A Network of Genetic Events Sufficient to Convert Normal Human Cells to a Tumorigenic State

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Abstract

Although great progress has been made at identifying and characterizing individual genes involved in cancer, less is known about how the combination of such genes collaborate to form tumors in humans. To this end, we sought to genetically recreate tumorigenesis in normal human cells using genes altered in human cancer. We now show that expression of mammalian proteins that inactivate the tumor suppressors Rb and p53 in conjunction with the oncoproteins Ras and Myc and the telomerase subunit hTERT is sufficient to drive a number of normal human somatic cells to a tumorigenic fate. This provides a blueprint of the events that lead to human cancer, allowing different cancers to be genetically modeled from normal human cells. (Cancer Res 2005; 65(21): 9824-8)

Introduction

There are numerous phenotypes that cells acquire to grow in a tumorigenic fashion. Such phenotypes include the ability to proliferate independent of external cues while also overcoming antiproliferative and apoptotic signals, in addition to cellular immortality, angiogenesis, and ultimately invasion and metastasis (1). Great progress has been made in identifying individual changes that foster these various phenotypes, such as loss of tumor suppressors, activation of oncproteins, restoration of telomerase activity, etc. However, it has been more challenging to reassemble how these pathways collectively drive the tumorigenic process. While mice models have been valuable in this regard, genetic alterations characteristic of specific human cancers often do not yield the same type of cancer in mice, mice are prone to different types of cancers, and their cells are far more easily transformed compared with humans (2–5). Recapitulating the set of genetic events that collectively are sufficient for tumorigenesis in human cells would thus help decipher the complex network of pathways required for human cancer.

It was recently shown that a number of human cells from either the epithelium (kidney, prostate, ovary, and mammary gland) or mesenchyme (fibroblasts and myoblasts) are converted to a tumorigenic state by the enforced expression of two viral proteins, SV40 T-Ag and t-Ag, and hTERT (6, 7). hTERT and the oncogenic (G12V) Ras, these proteins drive normal somatic cells to a tumorigenic fate (8) or fostering factor-independent cell proliferation, cell survival, invasion, angiogenesis, and metastasis in the case of Ras (9). Conversely, the SV40 viral proteins may, at most, be involved in the etiology of only a handful of human cancers (10). To complicate matters, both T-Ag and t-Ag are known to have a host of functions and interact with a wide range of cellular proteins (11–13). We therefore sought to identify a core set of cellular proteins that, in cooperation with hTERT and Ras, could replace viral oncoproteins to promote tumorigenic phenotypes, allowing cancer to be genetically dissected from its beginnings from a normal human cell.

A subset of functions of T-Ag and t-Ag are known to participate in oncogenesis. T-Ag binds to and inactivates the tumor suppressors Rb and p53, two genes commonly disrupted in human cancers, which allows cells to evade antiproliferative and apoptotic signals (12). Rb function can also be disrupted in human cells via constitutive phosphorylation by an activated cyclin/cyclin-dependent kinase (CDK) complex and both overexpression of cyclin D and activation mutants of CDK4 have been found in human cancers (14–16). p53 function can be impeded in human cells by expression of p53(DD), a dominant-negative form of the protein (14). In the case of t-Ag, the oncogenic activity of this protein has been attributed to inhibition of the function of protein phosphatase 2A (11, 13), which in turn exerts its oncogenic effects through, in large part, the stabilization of the proto-oncogene c-Myc (17). c-Myc is often up-regulated through either a stabilization mutation or gene amplification in a wide variety of human cancers and is known to play an important role in cancer (18, 19). Given that mutations or alterations in the expression or activity of cyclin D1, CDK4, Myc, and p53 all occur in cancer, and hence are considered bona fide tumorigenic changes (1), we tested whether the oncogenic activities of the SV40 viral proteins could be replaced by the cellular proteins cyclin D1, the R24C activated mutant of CDK4, p53(DD), and the T58A stabilization mutant of c-Myc and whether, in cooperation with hTERT and the oncogenic (G12V) Ras, these proteins drive normal human somatic cells to a fully tumorigenic state.

Materials and Methods

Cell lines. Low passage cultures of enriched mammary epithelial cells (Clonetics Cell Systems, Cambrex Corp., East Rutherford, NJ) were sequentially infected using established methodologies (7) with amphotrophic retroviruses derived from the plasmids pBabeHygro-FLAG-hTERT (20), pBABEneo-p53(DD) (14), pBABEpuro-cyclinD1-HA (14), pBABEbleo-CDK4(24C)-HA (14), and pBABEblast-c-Myc(T58A) (17) and sequentially selected for 7 to 10 days in media supplemented with 50 µg/ml hygromycin B, 250 µg/ml G418, 0.5 µg/ml puromycin, 800 µg/ml zeocin, and 10 µg/ml blastocidin, respectively (Sigma Chemical Co., St. Louis, MO and Life Technologies Invitrogen, Carlsbad, CA). Cultures were allowed to recover between selections for 5 days, except in the case of blastocidin, which required 14 days. Stably infected cultures were then infected with either pBABEhyfp-FLAG-H-Ras(G12V) or pBABEhyfp (6) and, 2 days later, were...
fluorescence-activated cell sorted for YFP+ cells, generating the cell lines Ma6 and Ma6-Ras, respectively. Enriched low passage human myoblast cultures (Clonetics Cell Systems) were infected with the aforementioned retroviruses encoding the six transgenes or all but RasG12V and selected in an identical fashion yielding cultures Mu6 and Mu6-Ras, respectively, with the exception that the pBABEneo-p53DD retrovirus was used to infect the primary culture before pBABEh2o-FLAG-hTERT. Lastly, human embryonic kidney cells were stably infected every 2 days without selection with retroviruses pBABE-hTERT-p53DD, pBABE-cyclinD1-CDK4R24C, and pBABE-c-MycT58A-H-RasG12V. These plasmids were generated by cloning FLAG-hTERT, cyclinD1, or c-MycT58A cDNAs with flanking EcoRI-Sall restriction sites added by PCR into the same sites of the polylinker of pBABEneo-p53DD and selected in media supplemented with 0.5 μg/mL puromycin, resulting in the polyclonal population termed He6. All retrovirus-infected cells were confirmed to be free of helper viruses by the horizontal spread assay.

Reverse transcription-PCR. Two micrograms of total RNA, prepared using the RNaZol B reagent (TEL-TEST, Friendswood, TX), were reverse transcribed using the Omniscript kit (QIAGEN, Valencia, CA) with OligoDT primer (Life Technologies Invitrogen); after which 4 μL of each reaction were PCR amplified using one primer specific to the transgene and another specific to a transcribed region of the pBABE plasmids or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as follows: 5′-GAGAGGAGGAAGAGGAGG (CDK4R24C), 5′-GAGGAGGAAGGAGGAGG and 5′-TTTCCACACCTGGTTGC (hTERT), 5′-GCTCTTACCTGGCTGCAATCCA (FLAG-hTERT), 5′-GCTCATGCTGCTGCTGCTG (p53DD), 5′-AGCTTGCCAAACCTACAGG (H-RasG12V). Expression of these mammalian transcripts (Fig. 1A) was confirmed by RT-PCR in the two cell lines and an empty vector, establishing a population termed Ma6 and Ma6-Ras cells; however, due to the cloning of transgenes at different sites in the pBABE plasmids encoding two transgenes, the following primer pairs were employed for reverse transcription-PCR (RT-PCR) amplification of He6 cells: 5′-TGCCAGTGCCACAGGAGC (hTERT), 5′-GTCTCAGTCAGGTGTGA and 5′-ATGCCTTGCAAATATGGG (p53DD), 5′-ACATGGACCATCCAAAGCC and 5′-TTCTGCTCTGGTGCTG (cyclinD1), 5′-GCTTGCTCTGGTGCTG (c-MycT58A), and 5′-GCACGACTTGGAATC and 5′-TAGCTGGCAAACTCAGAG (H-RasG12V). Transformation and tumorigenesis. Ma6 or Ma6-Ras cells (1 × 10⁶) or Mu6 or Mu6-Ras cells, were seeded in soft agar and 3 or 4 weeks later, respectively, the resultant plates were photographed and colonies >30 cells were scored as previously described (7). For xenograft assays, 1 × 10⁶ cells were suspended in 50 μL Matrigel (BD Biosciences, San Jose, CA) and injected s.c. into the flank of a severe combined immunodeficient/Beige mouse as previously described (7). Each cell line was tested in quadruplicate. All experiments were done under Duke Institutional Animal Care and Use Committee–approved protocols.

Results and Discussion

Tumorigenic conversion of mammary cells by expression of mammalian proteins. The Rh, p53, Myc, Ras, and telomerase pathways were perturbed in a fashion akin to that found in human cancers (1) by sequentially stably infecting (and selecting for an encoded selectable marker) a mixed population of cells established from human mammary tissue with retroviruses expressing hTERT (20), p53DD (14), cyclin D1 (14), CDK4R24C (14), c-MycT58A (17), and then either H-RasG12V (6), establishing a polyclonal population termed Mammary cells 6 gene (Ma6), or as a control an empty vector, establishing a population termed Ma6-Ras. Expression of the appropriate transgenes was confirmed by RT-PCR in the two cell populations using one primer unique to an expressed region of the retroviral vector and another anchored in the desired transgene to specifically amplify only the ectopically expressed versions of these mammalian transscripts (Fig. 1A).

Figure 1. Tumorigenic conversion of mammary tissue cells by expression of cellular proteins involved in human cancer. A, primary culture of enriched mammary epithelial cell was serially infected with and selected for retroviruses encoding hTERT, p53DD, cyclin D1, CDK4R24C, MycT58A and either RasG12V or an empty vector. RT-PCR amplification confirmed the expression of all six transgenes in the resultant polyclonal cell population Mammary cells 6 gene (Ma6) or five transgenes in the Ma6 cells lacking oncogenic Ras (Ma6-Ras). GAPDH serves as a loading control; uninfected (UN) cells serve as a negative control. Expressions vary for each reaction. B, photograph of colony growth in a representative plate of Ma6 or Ma6-Ras cells seeded in soft agar. C, photograph of a representative tumor (arrow) formed after Ma6 cells were injected into the flank of an immunocompromised mouse. D, H&E staining of a representative plate of Ma6 cells formed after Ma6 cells were injected into the flank of an immunocompromised mouse.
To assess the effect of expression of these six genes on the oncogenic process, both cell populations were suspended in soft agar to assay for anchorage-independent growth, a common characteristic of cancer cells. Whereas expression of five of six of these transgenes in the Ma6-Ras cells was unable to support anchorage-independent growth of mammary tissue cells, expression of all six (Ma6 cells) led to robust transformed cell growth in soft agar (Fig. 1B; Table 1). This level of transformation was similar to that seen when viral proteins were used to transform cells (not shown), argues that T-Ag and t-Ag functions could indeed be functionally replaced by p53<sup>DD</sup>, cyclin D1, CDK4<sup>R24C</sup>, and c-Myc<sup>T58A</sup>. Encouraged by these results, we next tested whether these cells would form tumors in immunocompromised mice, the most rigorous test for tumorigenesis. Ma6 cells, or as a control Ma6-Ras cells, were each injected s.c. into the flanks of four immunocompromised mice, after which tumor size was measured regularly. Control Ma6-Ras cells not only failed to grow in soft agar but also did not form tumors even after greatly extending the period of observation to 20 weeks (Table 1). In sharp contrast, all mice injected with the Ma6 cells expressing the six aforementioned proteins formed tumors at the site of injection, with an average latency period of 7 weeks (Fig. 1D; Table 1). Interestingly, two types of tumors grew in these mice, one which grew in a cystic by nevertheless neoplastic fashion and one that formed a solid mass, presumably reflecting a transformation of different cell types in the mixed population. The later tumors appeared as highly undifferentiated carcinomas with areas of local tissue invasion, characterized by cells with large nuclei with multiple nucleoli (Fig. 1C). Both the growth kinetics and morphology of tumors mimicked those generated by viral proteins T-Ag and t-Ag (not shown), arguing that the expressed cellular proteins replaced the functions of the aforementioned viral proteins.

**Tumorigenic conversion of muscle cells by expression of mammalian proteins.** To rule out cell type accounting for the described tumor growth and to confirm these observations, we tested whether ectopic expression of hTERT, p53<sup>DD</sup>, cyclin D1, CDK4<sup>R24C</sup>, c-Myc<sup>T58A</sup>, and H-Ras<sup>G12V</sup> could similarly promote the transformed and tumorigenic growth of a completely different normal human cell type. An enriched population of human skeletal muscle myoblasts was therefore infected with and selected for retroviruses encoding the described six, or as a control five, mammalian proteins in a fashion identical to that outlined above for the mammary cells. Cells confirmed by RT-PCR to express all six transgenes were termed Muscle cells 6 gene (Mu6) whereas the control cells lacking Ras<sup>G12V</sup> expression were termed Mu6-Ras (Fig. 2A). Both cell populations were seeded in soft agar to assay for anchorage-independent growth. As in the case of mammary cells, only expression of all six transgenes promoted transformed cell growth of the muscle cells in soft agar (Mu6 cells, Fig. 2B and Table 1). Similarly, Mu6 cells were also tumorigenic when injected s.c. into the flank of four immunocompromised mice; within 5 weeks palpable tumors formed, which were found to be highly undifferentiated and characterized by a high mitotic index and cells with large nuclei and macronucleoli (Fig. 2C; Table 1). In contrast, identically treated cells expressing the same transgenes except Ras<sup>G12V</sup> failed to form tumors, even after greatly extending the period of observation to 19 weeks (Mu6-Ras cells, Table 1). We conclude that independent of cell type, expression of mammalian proteins that suppress the p53 and Rb pathways and lead to the activation of c-Myc, H-Ras, and hTERT is tumorigenic in human cells.

**Rapid induction of tumorigenesis by expression of mammalian proteins.** It is possible that mutations were selected during the process of making the Ma6 and Mu6 cells that contributed to their tumorigenic phenotype. However, it is worth pointing out that care was taken to ensure that clonal populations were not generated during the introduction of the transgenes and that altered pathways already implicated to collaborate to form tumors, at least when viral proteins were used for transformation (6). Moreover, if a mutation did occur that was essential for tumorigenesis, it would have had occurred independently in the mammary and muscle cultures and only during the period the cells were being generated.

**Table 1. Transformed and tumorigenic phenotypes of engineered tumor cells.**

<table>
<thead>
<tr>
<th></th>
<th>Ma6-Ras</th>
<th>Ma6</th>
<th>Mu6-Ras</th>
<th>Mu6</th>
<th>He6</th>
</tr>
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<tbody>
<tr>
<td>Colonies in soft agar&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0 ± 0</td>
<td>247 ± 22</td>
<td>1.3 ± 0.6</td>
<td>52 ± 20</td>
<td>ND</td>
</tr>
<tr>
<td>Mice with tumors/ mice injected</td>
<td>0/4</td>
<td>4/4</td>
<td>0/4</td>
<td>4/4</td>
<td>4/4</td>
</tr>
<tr>
<td>Latency (wk)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>NG (&gt;20)</td>
<td>7</td>
<td>NG (&gt;19)</td>
<td>5</td>
<td>2</td>
</tr>
</tbody>
</table>

Abbreviations: NG, no growth. ND, not determined.

<sup>a</sup>Average number and SD of colonies growing in soft agar from triplicate plates.

<sup>b</sup>Latency was determined as the time taken before a palpable mass could be detected.
The desired six transgenes (Fig. 3c-MycT58A and H-Ras G12V. This was immediately followed by infection with a retrovirus derived from pBABEpuro-p53DD. The resulting cell population, termed human embryonic kidney-6 gene (He6) was confirmed to express these transgenes by RT-PCR. A water sample amplified with GAPDH-specific primers serves as a negative control; GAPDH serves as a loading control. B, photograph of a representative tumor (arrow) formed after He6 cells were injected into the flank of an immunocompromised mouse. C, H&E staining of a representative tumor formed after He6 cells were injected into the flank of an immunocompromised mouse. Note the prominent invasion of tumor cells into the adjacent muscle tissue (right).

Conclusion

We now show that expression of a set of six proteins altered in human cancers, which inactivate the tumor suppressors Rb and p53 and activate the Ras, Myc, and hTERT pathways, is sufficient to drive normal human somatic cells to a tumorigenic state. As tumor growth was reproducibly achieved by this approach in three very different cell types and could be rapidly induced, we argue that these changes, and not others, account for the tumor phenotype. Whereas it is clear that these exact changes are not found in every cancer, many mutations in human cancers are known to cause similar changes to those inflicted by one or more of these six proteins (1). Given this overlap, we speculate that these changes may serve as a general blueprint for the pathways that collectively must be altered in a similar fashion for the clinical growth of solid tumors.

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as both the colonies in soft agar and the tumors grew too fast to allow for the selection of additional events during these periods of growth. Nevertheless, to address this possibility, yet another cell type, normal human embryonic kidney cells, was infected over the course of 6 days in the absence of drug selection with three of the retroviruses derived from pBABE plasmids that we engineered to coexpress pairs of hTERT and p53DD, cyclin D1 and CDK4R24C, and c-MycT58A and H-RasG12V. This was immediately followed by infection with a pBABEpuro-p53DD-derived retrovirus and puromycin selection for another 6 days to ensure high expression of this dominant-negative protein, to enrich for infected cells, and, as a safety measure, to provide a means to easily detect helper virus. Thus, in the course of 2 weeks, cells were infected with retroviruses encoding the six transgenes shown to cooperate for promotion of tumorigenic growth of human cells. This cell population, termed human embryonic kidney-6 gene (He6), was confirmed to express the desired six transgenes (Fig. 3A) and injected into the flanks of four immunocompromised mice to assay for tumor growth. In all four mice, tumors were detected at, on average, 14 days post-injection (Fig. 3B; Table 1). These tumors were highly neoplastic and poorly differentiated, invading into adjacent muscle tissue. Cells displayed large pleomorphic nuclei with multiple nucleoli (Fig. 3C). The rapid introduction of these transgenes and formation of tumors supports the argument that expression of hTERT, p53DD, cyclin D1, CDK4R24C, c-MycT58A, and H-RasG12V is sufficient to drive normal human cells to a tumorigenic state.

References


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