p53 Mutations in Mouse Mammary Epithelial Cells: Instability in Culture and Discordant Selection of Mutations in Vitro versus in Vivo

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Abstract
The phenotypes of p53 mutations found in human and murine tumors often are analyzed using a variety of transformation assays in vitro, but data have not been available to correlate in vitro effects with in vivo activities. We have assessed the effects of p53 mutations using mouse mammary epithelial cell lines which can be analyzed both in vitro and in vivo. Parental mammary epithelial cell lines (FSK series) injected into cleared mammary fat pads of syngeneic mice frequently give rise to preneoplastic lesions (HAN) which can be reestablished in culture (TM lines) to permit analysis of genetic changes important in the development of preneoplasia. Characterization of the FSK3 cell line revealed a cell population mixed with respect to p53 genotypes. One subpopulation of mutant (Ser283–284) p53-expressing cells was selected in a preneoplastic mammary outgrowth in vivo (TM3), whereas another minor population of mutant (Pro235) p53-expressing cells was selected during culturing of FSK3 cells in vitro. When FSK3 cells were subdivided and passaged in parallel in vitro, p53 genotypes continued to evolve and diverge. These findings reveal that selective pressures exerted on mammary epithelial cell populations in vivo are different from pressures present in vitro. Selective forces may vary from one cell culture environment to another. The growth advantage conferred by a specific p53 mutation appears to differ in vivo versus in vitro. We propose that caution should be exercised when attempting to correlate the effects of p53 mutations assayed in cell culture systems with the in situ phenotypes of mutant p53 in cancer.

Introduction
A selective growth advantage conferred on a normal cell by some genetic mutation has been proposed to be the earliest step in tumor initiation (1). Subsequent selection by a multistep evolutionary process gives rise to subpopulations of cells that are increasingly abnormal, both genetically and biologically. Genetic alterations known to be important in the genesis of cancer include the activation of cellular protooncogenes and the functional loss of tumor suppressor genes (reviewed in Refs. 2 and 3). Immortalization of cells is also widely held to be an important step in tumorigenesis (for a review, see Ref. 4), although the biochemical and genetic mechanisms remain unclear. The stage-wise progression of neoplastic transformation has been clearly depicted in studies using cell culture models (reviewed in Ref. 5). Thus, many of the efforts made to understand carcinogenesis in vivo have been through the use of immortalized and transformed cells propagated in vitro.

In recent studies, independent laboratories have shown that the tumor suppressor gene p53 is commonly altered in certain spontaneously immortalized murine embryo cells (6, 7), indicating that this event may be significant in the immortalization process. Furthermore, mutations in p53 are commonly observed in primary tumors of human and animal origin (reviewed in Refs. 8 and 9). A number of studies have shown that although the WT p53 gene product can act to suppress the growth of many types of cells and tumors (10–24), MT forms of p53 often behave as dominant oncogenes capable of immortalizing primary cells in culture (25, 26) and cooperating with an activated ras oncogene to transform primary cells (27–31). Expression of certain p53 MTs is known to increase the growth advantage of cells in culture by increasing the plating efficiency of G418-resistant colonies (10, 32), stimulating oncogene-mediated focus formation (11), augmenting saturation density and colony formation in soft agar (22), and increasing resistance to the negative growth factor transforming growth factor β (32).

Few reports have considered the growth advantage that p53 MTs may have in vivo, aside from studies using nude mice. In one instance, expression of a mutated p53 gene in murine leukemia cells lacking endogenous p53 resulted in cells that were more malignant when injected into syngeneic animals (23, 33). A second study showed that transfection of a p53 MT increased the metastatic capacity of bladder carcinoma cells inoculated i.v. into syngeneic mice (34). A recent report described two MT p53 alleles from a case of human acute myelogenous leukemia. Each p53 allele was found to have different missense mutations, suggesting a recessive activity; however, when overexpressed in vitro, each MT was capable of acting as a dominant oncogene by co-
operating with an activated ras oncogene (35). Due to the lack of in vivo functional data on p53, it is difficult to correlate the phenotypes displayed by p53 MTs in an artificial culture system with phenotypes observed in the milieu of a whole animal.

In a recently developed murine system, genetic changes important in mammary preneoplasia can be investigated using MMECL in vivo and in vitro (36). When these cells are transplanted into the cleared mammary fat pad of a syngeneic mouse, preneoplastic lesions known as HAN result. The HAN outgrowths can be reestablished in vitro or can be serially transplanted to other animals. This system has been used to assess the potential role for p53 changes in the early stages of breast cancer (37, 38). These studies demonstrated that mutation of p53, although not necessary for the in vitro establishment or in vivo immortalization of MMECL, accompanied preneoplastic outgrowth in vivo (37–39). In addition, the p53 mutations selected in the outgrowths were also maintained in mammary tumors which developed from the preneoplasias in vivo (38). Although no p53 mutations were detected in the parental cell lines (FSK) which gave rise to the preneoplasias in vivo (37), it seemed plausible that a subpopulation of the parental cells might express MT p53 and that these cells would be efficiently selected following transplantation in vivo. We hypothesized that a minor population of MT p53-expressing cells which could be efficiently selected in an animal might also have a significant growth advantage when cultured in vitro. Such a finding would provide a more direct correlation of MT p53 phenotypes assayed in cell culture with MT p53 activities in a whole animal.

The purpose of this study was 2-fold: (a) to determine whether p53 mutations selected in cell lines derived from HAN and in mammary tumors preexist in a subpopulation of the parental lines prior to transplantation in vivo; and (b) if preexisting, to assess whether these mutations provide any selective growth advantage to the cells in vitro. Two independently derived parental MMECL (FSK3 and FSK4) were analyzed for p53 mutations which were characterized in their respective HAN outgrowth cell lines, TM3A and TM4 (37, 38). Our findings illustrate that the selective forces operating on a cell population in vivo are different from those forces exerted in vitro. We propose that the mutational evolution of the p53 gene continues until the greatest growth advantage conferred by a specific MT p53, in concert with changes in other growth-promoting or growth-suppressing gene products, is reached. This model for p53 is analogous to what has been proposed and shown for the evolution of tumor cell populations (1, 40).

Results

Analysis for Preexisting p53 Mutations in Subpopulations of Parental (FSK) Cell Lines. To determine whether the p53 mutations selected in HAN outgrowths and maintained in the outgrowth (TM) cell lines (37, 38) were present in a subpopulation of the parental (FSK) cell lines, MT p53-specific PCR was performed. The TM3A cell line overexpresses MT p53 due to a 3-bp insertion causing the addition of a serine residue between amino acids 233 and 234 (Ser233–234; see Fig. 1) (37). The TM4 cell line also overexpresses MT p53. TM4 synthesizes two MT p53 transcripts: one contains a point mutation (Trp134); the other has a 21-bp deletion resulting in the loss of amino acids 123–129 (37, 38). Primer 51A was shown to be specific for the 3-bp insertion (Ser233–234) mutation in p53 from TM3A cells (37), as it gave the appropriately sized product by RT-PCR when used with primer 27B (Fig. 2A; Fig. 2B, Lanes 2 and 3). A PCR product of identical size, albeit lower intensity, was also detected using primers 51A + 27B on cDNA from the parental FSK3 cells at P13 (vial A; Fig. 2B, Lane 4). As expected, no specific PCR product was observed when cDNA from a primary culture of MMEC was analyzed with primers 51A + 27B (Fig. 2B, Lane 1). These data demonstrated that the Ser233–234 p53 mutation found in the TM3A cell line was preexisting in the parental FSK3 cell line prior to transplantation in vivo. For the same purpose, primer 49A was synthesized to be specific for the 21-bp deletion in one allele of TM4 (38). Analysis of TM4 cell cDNA revealed that primer 49A, used in conjunction with 25B, was specific for the 21-bp deletion in p53, as an expected product of 86 bp was observed (Fig. 2A; Fig. 2C, Lanes 2 and 3). No specific product was seen when either the control cDNA from a primary culture of MMEC or the sample cDNA from FSK4 at P15 was analyzed using primers 49A + 25B (Fig. 2C, Lanes 1 and 4, respectively).

The parental FSK3 and FSK4 cell lines were transplanted in vivo at passages 11 and 8, respectively (Table 1), whereas passages 13 and 15 (respectively) were initially analyzed by RT-PCR for the preexisting mutations. For this reason, cells from lower passages were recovered from the freezer and were designated as vial B to maintain separate identities. RNA was extracted from a subset of FSK3-vial B cells at P9, whereas another subset of cells was passaged to P12 before RNA was harvested. FSK4-vial B cells were extracted for RNA at P8. Mutant p53-specific PCR was performed as described above on the cDNA samples from the lower passages of the cell lines. Results indicated that FSK3 P9 (Fig. 2B, Lane 6) and P12 (data not shown) and FSK4 P8 (Fig. 2C, Lane 5) did not express the respective p53 mutations that were selected when the parental FSK cell lines were transplanted in vivo. Southern transfer of the gels shown in Fig. 2 and hybridization using a random-primed, labeled murine p53
cDNA probe confirmed that no specific PCR products were amplified from the vial B cDNA samples (data not shown). The observation that FSK3 cells at P9 were negative for the Ser233→234 p53 mutation, whereas a second group of FSK3 cells at P13 was positive, suggests that the Ser233→234 p53 mutation occurred in vitro after P9 (see Fig. 1). Although the lower passages (P8, P15) of the FSK4 cell line tested negative for the 21-bp deletion mutation in p53 that was selected in the TM4 outgrowth cell line, it is possible that a rare mutant was below the detection level of the PCR assay.

Screening of FSK Cell Lines Passed in Vitro for Enrichment of the p53 Mutations Selected in Vivo. To investigate the possibility that p53 mutations expressed in subpopulations of the respective FSK3 and FSK4 cell lines might confer a significant growth advantage on those cells during propagation in vitro, the cells (designated as vial C; see Fig. 1) were passaged in culture, and RNA was extracted as described in "Materials and Methods." MT-specific PCR was used as described above. Results indicated that at high passages (P > 30), neither the FSK3-vial C cell line (Fig. 2B, Lane 7) nor the FSK4-vial C cell line (Fig. 2C, Lane 6) expressed the particular p53 mutations that were selected in their respective outgrowth cell lines, TM3A and TM4. Thus, although the Ser233→234 p53 mutation was present in the FSK3 cell line prior to transplantation in vivo, it was efficiently selected during formation of the HAN outgrowth in vivo, and was maintained in the TM3A outgrowth cell line in vitro, the mutation did not confer an observable growth advantage to the FSK3 cells during passage in vitro.

Protein Conformations and Stability of p53 Expressed by FSK Cell Lines. The FSK3 and FSK4 cell lines were analyzed at high passages (P > 30) to determine whether WT or MT forms of the p53 protein were expressed. The cell lines were metabolically labeled with 35S as indicated in "Materials and Methods." Cell extracts were analyzed by immunoprecipitation and sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Whereas the FSK3 and FSK4 cell lines at P < 15 expressed only the WT (PAb246*) form of the p53 protein (37), these cell lines at P > 30 synthesized both WT and MT (PAb240*) forms of p53 (Fig. 3A, Lanes 1–6), just as was observed in the respective outgrowth cell lines, TM3A and
Fig. 3. ^15S labeling and immunoprecipitation of p53 protein expressed in FSK cell lines at P > 30. Cells were either metabolically labeled (A) or pulse-chase labeled (B) as indicated in "Materials and Methods." A, cell extracts were immunoprecipitated with an irrelevant monoclonal antibody (MT3); negative control; Lanes 1, 4, 7, and 10] or anti-murine p53 monoclonal antibodies PAb240 (240; p53 MT specific; Lanes 2, 5, 8, and 11) or PAb246 (246; p53 WT specific; Lanes 3, 6, 9, and 12). B, FSK3 P32-vial C cell extracts were harvested at the times indicated (T, minutes). Extracts were immunoprecipitated either with PAb240 (240; Lanes 1, 4, 7, 10, and 13); PAb246 (246; Lanes 2, 5, 8, 11, and 14), or a mixture of PAb248 + PAb421 (MIX; specific for p53 protein amino terminus and carboxy terminus, respectively; Lanes 3, 6, 9, 12, and 15). Molecular weight standards in kilodaltons are indicated at left; migration of p53 is shown by arrow at right.

TM4 (37). The relative stability (i.e., half-life) of the p53 protein was estimated in cells from both FSK3 and FSK4 lines by pulse labeling. The p53 protein detected in FSK3 and FSK4 cell lines at P < 15 was shown to have a half-life on the order of 30 min (37); conversely, the same cell lines at P > 30 expressed p53 with a relative half-life of ~2.5 h (Fig. 38, and data not shown). In addition, use of the murine p53-specific polyclonal antiserum CM5 in conjunction with IHC confirmed that the cell lines at P > 30 expressed unusually high levels of the p53 protein in the nucleus (data not shown) in contrast to the undetectable levels of p53 in the cell lines at P < 15 (37). Taken together, these immunoprecipitation, half-life, and IHC data indicate that the FSK3 and FSK4 cell lines at P > 30-vial C express MT conformations of the p53 protein with increased stability.

Sequence Analysis of p53 cDNA in Late Passages of the FSK3 and FSK4 Cell Lines. To determine the genetic basis for the overexpression of MT p53 in the late-passage FSK cell lines, the p53 sequences across exons 5–9 were analyzed using a PCR-based cloning strategy (37). Direct RT-PCR sequencing (38) was used to verify the mutations identified. Single base substitutions in the p53 cDNA from both FSK3 and FSK4 cell lines at P > 30-vial C were identified. Neither mutation corresponded to those found in the TM4 outgrowth cell lines. The FSK3 cell line at P > 30 expressed a mutation in p53 (at nt 560) which caused a substitution of the WT codon 135 alanine for a proline (Ala135→Leu135; Fig. 4A). The p53 synthesized in the FSK4 cell line at P > 30 was mutated (at nt 550) such that a leucine was substituted for the WT phenylalanine codon 131 (Phe131→Leu131; Fig. 4B).

As illustrated in Fig. 4, both cell lines appear to express a WT allele of p53 in addition to the MT allele. In addition, sequencing of 6–10 p53 PCR-derived clones from each FSK cell line at P > 30-vial C confirmed that two p53 alleles, one WT and one MT, were expressed in each of these cell lines. These data concur with the observation that both cell lines at P > 30 express both WT and MT conformations of the p53 protein. Thus, passage of both the FSK3 and FSK4 cell lines in culture (P > 30) has afforded the selection of a population of cells expressing MT p53 alleles. The FSK3 cell line at P13 expressed the Ser^233→Thr^234 insertion mutation subsequently selected during outgrowth in vivo (TM3A); however, during passage of FSK3 in vitro, this Ser^233→Thr^234 insertion mutation was lost, and a second mutation (Pro^135) was selected by P32. Similarly, the FSK4 cell line was efficiently enriched for a population expressing a p53 mutation (Leu^131) by P34 distinct from the Trp^134 mutation and the 21-bp deletion found in TM4 cells.

Expression of the Pro^135 p53 Mutation in Sublines of FSK3 Cells. A new restriction enzyme site was created by the G to C transversion at p53 nt 560 (Pro^135) defined in the FSK3
cell line at P32-vial C. To deduce whether a subpopulation of the FSK3 cells prior to transplantation synthesized the Pro135 p53 mutation, FSK3 cells from P13-vial A were passaged as described in "Materials and Methods." RNA was extracted from these cells at P25, P27, and P29; PCR and restriction digestion were used to verify the mutation in the FSK3 cells at P32-vial C and to analyze the lower-passage FSK3-vial A cell line as well (see Fig. 1). The PCR primers 33A + 25B were used to amplify a 132-bp fragment from p53 cDNA which included the sequences of codon 135 (Fig. 5A). Fnu4HI digestion of the 132-bp PCR product from p53 cDNA of FSK3 P32-vial C cells resulted in partial cleavage to the predicted 77-bp and 55-bp MT fragments (Fig. 5B, Lane 7). This is consistent with both protein and sequencing data, verifying that these cells expressed both a WT allele of p53 and an allele containing the codon Pro135 mutation. Primary MMEC, TM3A cells, and FSK3 cells at P13-vial A did not detectably express this mutation (Fig. 5B, Lanes 1–3, respectively). However, from FSK3-vial A, cells passaged to P25, P27, and P29 expressed increasing proportions of the Pro135 allele of p53 (Fig. 5B, Lanes 4–6, respectively). Detection of the Pro135 MT from two independently passaged FSK3 lines indicates that cells expressing the Pro135 mutation likely were constituents of the total FSK3 cell population along with the Ser231–234 insertion mutation prior to transplantation in vivo. In addition, these striking observations show that the enrichment of the total FSK3 cell population for the Pro135 mutation occurred rapidly. In the FSK3 cells from P29-vial A, the Pro135 mutant p53 allele appears to be the only allele present (Fig. 5B, Lane 6). This is in contrast to the observation that the FSK3 cells from P32-vial C expressed a WT p53 allele, as illustrated by sequencing (Fig. 4A) and resistance to Fnu4HI digestion (Fig. 5B, Lane 7).

Analysis of Late-Passage FSK3 Cells for Loss of WT p53 Expression. To determine whether the FSK3 cells at later passages were predisposed to the loss of the WT p53 allele, cells from FSK3 P32-vial C which expressed both a WT and a Pro135 MT p53 allele were divided into two populations. One population of FSK3-vial C cells was passaged to P > 45 (C1); the other population was propagated to P > 70 (C2; see Fig. 1). These cells were analyzed at both the protein and nucleic acid level for p53 synthesis. Metabolic labeling and immunoprecipitation indicated that both the FSK3 P > 45-vial C1 cells and the FSK3 P > 70-vial C2 cells continued to synthesize both WT and MT forms of the p53 protein (Fig. 3A, Lanes 7–12). However, RT-PCR using primers 33A + 25B followed by restriction enzyme digestion with Fnu4HI, as described above, yielded slightly dissimilar results. FSK3 P > 70-vial C2 cells continued to express both Pro135 and WT (at this codon) alleles of p53, whereas the FSK3 cells at P > 45-vial C1 synthesized only the Pro135 p53 allele (Fig. 5B, Lanes 9 and 8, respectively). These results show that the FSK3 cell line was not dramatically predisposed to loss of a p53 allele; however, loss (or conversion) of the WT allele did occur with some frequency during passaging of the FSK3 cell line in vivo.

Growth Properties of MMECL in Vitro. Many p53 mutations which confer a selective advantage to cells in vitro have been described (10, 11, 22, 32). To assess the relative growth enhancement provided by the p53 MT Pro135 selected in the FSK3 cells in vitro, or Ser231–234 selected in the TM3A outgrowth in vivo, growth rate and saturation density analyses were performed with the MMECL. When the FSK3 P36-vial C cells which expressed one WT and one Pro135 MT p53 allele were compared to the FSK3 P14-vial A cells (an apparently heterogeneous population of cells with respect to p53; see Fig. 1), no significant differences were observed (Fig. 6A). However, the TM3A cell line, which synthesized a single MT allele of p53 (Ser231–234), grew at a slower rate and to a lower saturation density than did the FSK3 P > 30-vial C (Ala/Pro135) cell line (Fig. 6B). This suggests that the Pro135 p53 MT may confer a growth advantage in vitro to (FSK3) cells relative to those (TM3A) cells expressing a Ser231–234 p53 MT. It is unknown whether the Pro135 MT confers a growth advantage to cells transplanted in vivo, owing to the difficulty in obtaining outgrowths when cells from late passages of the MMECL are injected. This technical problem is not well understood at present.

Discussion
Carcinogenesis is widely held to be a multistep process, and cell culture systems have established the stage-wise progression of neoplastic transformation (for a review, see Ref. 5). The studies of Newbold et al. (41) provided initial evidence that immortalization might be essential for tumorigenesis. Additional support came from the finding that expression of certain oncogenes was sufficient to immortalize normal cells (reviewed in Ref. 2). One such oncogene is a MT p53 gene (25, 26). In addition, mutation of p53 appears to be impor-
The primary objective of this investigation was to determine whether p53 mutations that were detected in TM cell lines derived from HAN in vivo (and selected in mammary tumors) were preexisting in a minor population of their parental (FSK) cell lines before transplantation in vivo. A parental MMECL (FSK3), which expressed only WT p53 as determined by the assays of protein immunoprecipitation and direct PCR sequencing of cDNA (37), was found using a more sensitive MT-specific PCR approach to contain a minor population of cells that synthesized MT (Ser(233-234) p53). Although not quantitative, the reproducibly lower yield of product from the MT-specific p53 PCR indicated that this mRNA species was a minor population of the total p53 mRNA pool. This probably explains why the mutation was not detected upon direct sequencing of the p53 cDNA from the FSK3 cell line at P13. Injection of the parental FSK3 cells into the cleared mammary fat pad of a syngeneic mouse resulted in a preneoplastic (HN) outgrowth (36). Two independently derived cell lines from this outgrowth (TM3A and TM3B; see Table 1) were shown to express p53 with the Ser(233-234) MT4 (37). A similar approach used to screen the parental FSK4 cell line for the 21-bp deletion MT found in TM4 yielded negative results. However, the detection of the mutation in the FSK4 cells could have been impeded in several ways. First, lack of sensitivity of the MT-specific PCR could preclude detection of the p53 MT in FSK4. Estimations of the sensitivity of this technique by dilution of TM3A RNA with normal primary MMECL RNA indicated that the Ser(233-234) MT could be detected at levels of approximately 5–10% (data not shown). Second, the transient nature of the Ser(233-234) MT in the FSK3 cell line during passage in vitro prompts us to question whether the FSK4 cells available for analyses may have been a distinct subpopulation compared to those transplanted in vivo. Finally, the 21-bp deletion MT in p53 may have arisen in vivo, following transplantation.

Because introduction of MT p53 can increase the proliferative capacity of cells in vitro (10, 11, 22, 32), we surmised that a subpopulation of cells expressing MT p53 (which was effectively enriched in vivo) would likely overtake the WT p53-expressing cells in the population when passed in vitro. This hypothesis proved incorrect. During passage of the FSK3 cell line in culture, the Ser(233-234) p53 MT was completely undetectable by P25 (Fig. 2B, Lane 5). Thus, the parental FSK3 cell line contained a subpopulation of cells that was efficiently selected during development of the preneoplastic outgrowth in vivo, but these cells were lost during passage of the cells in vitro. Not only was the Ser(233-234) MT undetectable in the FSK3 cells at later passages, but completely unrelated p53 mutations were observed in both FSK3 and FSK4 cell lines. Both FSK lines at the higher passages (vial C) were found to express a WT and a MT p53 allele. The FSK3 P32 cells expressed a Pro(335) MT allele, whereas the FSK4 P34 cells synthesized a Leu(331) MT allele. IHC analysis showed that every cell from both cell lines at P > 30-vial C overexpressed p53 protein in the nucleus (data not shown), arguing homogeneity with respect to p53 genotype (i.e., the cell lines did not contain two separate populations of cells, one expressing WT p53 alleles and the other expressing MT p53). These findings indicate that different p53 MTS which apparently conferred some growth advantage in vitro were selected in the MMECL.

* M. A. Ozbun, unpublished observations.
When the FSK3 P13-vial A cells that showed the Ser$^{233-234}$ MT in p53 were passaged to P25, the Pro$^{135}$ p53 MT was again detected. A comparison of the relative growth potential conferred by the respective p53 MT showed that the FSK3 P > 30 cells expressing the Pro$^{135}$ MT grew at a faster rate and to a higher saturation density than did the TM3A cells harboring the Ser$^{233-234}$ p53 MT. Although this is not a direct comparison of the difference in p53 MTs (which could be accomplished by individually placing the different p53 MTs into the same cell line to provide a uniform background), this experiment suggests that the two MTs may confer different phenotypes on the cells in vitro. Identification of the same Pro$^{135}$ p53 MT in independently cultured FSK3 cells from a second vial of frozen stock suggests that, in addition to the Ser$^{233-234}$ p53 MT, the Pro$^{135}$ MT may also have been present in a subpopulation of FSK3 cells prior to their transplantation in vivo. Together, these data indicate that the Ser$^{233-234}$ p53 MT may provide the cells with an advantage over the Pro$^{135}$-expressing cells in vivo, whereas in vitro the cells expressing the Pro$^{135}$ MT may have the advantage.

Recent reports indicate that p53 MTs frequently occur in the establishment of murine cell lines (6, 7, 42); however, to our knowledge, this report is the first to demonstrate how quickly MT p53-expressing cells can dominate a population. Although the Pro$^{135}$ MT was present at a ratio of ≈1:1 with the WT allele in FSK3-vial A at P25, by P29 the WT p53 allele was virtually undetectable. From these findings, we infer that two different cell types were present in FSK3 P25, one expressing WT p53, the other only the MT Pro$^{135}$ p53 allele, and that the p53 Pro$^{135}$-expressing cells exerted a vast proliferative advantage over the WT p53-expressing cells in culture. These data seem to conflict with the finding that no growth rate difference was observed when the FSK3 P14-vial A cells were compared with FSK3 P36-vial C cells. One possible explanation for this apparent discrepancy is that the FSK3 P36 cells express both Pro$^{135}$ MT and WT p53 alleles in the same cells, in contrast to the FSK3 P25–P29 cells (vial A) which we believe expressed the Pro$^{135}$ MT and WT p53 alleles in different cell populations. Within an individual cell, expression of the WT p53 allele may serve to restrain the growth-promoting effects of a Pro$^{135}$ MT p53 allele.

That the p53 genotype evolves in cells in vitro might be expected in light of the theory of clonal selection as proposed by Nowell (1); however, we believe this to be the first report of the evolution phenomenon in a cell culture system with the p53 gene as the indicator. Others have shown that cells in vitro expressing a MT p53 have a growth advantage over cells expressing only WT p53 (10, 11, 22, 32). However, we have illustrated that a specific p53 MT apparently can confer a growth advantage to a cell in vitro in either the presence or absence of the WT p53 allele. Whereas FSK3 P7-vial C$_2$ continued to synthesize both the WT and Pro$^{135}$ p53 alleles, the FSK3 P49-vial C$_1$ and P29-vial A sublines were found to have lost the WT p53 allele. It has been noted that a fraction of p53 proteins expressed from MT genes were PAb246 reactive ("pseudo-WT p53") (43, 47). This would explain why both PAB240- and PAB246-reactive forms of p53 were recovered from the FSK3-C$_2$ subline (Fig. 3A, Lanes 8 and 9). Hence, the P > 70-vial C$_2$ cell population appears to have reached a maximum growth advantage while continuing to express two p53 alleles, whereas the P > 45-vial C$_1$ and P > 29-vial A cell populations selected for the loss of the non-Pro$^{135}$ MT (Ala$^{135}$) allele. We cannot exclude the possibility that the FSK3 P > 70 cell population, which expresses both Ala$^{135}$ (presumably WT) and Pro$^{135}$ p53 alleles, has sustained a mutation elsewhere in the Ala$^{135}$ allele. Nonetheless, a second p53 MT occurring in this "WT" allele would only strengthen the argument that p53 is unstable in vitro. Thus, it appears that passage of the FSK3 cells in vitro facilitated a continual evolution of p53 mutation.

Previous investigations have suggested that the cellular and molecular changes that occur in the neoplastic conversion of cells in vivo and in vitro are the same (reviewed in Ref. 5). These studies have argued that the benefits of cell culture systems include the ability to quantitatively investigate cellular, biochemical, and molecular aspects of defined cell populations using techniques which are impractical for use in vivo. However, the data presented here indicate that the p53 genotype is unstable and prone to evolution of mutations in MMECL passed in vitro. Therefore, we propose that, with respect to certain growth suppressor genes (i.e., p53), a cell line may not be well defined until it has had sufficient time in culture to accumulate the mutations necessary for conferring the growth properties needed in the in vitro microenvironment. We also predict that different mutations may be selected in different microenvironments. However, it is difficult experimentally to address the possibility that subpopulations of subcultured cells expressing various p53 mutations are battling for dominance in the population. In contrast, it would seem that after a p53 mutation has been rigorously selected in a population of cells in vivo, the p53 genotype becomes stable: analyses using p53 MT-specific PCR showed that both TM3A and TM4 continued to express the same MT p53 following in vitro passage to P26 and P51, respectively (Fig. 1; Fig. 2B, Lane 3; Fig. 2C, Lane 3).

From this study, two rather striking observations should be noted. The first is the number of MT p53-expressing cell populations that were identified in the FSK3 cell line; the second is the apparent rate at which the p53 MT populations evolved (Fig. 1). The FSK3 population at P13 was found to synthesize the Ser$^{233-234}$ insertion p53 MT, whereas another minor population presumably expressed the Pro$^{135}$ MT; however, only the Ser$^{233-234}$ p53 MT was detected in the preneoplastic outgrowth cell line TM3A. By P25 in FSK3, the Ser$^{233-234}$ insertion p53 MT was not detected, whereas the proportion of Pro$^{135}$-expressing cells increased. Over the course of four passages of FSK3-vial A (P25–P29) in vitro, the WT p53-expressing cells disappeared. We conclude that the selective pressures placed upon the MMECL vary, depending upon the milieu of cellular propagation. Likewise, specific p53 mutations may provide different growth effects, depending upon the environment in which they are expressed (i.e., a whole animal versus a cell culture flask). Although the relevance and usefulness of cell culture systems as models for multistep tumorigenesis must not be underestimated, it is important to keep the information gleaned from such systems in perspective. We reiterate the point made by Slingerland and Benchimol (35) that the classical transformation assays, in which p53 is typically overexpressed by a heterologous promoter, may not accurately reflect the significance of MT p53 expression in primary tumors. Our observations suggest that great caution should be exercised when attempting to extrapolate from analyses of p53 mutations in cultured cells to predicting the activities of mutant p53 in cancers in situ.
Materials and Methods

Mouse Mammary Epithelial Cell Lines. Establishment of the MMECL has been described (Ref. 36; Table 1). The cell line designated TM3A is the same as the TM3 cell line, which has been described previously (36–38, 44, 45). The cell lines were maintained in Dulbecco’s modified Eagle’s medium and Ham’s nutrient mixture F-12 (Sigma Chemical Co., St. Louis, MO) supplemented with 2% adult bovine serum (HyClone Laboratories, Logan, UT), insulin (10 μg/ml; Sigma), epidermal growth factor (5 ng/ml; Sigma), and 10 μM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Calbiochem, La Jolla, CA). Separate lineages of a cell line, derived from different vials of frozen stocks maintained in a liquid nitrogen freezer, are designated as vial A, B, C, C’, or C’ (Fig. 1).

Cell Labeling and Condition Proteins Analyses. Subconfluent cells (≈107/75-cm2 flask) were either metabolically labeled or pulse-chase labeled. Metabolic labeling was for 3 h with 200 μCi Tran35S-label (>1000 Ci/mmol; ICN Biomedicals, Irvine, CA) in 2% MFEM. Pulse-chase labeling included a 2-h starvation with 2% MFEM lacking methionine followed by pulse labeling for 30 min with 200 μCi Tran35S-label in 2% MFEM. The 35S-containing media were removed, cell monolayers were washed three times in warm Tris buffer, and cells were then “chased” in standard cell culture media lacking 35S label for varying times.

Labeled cells were harvested with Nonidet P-40 lysis buffer as described (37), extracts were clarified by centrifugation (~15,000 × g), and supernatants were immunoprecipitated. M73 (46), specific for the adenosine 5’EIA protein, was used as a negative control monoclonal antibody. Monoclonal antibodies to p53 were PAb240 (47), PAB246 (48), PAB248 (48), and PAB241 (48, 49). Immunoprecipitations and separation of proteins by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis were performed as previously described (37, 50). Detection of p53 expression immunohistochemically using CM5 antisera (a gift of D. Lane) has been detailed elsewhere (37, 38).

RNA-PCR. RNA was extracted and reverse transcribed using oligo-d(T)18 primers as described (37) with modifications using the GeneAmp reverse transcription-PCR system (Perkin-Elmer Cetus, Norwalk, CT). All PCR primers were synthesized at the Institute for Molecular Genetics (Baylor College of Medicine, Houston, TX) and were used at 50 pmol/100-μl reaction. The WT p53-specific primers 33A, 25B, and 27B have been reported (37). TM3A 3-bp insertion MT-specific primer: 51A, 5’-CAC CAT CCA CTA CAA GAT CAG CAG C-3’, exon 7, nt 838–856. TM4 21-bp deletion MT-specific primer: 49A, 5’-AAG TCT GGT ATG TGC ACG CTA C-3’, exon 4–5, nt 506–547. For amplification of p53 using WT p53-specific PCR primers, the thermocycling profile follows: 5-min time delay at 95°C; 30 cycles including 95°C for 30 s; 62°C for 1 min; 72°C for 1 min; concluding with a 7-min extension at 72°C. MT-specific PCR was further modified in accordance with the Perfect Match protocol (Stratagene, La Jolla, CA). Following an initial denaturation at 95°C for 5 min, 1 unit of Perfect Match and 2.5 units of AmpliTaq polymerase (Perkin-Elmer Cetus) were added to each 100-μl PCR reaction and held at an annealing temperature of 62°C for 5 min. The samples underwent 30 cycles of PCR as follows: 72°C for 1 min; 95°C for 1 min; 62°C for 2 min.

Nucleic Acid Analyses. The conditions for cloning and sequencing of the p53 CDNA, including exons 5–9, have been detailed elsewhere (37). PCR products used for cloning were screened for contamination with the murine pseudo-gene DNA as indicated (37), and direct sequencing of PCR products was used to confirm mutations defined from sequencing the clones (38). p53 sequence data were aligned to WT murine p53 (51, 52).

PCR products were analyzed by electrophoresis of 10–15 μl of a 100-μl reaction on a 20% nondenaturing polyacrylamide gel (Bio-Rad Laboratories, Richmond, CA). Gels were stained in 5 μg ethidium bromide/ml (Boehringer Mannheim Biochemicals, Indianapolis, IN) and were photographed using Polaroid 55 film (Polaroid Corp., Cambridge, MA). The Pro130 p53 mutation was screened by digesting 10 μl (~500 ng) of DNA from a 100-μl PCR reaction for 2 h with 3 units of Fnu4HI (Boehringer Mannheim Biochemicals), followed by a second addition of enzyme under identical conditions to ensure complete digestion. Restriction enzymes were used in accordance with the manufacturer’s recommendations.

Growth Properties of MMECL. FSK3 cells at P14–vial A and P36–vial C cells were seeded at a density of 2.5 × 104 viable cells/25-cm2 tissue culture flask; FSK3 cells at P36–vial C and TM3A P13 cells were seeded at a density of 105 viable cells/35-mm tissue culture dish. Media were changed every 3 days. Duplicate flasks of cells were trypsinized and counted each day, and the log of total cell counts was plotted versus days in culture.

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References


