR

The presence of the human HRAS oncogene was confirmed by polymerase chain reaction (PCR) amplification with the use of human specific HRAS primers that do not amplify the mouse h-ras proto-oncogene, but do amplify regions of the human proto-oncogene or oncogene (Figure 2). To determine if any tumors arose from spontaneous tumors and therefore were unrelated to the original GhrasT-NIH/3T3 cell line four primers for two PCR assays were designed. This spontaneous occurrence was a possibility, since one spontaneous tumor did occur in the nude NIH Swiss mouse colony used to generate nude mice for these studies. Although the coding portion of the c-H-ras-1 gene in mouse, human, rat and hamster are extremely similar, there are significant differences among these species in their gene’s three introns (Przybojewska & Plucienniczak, 1996). The two PCR primer sets described in the material and methods were able to successfully discriminate between mouse and human h-ras sequences. The human primers only detect either the human c-H-ras-1 oncogene and the human normal proto-oncogene (yielding a 1152
bp PCR product from the 3’ non-translated region). The mouse primers detect only mouse h-ras proto-oncogene (the *M. musculus* gene for C-H-Ras; yielding a 216 bp PCR product coded in the third intron) present in the NIH/Swiss genome. The normal mouse ras proto-oncogene should be present in the mouse liver, mouse cells and cell lines and the H-ras oncogene should only be in the GhrasT-NIH/Swiss, T1-A, T2-A, T3-HA and T4-PA cell lines and in human placenta DNA but not in the NIH/Swiss cells or the NIH/3T3 cell line. This pattern of ras detection was confirmed (Figure 2). The PCR results confirm that the tumors used to propagate the cell lines in this tumorigenic model were all derived from the GhrasT-NIH/3T3 cells and not produced simply by random spontaneous tumorigenic events.

**Figure 2**

![DNA gel image](image)

**Growth Rates of each Cell Line:** The growth rates (Figure 3) for each cell line were determined from photomicrographs taken at different times of six pre-specified 2x2 mm grids on gridded plates in each experiment (Fig 3 insert). The doubling time in log phase growth for each cell line determined by this new method compared well with values obtained from counting cells by hemocytometry in which cells were harvested from multiwell plates on a daily bases to obtain doubling times (Table 3).

The advantages of this new method include the ability to quantify the cell growth within six defined areas within a single dish over the period of the experiment. This is accomplished with fewer dishes.
plated initially and eliminates the variations in cell counts inherent in recovering an equal percentage of cells from separate dishes in the preparations required to use the hemocytometer. Moreover the cells can be recounted from the photomicrographic records if necessary. The average doubling time of the transformed cells GhrasT-NIH/3T3 (17 hrs), T1A (17.5 hrs), T2A (15.5 hrs), T3-HA (17.5 hrs) and T4-PA (18.5 hrs) was 17.2 hrs and was significantly faster (P=0.006) than the doubling times for the NIH Swiss primary cells and NIH/3T3 cells (22 hrs each). In cell growth inhibitor studies ethanol or DMSO needed to solubilize test compounds can be as high as 0.1% in control cultures without significant growth rate effects compared to growth rates in media without added ethanol or DMSO. (D.B. Ray, pers. comm.).

Summary:
As these cell lines were developed and characterized, it appears that they do have variable abilities to produce primary tumors and metastatic lesions. Most noticeable is the difference in the rate and occurrence between the tumorigenic capacity of the GrasT-NIH/Swiss cells and the T1-A cells. It appears that as the GhrasT-NIH/3T3 established the tumor that produced growth of the T1-A and T1-C tumors, cellular adaption and tumorigenic capacity increased dramatically. This could be explained by exposure to the microenvironment in vivo as well as haplotype genomic changes and associated gene expression changes leading to a more aggressive tumorigenic capacity. This type of change may be similar to the ETM transition known to occur in epithelial cancer cell metastasis. In fact micro array studies comparing RNA levels in NIH Swiss embryonic cells to these T4-PA cells indicate several key genes are significantly up or down regulated which are similar to the changes seen in the EMT transitions in breast cancer cell metastasis (A. W. Sodergren, pers. comm.). The other downstream cells in this tumor progression model show some interesting properties. The T2A cells apparently are more able to metabolize to more sites than the T3-HA cells. The T4-PA cells seem to be more adapted to producing metastatic lesions at more numerous sites than the T3-HA cells. (Table 2). Whether these apparent differences are inherently stable will require further studies with a larger group of animals.

Current studies of transformation and tumor progression studies often use the primary NIH Swiss embryonic cells and/or the NIH/3T3 cell line (ATCC- 1658) paired with the EJ-6-2-Bam-6a cell line (ATCC-1888™) or other cells derived from NIH/3T3 cells transfected with the pT24-C3 plasmid (ATCC 41000™) as a model to evaluate the myriad effects of various treatments on cells progressing from normal to pre-malignant to malignant and even metastatic potential. The EJ-6-2-Bam-6a cell line and NIH/3T3 cells cell lines are readily available for investigators throughout the world from the ATCC repository and have been used for over 4 decades to evaluate the effects of H-ras-dependent transformation and related chemotherapeutic, gene knockout or knockdown protocols. Since the original transfection to create the EJ-6-2-Bam-6a cell line involved a transfer of the BamH1 fragment of the EJ-6-2-Bam-6a, transfection into a unique site or sites within one cell, it may only represent one set of oncogene integration events that led to a transformed cell. The development of other models such as the one described here using the T24-Ha-ras1 oncogene DNA at a different time to transfect a second NIH/3T3 cell to produce the GhrasT-NIH/3T3 cell line offers the opportunity to compare molecular outcomes that led to different but similar transformations of the same NIH/3T3 cell line. DNA and transcriptome sequencing studies, protein expression studies in combination with Spectral karyotyping (KEY) comparing the EJ-6-2-Bam-6a and GhrasT-NIH/3T3 cell lines along with the T1-A, T2-A, T3-HA and T4-PA cells would be helpful to elucidate the role of the microenvironment influences along with the transcriptome and chromosomal changes that occur to cause tumorigenic and metastatic capabilities.

Since ras oncogene changes or their effects are known to occur in a large percentage of spontaneous human malignancies, this model system may also be helpful in evaluating new strategies to repair or overcome ras oncogene dependent mechanisms to control cancer cell growth and metastasis.

This ras-transformed NIH/3T3 tumorigenic progression model may be useful for studies of transcriptional changes and chromosomal rearrangements in a series of cells known to be derived in a series of events beginning with a single origin with each cell line related to the preceding cell and giving rise to the subsequent cell in this series as the cells display progressively increasing tumorigenic potential.
Although quite abnormal, the HeLa cell line genome is relatively stable since apparently few new mutations have occurred after early passaging (Adey et al., 2013). Thus, this series of mouse cell lines may be relatively stable once they have been passaged a few times. Their availability to study multiple single cells in vitro and/or in vivo makes them an attractive model to snapshot the progressive stages represented by each cell line in this series.

To our knowledge no other series of HRAS transformed cells are available. Other cell lines a derivatives of The EJ-6-2-Bam-6a cell line and have not been available in a series similar to this new one derived from the T24 human bladder carcinoma. DNA available in the pT24-C3 plasmid.

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References


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Figure Legends

Figure 1: Photomicrographs of Normal and Transformed Cells

Figure 10 A and B: NIH Swiss embryonic 100X, lower and higher cell densities, respectively

Figure 10 C and D: NIH/3T3 100X, lower and higher cell densities, respectively

Figure 10 E and F: GhrasT-NIH/3T3 100X, lower and higher cell densities, respectively

Figure 10 G and H: T1-A 100X, lower and higher cell densities, respectively

Figure 10 I and J: T2-A 100X, lower and higher cell densities, respectively

Figure 10 K and L: T3-HA 100X, lower and higher cell densities, respectively

Figure 10 M and N: T4-PA 100X, lower and higher cell densities, respectively

Figure 2: Detection of Mouse and Human Ras Genes by PCR Analysis

Agarose Gel Electrophoresis of Mouse and Human HRAS PCR products: Total DNA was isolated from NIH/3T3, T-2 and T4-PA cell lines and mouse liver. Human placenta DNA was purchased commercially. PCR Reactions were performed using human primer set (HN-3630 with HT-4781) or mouse primer set (MN-1121 with MT-1336) on each DNA sample. Products were analyzed by agarose gel electrophoresis and portions of each gel photograph are shown at the human 1152 bp and mouse 216 bp size regions detected in ethidium bromide stained slab gels. Lanes 1,2,5,6,9,10,13,14,17 & 18 show results from the mouse primer set and lanes 3,4,7,8,11,12,15,16,19 & 20 show results from the human primer set. No products appeared in controls with each primer set without added DNA (not shown). M = primer set, H = Human primer set.

Figure 3: Using a Gridded Plate Method to Determine Growth Rates for Seven Cell Types.

Each cell type was plated, allowed to grow at least one day and fresh media was added. Photomicrographs were recorded at 100X of 6 predetermined 2x2 mm grids on each plate at the various time intervals indicated. A depiction of selecting random grids for a plate is illustrated in figure 3 insert. The average number of cells in the 6 grids counted in the plate for each cell type each day was determined along with a standard deviation. Relative growth fold increase was determined by normalizing each average cell count to the cell count average of the earliest time point shown in the semi-log graph. To plot all the data on the same graph, the first time point for each cell type was displaced by a one-day on this graph. The graph for each cell type includes some portion of the log phase of growth from which the doubling times were determined as listed in Table ??? . The R² value for each growth curve was determined when possible. Doubling times were determined from each curve and compared, in the case of the NIH/3T3 cells, to the doubling times determined from two experiments using the standard hemocytometer method of counting viable cells each day from 4 multiple plates.

Figure 3 (insert)
Six random grids marked on the bottom of a 60 mm diameter plate with 2x2 mm grids is depicted here and grids are not shown to scale.