Immortal DNA Strand Cosegregation Requires p53/IMPDH–Dependent Asymmetric Self-renewal Associated with Adult Stem Cells

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Abstract

Because they are long-lived and cycle continuously, adult stem cells (ASCs) are predicted as the most common precursor for cancers in adult mammalian tissues. Two unique attributes have been proposed to restrict the carcinogenic potential of ASCs. These are asymmetric self-renewal that limits their number and immortal DNA strand cosegregation that limits their accumulation of mutations due to DNA replication errors. Until recently, the molecular basis and regulation of these important ASC-specific functions were unknown. We developed engineered cultured cells that exhibit asymmetric self-renewal and immortal DNA strand cosegregation. These model cells were used to show that both ASC-specific functions are regulated by the *p53* cancer gene. Previously, we proposed that IMP dehydrogenase (IMPDH) was an essential factor for p53-dependent asymmetric self-renewal. We now confirm this proposal and provide quantitative evidence that asymmetric self-renewal is acutely sensitive to even modest changes in IMPDH expression. These analyses reveal that immortal DNA strand cosegregation is also regulated by IMPDH and confirm the original implicit precept that immortal DNA strand cosegregation is specific to cells undergoing asymmetric self-renewal (i.e., ASCs). With IMPDH being the rate-determining enzyme for guanine ribonucleotide (rGNP) biosynthesis, its requirement implicates rGNPs as important regulators of ASC asymmetric self-renewal and immortal DNA strand cosegregation. An *in silico* analysis of global gene expression data from human cancer cell lines underscored the importance of p53-IMPDH-rGNP regulation for normal tissue cell kinetics, providing further support for the concept that ASCs are key targets for adult tissue carcinogenesis. (Cancer Res 2005; 65(8): 3155-61)

Introduction

Recently, there has been a revival of the concept that many human cancers may arise from mutated adult stem cells (ASCs; refs. 1, 2). The revival of this idea is based on recent reports of rare cells in cancers that express markers associated with ASC-enriched cell preparations from heterologous tissues. However, the concept was developed years earlier based on the well-known cell renewal of adult mammalian tissues (3–6). With the exception of ASCs, the cells of most adult tissues are born by division, mature, expire, and are removed from the tissue by apoptosis or physical loss. The rapid time scale for this cell turnover compared with the much slower rates of tumor development is the basis for the hypothesis that non–stem cells cannot effectively initiate cancers (7). This tissue cell kinetics concept contrasts the commonly held idea that cancers may arise from any tissue cell with equal likelihood.

Based on tissue cell kinetics concepts, carcinogenesis has been attributed by some investigators to the accumulation of carcinogenic mutations in ASCs that are long-lived in mammalian tissues (3, 7). In fact, the cancer risk of a tissue may be related to its ASC number (3). Two special properties of ASCs have been proposed to constrain their potential to initiate cancers. The first is asymmetric self-renewal (3, 5, 6, 8). Asymmetric self-renewal is characterized by ASC divisions that produce a new ASC and a non–stem cell sister. The non–stem cell sister becomes the progenitor for short-lived, differentiating, functional cells that in most cases mature to a terminal division arrest (9). Whereas asymmetric self-renewal by ASCs is responsible for maintaining normal tissue architecture and cell renewal, it also limits ASC number. Because ASCs are the main carcinogenic target, limiting their number will limit cancer risk (3).

The second property of ASCs that limits their carcinogenic potential is immortal DNA strand cosegregation, which reduces their rate of mutation accumulation (3, 10–12). For immortal DNA strand cosegregation, at each cell division, ASCs continuously nonrandomly segregate to themselves the set of chromosomes with the oldest template DNA strands (i.e., immortal DNA strands). By this molecular maneuver, ASCs avoid all mutations that arise from unrepaired or misrepaired replication errors. The effect of immortal DNA strand cosegregation on ASC mutation rate, compared with their symmetrically renewing progeny, is estimated to be a >1,000-fold reduction.[1]

Evidence for the existence of immortal DNA strand cosegregation in crypt stem cells of the adult small intestinal epithelium in the mouse has been reported (11). More recently, we reported the first direct demonstration of immortal DNA strand cosegregation in cultured mammalian cells in association with asymmetric self-renewal (12). We used genetically engineered murine cell lines that shift from symmetric self-renewal to asymmetric self-renewal due to conditionally expression of wild-type p53 protein at physiologic levels. Asymmetric self-renewal by these cells is characterized by continuous cell divisions that produce another stem-like cycling cell and a nondividing daughter cell. This p53-dependent shift to adult stem cell-like asymmetric cell kinetics was observed for several independent cell lines that were derived

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[1] J.L. Sherley, in press.

with different types of inducible promoters (zinc dependent and temperature dependent) and with parental cells of different tissue origin (embryo fibroblasts and adult mammary epithelium, respectively) and p53 genotype (null and wild type, respectively; reviewed in ref. 13).

Using asymmetrically self-renewing cell lines derived with either immortalized embryonic fibroblast or adult mammary epithelium cells, it was possible to detect immortal DNA strand cosegregation (12). The most illustrative demonstration was obtained with Zn-responsive p53-inducible embryo fibroblast lines. Under conditions for symmetric self-renewal (i.e., Zn free; p53 off), cell cultures were allowed to incorporate the thymidine base analogue bromodeoxyuridine (BrdUrd) for one cell cycle. This is sufficient time for all chromosomes to become hemi-labeled, containing one BrdUrd-substituted DNA strand. Thereafter, when the cells were shifted to conditions that induced asymmetric self-renewal (i.e., Zn supplementation; p53 on), the induced, asymmetrically self-renewing stem-like cells were shown to cosegregate a complement of hemi-labeled chromosomes for as many as six cell divisions. The result showed retention of older labeled template DNA strands by nonrandom chromosome cosegregation. This property was not detected in isogenic p53-null cell lines that underwent symmetric self-renewal at all times during the analysis. This feature was consistent with the original proposal that immortal DNA strand cosegregation would be limited to cells with asymmetric self-renewal (3).

Here, we report our use of cultured cell models to further elucidate the nature of the relationship between asymmetric self-renewal and immortal DNA strand cosegregation and their regulation by p53-dependent cellular pathways. Our earlier studies have established that "growth suppression" by the wild-type *p53* gene in cultured cells is due to the ability of p53 to switch individual cells from symmetric self renewal (i.e., all divisions producing two "stem cell-like" sisters) to asymmetric self-renewal (reviewed in ref. 13). Now, we confirm that the ability of p53 to induce this switch requires down-regulation of the rate-limiting enzyme for guanine nucleotide biosynthesis, IMP dehydrogenase (IMPDH, IMP/NAD oxidoreductase, EC 1.2.1.14). Moreover, this down-regulation is also essential for immortal DNA strand cosegregation. Thus, this study identifies p53, IMPDH, and guanine ribonucleotide (rGNPs) as key players in mechanisms that govern the emergence of cancers from ASCs.

Materials and Methods

Cell culture. Temperature-dependent p53-inducible line 1h-3 and paired control line 1g-1; Zn-responsive p53-inducible fibroblasts (lines Ind-4 and Ind-8) and p53-null control fibroblasts (Con-3); and Zn-responsive, p53-inducible, impdh-transfectant cells (tI-1, tI-3, and tI-5) and control vector-transfectant cells (tC-2 and tC-4) were maintained as previously described (5, 14–16).

Bromodeoxyuridine-Hoechst quench assay. After 24 hours of growth under control noninducing conditions or p53-inducing conditions in 6-well culture plates, BrdUrd was added to 0.5 μmol/L final concentration and culture continued for an additional 48 hours. At the end of this period, cells were washed briefly in PBS warmed to 37°C, fixed for 15 minutes in absolute methanol chilled to −20°C, and air-dried at room temperature. Dried fixed cells were stained for 40 minutes in the dark at room temperature with 0.5 μg/mL Hoechst 33258 dissolved in PBS. After washing with PBS at room temperature, the cells were examined for UV epifluorescence. Fluorescence micrographs were prepared at 40× magnification.

Micrographs were used to determine the percent of cells with brightly fluorescent nuclei. One hundred to 200 cells were counted for epithelial cell line analyses and at least 500 were counted for fibroblast lines. Cells with brightly fluorescent nuclei correspond primarily to arrested asymmetric daughter cells produced during the first 24 hours of p53 induction. A small fraction is also due to noncycling cells produced early in the BrdUrd incorporation period.

Calculation of the dependency of P_{ack} on changes in p53 or IMP dehydrogenase ($\Delta P_{ack}/\Delta y$). The change in P_{ack}, the probability of asymmetric cell kinetics (ΔP_{ack}), with respect to change in p53 or IMPDH expression was calculated using the chain rule of differentials. The following equations were used as defined in Results and Discussion:

$$\Delta P_{ack}/\Delta p53\ \text{protein} = \Delta P_{ack}/\Delta F_d \times 1/(\Delta p53/\Delta[Zn]) \times \Delta F_d/\Delta[Zn] \quad (A)$$

$$\Delta P_{ack}/\Delta IMPDH\ \text{protein} = \Delta P_{ack}/\Delta F_d \times 1/(\Delta IMPDH\ \text{protein}/\Delta[Zn]) \times \Delta F_d/\Delta[Zn] \quad (B)$$

$$\Delta P_{ack}/\Delta IMPDH\ \text{activity} = \Delta P_{ack}/\Delta F_d \times 1/(\Delta IMPDH\ \text{activity}/\Delta[Zn]) \times \Delta F_d/\Delta[Zn] \quad (C)$$

Label retention assay for detection of immortal DNA strand cosegregation. Label retention assays were done as previously described (12) with the following modifications. Evaluated cells were plated at 500 cells per 1.7-cm^2 chamber slide in 0.5 mL Zn-free culture medium. Twenty-four hours later, BrdUrd was added to achieve a concentration of 20 μmol/L. Twenty-four hours later, the culture medium was replaced with BrdUrd-free medium supplemented with 65 μmol/L $ZnCl_2$. Ninety-six hours later (approximately four population division cycles; ref. 12), cytochalasin D was added to a concentration of 2 μmol/L. Twenty-four hours later, cells on slides were fixed and examined by *in situ* immunofluorescence with FITC-conjugated anti-BrdUrd antibodies (PharMingen, San Jose, CA) and 4′,6-diamidino-2-phenylindole dye fluorescence using a Nikon E800 epifluorescence microscope.

***In silico* analyses.** The NCI60 database was downloaded from http://genome-www.stanford.edu/cgi-bin/sutech/data/download/nci60/index.html. The normalized Cy5/Cy3 ratio variable "RAT2N" was used for all analyses (17). All statistical analyses were done using the statistical analysis software StatView (SAS Institute, Inc., Cary, NC).

Results and Discussion

IMP dehydrogenase down-regulation is required for p53-dependent asymmetric self-renewal. Previously, we have reported evidence that *IMPDH* gene regulation plays a critical role in p53-dependent asymmetric self-renewal. We showed that the purine nucleoside xanthosine could suppress asymmetric self-renewal by p53-inducible cells (5, 14). Xanthosine is converted into xanthosine-5′-monophosphate, the product of the IMPDH reaction, in one step by ubiquitous nucleoside kinases (18). When added to asymmetrically self-renewing cells, xanthosine induces a transition to symmetric self-renewal, which promotes exponential proliferation (5, 19).

The cell kinetics effects of xanthosine reflect the essential role of IMPDH regulation in self-renewal symmetry. Down-regulation of IMPDH by wild-type p53 is required for p53-dependent growth suppression. In both murine epithelial cells and fibroblasts engineered for conditional p53 expression, cellular IMPDH mRNA, protein, and activity decline in response to p53 expression in the physiologic range (14). Down-regulation of IMPDH (and guanine ribonucleotides under its control) by p53 is required for p53-dependent growth suppression (5, 14, 16, 20). This requirement was established in studies with isogenic p53-inducible cell lines that contain a constitutively expressed IMPDH transgene (16). These "impdh-transfectant" lines are isogenic to p53-inducible cells derived from p53-null murine embryo fibroblasts. They express physiologic levels of wild-type p53 protein in response to zinc chloride, which activates the modified metallothionein promoter that controls expression of their p53 transgene. However, they maintain IMPDH expression at levels comparable to that of cells that do not express p53. Impdh-transfectants show little or no p53-dependent growth suppression, although they retain inducible wild-type p53 function (16).

In the present study, we confirmed that the effect of constitutive IMPDH expression on p53-dependent growth suppression was due to prevention of asymmetric self-renewal. Failure to down-regulate IMPDH activity sufficiently in isogenic impdh-transfectants might prevent "growth suppression" by one of two different mechanisms: (a) continued symmetric cell kinetics or (b) induction of a shorter cell cycle time for asymmetrically self-renewing cells. We used a bromodeoxyuridine-Hoechst dye quench (BrdUrd-HO quench) procedure (21) to distinguish between these two mechanisms.

The BrdUrd-HO quench procedure is done by culturing cells for one generation period with the thymidine analogue BrdUrd and then examining their UV-excited nuclear fluorescence after staining with Hoechst dyes. Because noncycling daughters produced by asymmetric self-renewal are nonreplicative (5, 12), they are distinguished from cycling daughters by their failure to incorporate BrdUrd. When cycling cells, which incorporated BrdUrd during S phase, are stained with Hoechst dye, their nuclear fluorescence is quenched by the BrdUrd and seems dim compared with the bright fluorescence of nuclei in cells that have not incorporated BrdUrd. When cultured for at least one cell cycle period, all nuclei of cells dividing with symmetric cell kinetics take up BrdUrd and therefore exhibit uniform dim nuclear HO fluorescence. Under either condition, nuclei from p53-null control cells are uniformly dim (Fig. 1*A* and *B*). Similarly reflecting their symmetric cell kinetics, p53-inducible cells grown under noninducing conditions exhibit uniformly dimly fluorescent nuclei (Fig. 1*C*).

In contrast, cells undergoing asymmetric self-renewal (conditions of p53 expression; Fig. 1*D*) exhibit both dim nuclei and bright nuclei. The dim nuclei correspond to cycling asymmetric stem-like sister cells, whereas the brightly fluorescent nuclei correspond to asymmetric noncycling sisters. For several different independently derived p53-inducible cell lines, the mean % bright fraction after 48 hours of incubation in BrdUrd under conditions of p53 expression was 37 $\pm$ 12% (n = 5; P = 0.002; Table 1). This experimental value is in good agreement with the expected value for ideal asymmetric cell kinetics of 40% after 48 hours of BrdUrd labeling (5, 15). Quantification of the % bright fraction by both CCD digital imaging and flow cytometry was used to confirm results of fluorescent microphotography (data not shown). Compared with control p53-inducible vector-transfectants, impdh-transfectants showed a marked reduction in the % bright cell fraction (compare Fig. 1*D* and *F*; Table 1; tC-X lines versus tI-X lines). This finding shows that unless IMPDH is reduced below its basal level, p53 cannot initiate an asymmetric self-renewal program.

Asymmetric self-renewal is sensitive to modest changes in p53 and IMP dehydrogenase expression. We did analyses to quantify the degree to which changes in p53 and IMPDH expression effect asymmetric cell kinetics, as an indicator of asymmetric self-renewal. Previously, we developed a method to quantify asymmetric cell kinetics in terms of a mathematical variable F_d, the fraction of new daughter cells that divide (15, 22). For asymmetric cell lineages, F_d approximates 0.5; whereas for exponential cell lineages, F_d approaches 1.0. On the time scale of our experiments, individual cell lineages adopt discretely one or the other of these two cell kinetics programs (5, 15). Therefore, the average F_d of a cell population gives an estimate for P_{ack}, the probability that any cell in the population will initiate deterministic asymmetric cell kinetics.

We have described methods for determination of the average F_d of cultured cell populations (22). Theoretically, in the range of F_d = 0.5 to 1.0, $\Delta P_{ack}/\Delta F_d = -2$ (Δ is "change in"). For exponentially dividing p53-null fibroblasts and asymmetrically dividing p53-expressing fibroblast, we independently determined

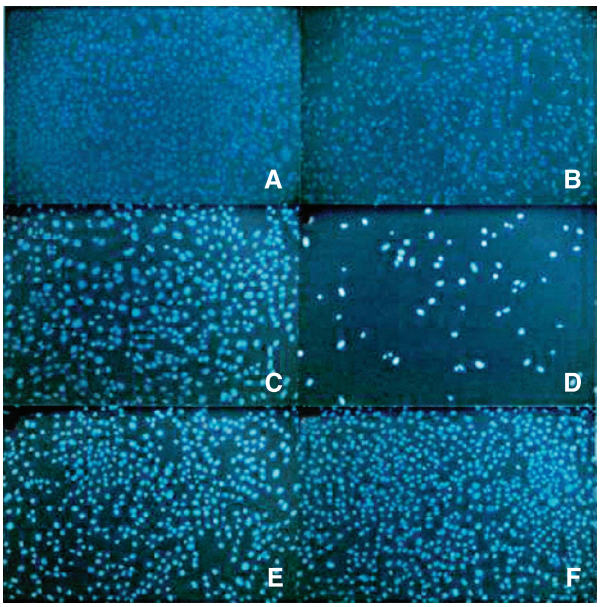

Figure 1. Constitutive IMPDH expression prevents p53-dependent asymmetric cell kinetics. Brightly fluorescent Hoechst dye–stained nuclei were detected as an indicator of asymmetric cell kinetics in cells expressing wild-type p53 protein. Zn-responsive fibroblast lines were grown for 72 hours under either control conditions for symmetric kinetics (0 μmol/L $ZnCl_2$; *A*, *C*, and *E*) or p53-expressing conditions for asymmetric kinetics (75 μmol/L $ZnCl_2$; *B*, *D*, and *F*). During the last 48 hours (equivalent to approximately two cell cycle periods), BrdUrd (0.5 μmol/L) was added. At the end of the labeling period, cells were fixed, stained with Hoechst 33258 and examined for UV-excited nuclear fluorescence. Indicative of their exponential kinetics, p53-null cells (Con-3; refs. 14, 16) exhibit uniform dimly fluorescent nuclei under nonexpressing (*A*) or p53 expressing conditions (*B*). In contrast, p53-inducible vector-transfectant cells (*C* and *D*; tC-4; ref. 16) exhibit conditional asymmetric cell kinetics. *D,* reduced number of cells and the presence of both bright and dim nuclei are indicative of asymmetric cell kinetics. p53-inducible impdh transfectant cells (*E* and *F*; tI-3; ref. 16) exhibit a nuclear fluorescence pattern like that of p53 null cells (compare *A* and *B*) indicating complete abrogation of asymmetric cell kinetics. Magnification, 300×.

Table 1. Percent Hoechst-bright nuclei fraction as an indicator of asymmetric cell kinetics in cell lines with different levels of p53 and IMPDH expression

Cell line	Percent Hoechst-bright nuclei*	
	Noninduced	p53 Induced
Epithelial lines†		
Noninducible control, *1g-1*	0.0	5.0
p53 inducible, *1h-3*	0.0	24.0
Fibroblast lines‡		
p53-null control, *Con-3*	0.0	0.9
p53-inducible		
Ind-4	0.3	43.0
Ind-8	0.3	46.0
Vector-transfectant derivatives of Ind-8		
tC-2	0.2	47.0
tC-4	0.0	24.0
Impdh-transfectant derivatives of Ind-8		
tI-1	0.2	3.0
tI-3	0.2	0.2
tI-5	0.2	6.3

*After 24 hours of growth under control noninducing conditions or p53-inducing conditions, BrdUrd was added and culture continued for an additional 48 hours. At the end of this period, cells were fixed, stained with Hoechst dye, and UV epifluorescence micrographs prepared at 40× magnification. % cells with bright fluorescent nuclei was determined from fluorescent micrographs. One hundred to 200 cells were counted for epithelial cell line analyses and at least 500 were counted for fibroblast lines. Cells with brightly fluorescent nuclei correspond primarily to nondividing asymmetric daughter cells produced during the first 24 hours of p53 induction, as well as an indeterminate fraction produced early in the BrdUrd incorporation period.

† p53-inducible mammary epithelial cell lines induced by culture at 32.5°C (20).

‡ p53-inducible murine embryo fibroblast cell lines induced by culture in medium containing 75 μmol/L $ZnCl_2$ (14, 16).

F_d (15) and estimated values for P_{ack} from published time lapse data (15). These data (F_d = 0.79 and 0.49, respectively; and P_{ack} = 0.09 and 0.72, respectively) yield −2.1 as the experimentally determined $\Delta P_{ack}/\Delta F_d$, in excellent agreement with the theoretical value of −2. Based on this determination, $\Delta P_{ack}/\Delta F_d$ = −2.1 was used to estimate ΔP_{ack} from experimentally determined ΔF_d values.

With published data from the Zn-responsive model cells (14, 15), simple linear regression analyses were used to estimate independent rates of change in F_d, p53 protein, IMPDH protein, and IMPDH activity with respect to the common variable of Zn concentration (Table 2). The chain rule of differentials was then applied to yield estimates of the dependency of P_{ack} on changes in p53 and IMPDH (see Materials and Methods for calculations). The results of this analysis show that the probability of asymmetric self-renewal, as measured by induction of asymmetric cell kinetics, increases dramatically in response to small changes in p53 and IMPDH. The averaged absolute value of 12 for $\Delta P_{ack}/(\Delta$p53 or ΔIMPDH) is consistent with our observations that a 30% to 50% reduction in IMPDH in response to modest levels of wild-type p53 precipitates a nearly quantitative shift from symmetric to asymmetric self-renewal (5, 15, 22).

Immortal DNA strand cosegregation requires down-regulation of IMP dehydrogenase and asymmetric self-renewal. We next investigated whether immortal DNA strand cosegregation occurred in p53-expressing impdh-tranfectants. For this analysis, we modified the previously described BrdUrd retention assay (12) to increase its sensitivity for detecting immortal DNA strand cosegregation if it occurred in symmetrically cycling cells (see Materials and Methods). Under conditions that do not induce p53 expression, impdh-transfectants were cultured for one-generation period in BrdUrd to allow DNA to become hemi-substituted with BrdUrd. Thereafter, they were induced to express p53 while cycling in BrdUrd-free medium for four population division cycles. This shorter period of BrdUrd-free cycling permitted sufficient BrdUrd to remain for detection in cells that diluted it by random chromosome segregation. Treatment of cells with cytochalsin D then allowed an *in situ* evaluation of whether BrdUrd-labeled DNA strands were retained at a high level by cycling cells; and if so, how they were segregated between sister cells (12).

As shown in Fig. 2*B* (tI-3 cells), impdh-transfectant cells showed a pattern of BrdUrd retention and segregation indicative of random chromosome segregation. Like p53-null cells (Fig. 2*A*, Con-3 cells), they had a low level of anti-BrdUrd immunofluorescence that was uniformly distributed between sister nuclei trapped in cytochalasin D–induced binucleated cells. In contrast, parental p53-expressing cells (Fig. 2*C*, Ind-8 cells) and vector-transfectant control cells (tC-2 cells; data not shown) showed a high level of anti-BrdUrd fluorescence that was asymmetrically localized to one of the sister nuclei, indicative of immortal DNA strand cosegregation (12).

This result has several significant implications. It indicates that like asymmetric self-renewal, immortal DNA strand cosegregation requires down-regulation of IMPDH. This requirement implicates rGNPs as possible components of the mechanism(s) for nonrandom chromosome segregation. In the absence of IMPDH/rGNP regulation and asymmetric self-renewal, even though p53 is independently expressed, immortal DNA strand cosegregation does not occur. Thus, this result shows that p53 expression alone is not sufficient for immortal stand cosegregation. Moreover, it further emphasizes the idea that asymmetric self-renewal and immortal DNA strand cosegregation are tightly coupled mechanisms.

***In silico* analysis of relationships among p53, IMP dehydrogenase, and cell kinetics in human cancer cells.** IMPDH is a ubiquitously expressed essential cellular enzyme (23, 24). In human cells, two different single-copy genes express two highly homologous isoforms (types I and II; 84% amino acid identity; ref. 25). Whereas the type I gene shows little regulation with cell growth state, changes in the expression of the type II gene have been associated with cell proliferation, cell differentiation, and malignant transformation of rodent and human cells (26–28). The type II IMPDH is also highly conserved during evolution (25). Immortal cells in culture express high levels of IMPDH activity whether actively dividing or arrested by growth factor removal. Based on this observation, we have suggested that increased IMPDH gene expression indicates that cells have acquired the capacity for symmetric self-renewal, as opposed to activation of cell division per se (23).

We did an *in silico* (29, 30) microarray database analysis to investigate the significance of p53-IMPDH interactions in the growth regulation of human cancer cells. Linear regression analyses were used to look for statistically significant associations between type II IMPDH mRNA expression, p53 mRNA expression, cell population doubling time, and p53 genotype in microarray data extracted from the Stanford NCI60 database (17). This database contains normalized mRNA expression data for ~8,000 gene sequences for 60 cell lines derived from tumors of different human tissues.

No significant association was detected between wild-type p53 mRNA expression and type II IMPDH mRNA expression (n = 20, R^2 = 0.016, P = 0.597). Because of the high degree of post-translational regulation that p53 is known to undergo (31), a relationship between its mRNA and regulated target genes may be difficult to detect. Consistent with this explanation, only a weak association was detected between wild-type p53 mRNA and p21waf1 mRNA (n = 20, R^2 = 0.117, P = 0.031), a highly p53-induced mRNA (32). Moreover, the regression coefficient from this analysis is negative (−1.15), a result that is contrary to the known positive correlation between wild-type p53 protein expression and p21waf1 mRNA level.

Although no association was detected between the expression level of p53 mRNA and type II IMPDH mRNA, p53 genotype had a major effect on the association between type II IMPDH mRNA expression and cell proliferation rate. The developers of the NCI60 microarray database have reported an association between type II IMPDH mRNA and population doubling time (17). The association was detected in a cluster image map including data for all 60 cell lines; and the analysis was not stratified by p53 genotype. By simple regression analysis, we found that, in cells of wild-type p53 genotype, type II IMPDH mRNA showed a significant association with population doubling time (n = 18, R^2 = 0.427, P = 0.003; Fig. 3*A*). Cell lines with higher proliferative rate (i.e., shorter doubling time) exhibit higher levels of type II IMPDH mRNA expression. Our studies indicate that in murine cells with wild-type p53 protein expression, IMPDH mRNA, protein, and activity vary coordinately (14); and the single murine form of IMPDH is most homologous to the human type II protein (33). Therefore, the observed association is consistent with the hypothesis that type II IMPDH functions as a rate determining factor for cell proliferation in human cancer cells that express wild-type p53.

In contrast to cells that express wild-type p53 protein, cell lines expressing mutant p53 proteins showed no significant association between type II IMPDH mRNA and population doubling time (n = 37, R^2 = 0.001, P = 0.868; Fig. 3*B*). Thus, wild-type p53 seems to be an important determinant of this relationship in human tumor cells (compare Fig. 3*A* and *B*). The loss of the association may reflect deregulation or malregulation of the type II *IMPDH* gene

Table 2. Estimate of the magnitude of the change in the probability of asymmetric cell kinetics (ΔP_{ack}) associated with changes in p53 and IMPDH expression

A. Results of linear regression slope analyses to estimate of rates of change of specific cell kinetics determinants with respect to Zn concentration*

Rate	Regression slope ($\Delta x/\Delta$[Zn])	n	R^2	P
$\Delta F_d/\Delta$[Zn]	−0.017 per μmol/L	5	0.967	0.007
Δp53/Δ[Zn]	0.006 per μmol/L	8	0.667	0.013
ΔIMPDH protein/Δ[Zn]	−0.003 per μmol/L	6	0.765	0.023
ΔIMPDH activity/Δ[Zn]	−0.002 per μmol/L	5	0.818	0.035

B. Results of calculations of $\Delta P_{ack}/\Delta y$†,‡

$\Delta P_{ack}/\Delta$p53 protein = 6
$\Delta P_{ack}/\Delta$IMPDH protein = −12
$\Delta P_{ack}/\Delta$IMPDH activity = −18
Mean $|\Delta P_{ack}/\Delta y|$ = 12 (P = 0.037)§

*Data from published studies with Zn-responsive p53-inducible cells (14, 15) were used in linear regression analyses to develop estimates for the rate of change of specific cell kinetics determinants with respect to increasing Zn concentration.
†These rates were used, along with the experimentally determined value of $\Delta P_{ack}/\Delta$[Zn] = −2.1 (see Results and Discussion), to calculate ΔP_{ack} as a function of changes in *p53* or *IMPDH* gene expression as described by Eqs. A-C in Materials and Methods.
‡Δy, change in p53 protein or change in IMPDH protein or activity.
§One-tail Student's t test.

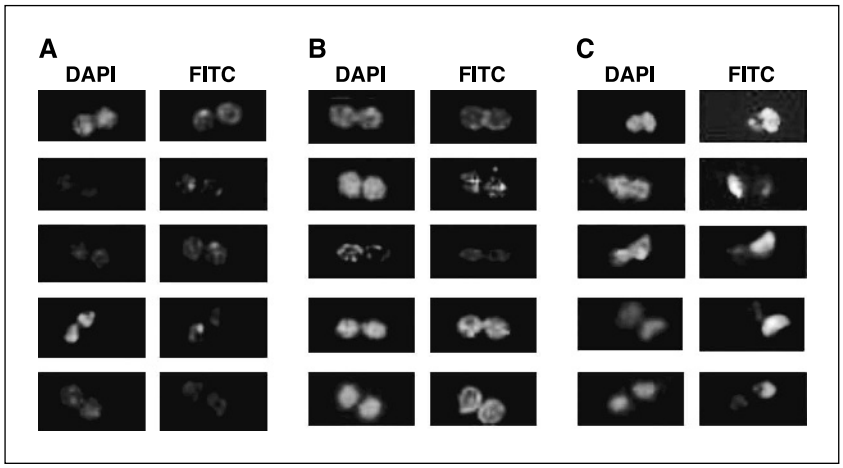

Figure 2. IMPDH down-regulation is required for immortal DNA strand cosegregation. Fluorescent micrographs of cytochalasin D–induced binucleated cells after completion of the BrdUrd label retention procedure described in Materials and Methods. Images are paired for 4′,6-diamidino-2-phenylindole (*DAPI*) fluorescence (to localize total nuclear DNA) and FITC fluorescence indicative of specific binding or anti-BrdUrd antibodies. *A,* p53-null Con-3 cells: symmetric self-renewal. *B,* p53-induced, tI-3 impdh-transfectants: symmetric self-renewal. *C,* p53-induced Ind-8 cells, the nontransfected parents of tI-3 cells: asymmetric self-renewal.

due to loss of the input from normal p53 protein. Of course, all of the cells in this study have undergone neoplastic transformation. The available data do not allow us to determine whether each tumor line expresses a higher level of IMPDH than its tissue of origin. However, elevated expression of type II IMPDH in malignant cells is a well-described property of the enzyme (28).

Given the common property of human cancers with a wild-type 53 genotype to display another form of p53 pathway inactivation (e.g., human papillomavirus E6 transduction, mdm2 up-regulation; ref. 31), it is a curiosity that tumor cell lines with wild-type p53 preserve an association between IMPDH expression and population doubling time that is lost in lines with mutant p53 proteins. This feature may indicate that p53 cell kinetics regulation via IMPDH is distinct from p53's other functions in checkpoint arrest and apoptosis. Another possible explanation is that mutant p53 proteins may function actively in tumor cells to disrupt IMPDH-dependent cell kinetics control.

It is noteworthy that the distributions of type II IMPDH mRNA levels in cells with wild-type p53 versus mutant p53 are quite similar (mean normalized expression level ± SD = 1.1 ± 0.39 versus 1.1 ± 0.47, respectively). Because IMPDH is an essential enzyme, the finding of similar levels of expression in populations of either genotype may indicate a narrow range of elevated IMPDH expression that promotes tumor formation. In cells that retain wild-type p53 expression, such a requirement for IMPDH up-regulation for tumor growth must be accomplished by other mechanisms. The elucidation of such mechanisms is likely to reveal components of pathways that control self-renewal symmetry in human ASCs.

Presently, our knowledge of the detailed molecular functions of p53 and IMPDH in asymmetric self-renewal control is limited. For example, although IMPDH down-regulation is required for asymmetric self-renewal, whether it is sufficient has not been established. It may be that changes in the expression of other p53-regulated genes (e.g., the cyclin-dependent kinase inhibitor p21waf1) are also required for p53-dependent asymmetric self-renewal and associated immortal DNA strand cosegregation. Similarly, regulation of IMPDH may not occur via direct gene repression by p53 but indirectly via other p53-responsive factors.

The self-renewal terrain of cultured cancer cells also needs additional exploration. Asymmetric self-renewal in culture is easily overlooked (15, 34) especially if it occurs mixed with symmetric self-renewal. Although the accumulation of sufficient cells to form tumors in humans requires symmetric self-renewal (3, 6, 8, 35), some tumor-derived cell lines may still exhibit residual asymmetric self-renewal in culture, resulting in longer population doubling times (15, 22). Of course, cell populations with protracted doubling times (>30 hours) may simply suffer from nonoptimal culture conditions. The BrdUrd-HO quench procedure is one of several methods that we have developed to detect asymmetric cell kinetics by cultured cells (5, 15, 22). The application of these methods to the evaluation of cancer cell lines of wild-type versus mutant p53 genotype may elucidate the significance of the association between IMPDH and population doubling time to cancer cell self-renewal programs.

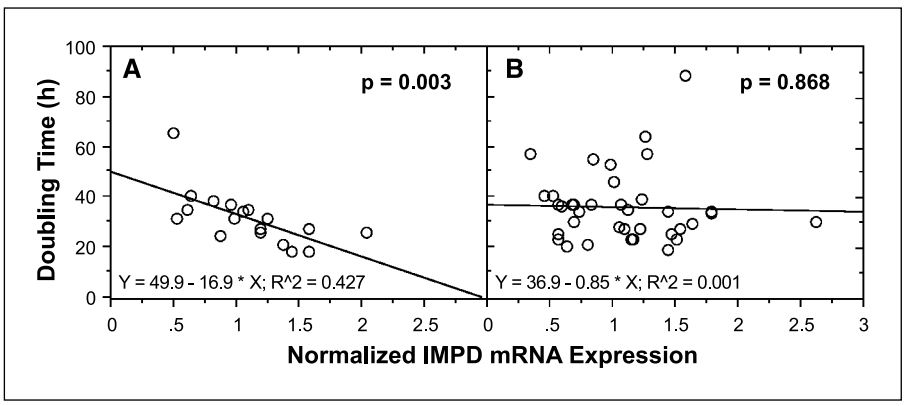

Figure 3. *In silico* analysis of the effect of p53 genotype on the association between type II IMPDH mRNA expression and proliferation by human cancer cells. Linear regressions were performed for type II IMPDH mRNA expression versus population doubling time (38). Analyses were performed with gene expression microarray data for the NCI60 cancer cell line panel stratified for either wild-type p53 protein expression (*A*) or homozygous mutant p53 protein expression (*B*).

Adult stem cell–based cancer mechanisms. Although the presented studies were done in engineered cultured cells, there is substantial evidence that the findings are relevant to processes in ASCs *in vivo*. Since our first demonstration of asymmetric self-renewal by murine mammary epithelium cells, we and others, respectively, have documented this program in rat (19) and human cell populations enriched for ASCs (36, 37). We have shown that manipulation of IMPDH-dependent pathways can be used to expand asymmetrically self-renewing ASCs from normal tissues (19). Our findings lead us to propose that a major of effect of *p53* gene mutations is a combined loss of ASC self-renewal control and a critical mutation avoidance mechanism. IMPDH and the rGNP pools that it controls are also important regulators of these critical ASC functions. Continued elucidation of these molecular, genetic, and cell kinetics relationships may hold the key to a new understanding of normal tissue function and the origin of human cancers.

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