MUTAGENESIS OF HUMAN p53 TUMOR SUPPRESSOR GENE SEQUENCES IN EMBRYONIC FIBROBLASTS OF GENETICALLY-ENGINEERED MICE

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INTRODUCTION

Mutagenesis assays in mammalian cells and prokaryotic organisms were indispensable tools contributing to the elucidation of basic principles and molecular events underlying sequence changes in DNA. Two facts emanating from decades of research on molecular mechanisms of mutagenesis, e.g., with the *E. coli lacI* system, or by characterizing HPRT (hypoxanthine phosphoribosyltransferase) mutations in rodent cells and human fibroblasts, is that the chemical properties of the DNA bases and the base sequence context, in addition to biological selection, are crucial determinants of both spontaneous and carcinogen-induced mutation spectra (1-3). A mutation assay that would allow induction and selection of tumorigenic point mutations in human p53 tumor suppressor gene sequences, a major target of mutations in development of human cancer (4, 5), would be valuable because with such an experimental system it would be possible to test various hypotheses on the origins of disease-causing sequence changes, and to compare directly the human tumor p53 mutation spectra in the IARC

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database (6, 7) with experimentally-generated mutation patterns . The keystone in design of a mutation test is the strategy that permits recovery of the cell, the organism, or the plasmid that harbors a mutation in a chosen target sequence. Selection typically is accomplished by manipulating growth conditions or other parameters such that all entities that are not mutated are lost, that is, they do not survive or are not retrieved. In the widely used HPRT locus mutation protocol, the rare cell with a dysfunctional mutation in the HPRT gene is recovered from the population of wild-type cells by cultivating the cells in a selection medium containing drugs that are toxic only to those cells (the vast majority) still harboring a functional HPRT locus. Development of a comparable mammalian mutation assay with human p53 sequences as the mutagen target offers special challenges, such as the task of devising a successful strategy to separate cells in culture that have p53 mutations away from cells that do not, and foster their proliferation.

To achieve this goal, we took advantage of the propensity of mouse, but not human, fibroblasts to undergo immortalization when the p53 locus is functionally inactivated (discussed below), and employed gene-targeting technology to introduce the exact human p53 sequence into mouse fibroblasts. In this way, it is possible to have a human sequence as mutagen target in mouse cells. Thus, we have combined an advantage of working with mouse cells (ability to immortalize by p53 point mutation) with the advantage of having a human p53 target sequence in the mutation assay (i.e., the exact sequence in human cells most frequently mutated in human cancers). Mouse and human p53 are highly similar, especially at the level of amino acid sequence, but due to the third wobble base of the genetic code, divergence of the DNA sequences between the 2 species is about 15%.

First, we created a human p53 knock-in mouse strain by constructing a gene-targeting vector that has the human p53 DNA-binding domain-encoding sequence. The plasmid also harbors a *loxP*-flanked neomycin phosphotransferase gene for selection of homologous recombinant mouse embryonic stem cells that can be removed subsequently by cre recombinase *in vivo* or *in vitro*. (Figure 1, and ref. 8). In the **hu**man **p**53 **k**nock-**i**n (Hupki) mice derived from the ES cells in which proper targeting by the vector occurs, exons 4-9 of endogenous mouse p53 allele are replaced with the corresponding human p53 gene sequences. Exons 4-9 encompass the segment in which most human tumor mutations arise. We included the adjacent polyproline domain (PPD) in the exchanged DNA segment also, so that the Hupki model could serve additionally to investigate the role of PPD polymorphic variants in the human population on p53 function *in vivo* (discussed in ref. 9).

The homozygous Hupki strain is phenotypically "wild-type": the mice develop normally, and do not show the biochemical and biological abnormalities that have been reported in p53 deficient and p53 knockout mice. The chimaeric transcript of the Hupki p53 gene is correctly spliced, present at normal levels in various (murine) tissues, and encodes protein that binds to p53 consensus sequences. The Hupki p53 protein also has the biological properties of functional, normal p53, such as the ability to accumulate following stress, and to activate transcriptionally known p53 target genes controlling cell-cycle checkpoints and apoptosis (8, 10, 11). The kinetics and dose response of gamma

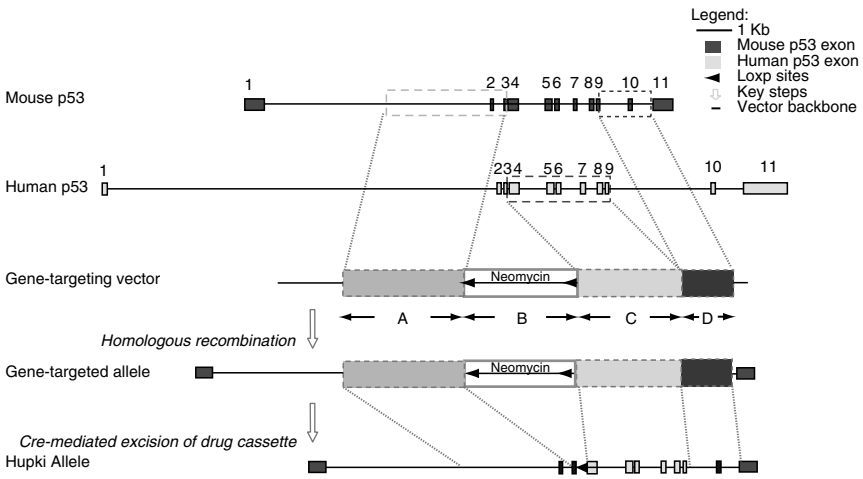

Figure 1. Gene-targeting strategy generating Hupki strains.

irradiation-triggered apoptosis in thymocytes, which is a strictly p53-dependent process (12, 13), are similar in Hupki mice and mice with a normal mouse p53 gene (8). Since typical p53 wild-type responses to DNA damage and other apoptotic stimuli are intact in Hupki prototype mice, the strain and its mutant derivatives can be applied to the study of human p53 PPD and DBD structural/functional properties *in vivo*. Second, the Hupki mouse model provides a unique experimental tool for elucidation of human tumor p53 mutation spectra, both *in vivo* and in Hupki fibroblasts *in vitro.* This latter application and its ramifications are the subject of this chapter.

IMMORTALIZATION MECHANISMS OF MURINE EMBRYONIC FIBROBLASTS(MEFs): CRUCIAL ROLE OF p53 POINT MUTATIONS

Under standard cell-culture conditions, primary murine embryonic fibroblasts from wild-type mice stop proliferating after 10 or more population doubling. At this stage, cells become senescent, acquiring an irregular, and often enlarged, flattened morphology. Functional inactivation of the ARF/p53 tumor suppressor pathway allows cells to bypass proliferation block (14-16). Cell division is resumed, and the recovering cell population regains a homogeneous morphology. Spontaneous bypass of senescence and death is a relatively rare event ($<1/10^6$) in murine embryonic fibroblast (MEF) cultures; nevertheless, spontaneous immortalization of primary mouse cells is orders of magnitude more common than immortalization of human cells (15, 17). Inactivation of the p19ARF-p53 pathway, but not p16INK4a, appears to be sufficient for MEF immortalization, which typically occurs by mutation of p53 or by loss of INK4a/ARF gene sequences (14, 18-20). The INK4a/ARF locus encodes two distinct

tumor suppressors, p16INK4a and p19ARF. These two genes have a different transcription start but share exons 2 and 3, processed in different reading frames, thus encoding proteins with unrelated amino acid sequences (21). p16INK4a is a core component of the cell-cycle control machinery and responds to both positive and negative growth regulatory signals. It regulates the activities of CyclinD-Cdk4/6 complex, and consequently affects pRB tumor suppressor function and E2F responsive genes (22). ARF, the alternative reading frame product, is a key mediator of p53-dependent growth suppression. It exists at a low or undetectable level in most normal cell and tissues types (23). ARF can be directly or indirectly induced in response to abnormal proliferation signals, such as continued *in vitro* culturing (19), and inappropriate expression of proliferative oncogenes, including Ras (24, 25), c-myc (26) and E2F1 (27). Although ARF is thought to function mainly as an activator of p53 by neutralizing the activities of Mdm2, ARF has other p53-independent functions impinging on growth control and apoptotic decisions of cells (28, 29).

HUF (HUPKI EMBRYONIC FIBROBLAST) IMMORTALIZATION AS A METHOD TO SELECT FOR DYSFUNCTIONAL POINT MUTATIONS IN HUMAN P53 GENE SEQUENCES

HUFs (Hupki embryonic fibroblasts), like MEFs, are mouse cells, and immortalize readily as anticipated. Under normal culture conditions, the serially passaged embryonic fibroblast cultures become senescent by passage 5, but then almost all cultures (<90%) recover, generating immortalized cell lines when the protocol described here is followed (Figure 2 Flow Diagram and Short Protocol below, page opposite). At passage 3, and again after the senescent phase (usually >passage 8), the morphology becomes uniform and cell doubling time is short (1-2 d) (Figure 3, Panels A and C), at passage 5-6 , however, as senescent features develop in the population, division stops for up to several weeks, cells are sparse and become flattened or irregular in shape (Figure 3 Panel B). When the primary cultures are not exposed to mutagen at early passage, but instead are allowed to immortalize spontaneously according to the protocol below, approximately 10% of the cell lines recovered will harbor a p53 mutation (11, 30, 31 and unpublished observations). When we expose primary cells at passage 1-2 to a carcinogenic mutagen (UV light, aristolochic acid, benzo(a) pyrene), up to 40% of the recovered immortalized cell lines can harbor one or even 2 p53 mutations. The mutagen-induced *p53* mutations in HUFs we have characterized thus far display various features of human tumor *p53* mutations recorded in the IARC database (6, 7). For example, cell lines recovered from cultures initially exposed to the tobacco carcinogen benzo(a)pyrene frequently harbor a p53 gene G to T transversion on the non-transcribed strand (11), as do lung tumors of smokers (6). To optimize screening of immortalized HUF cell lines for the presence of mutant p53, high throughput procedures can be performed, such as the p53GeneChip protocol developed by Affymetrix (30, 32).

The protocol we currently use for performing mutagenesis experiments with HUFs is as follows (see also Figure 2, and accompanying footnotes).

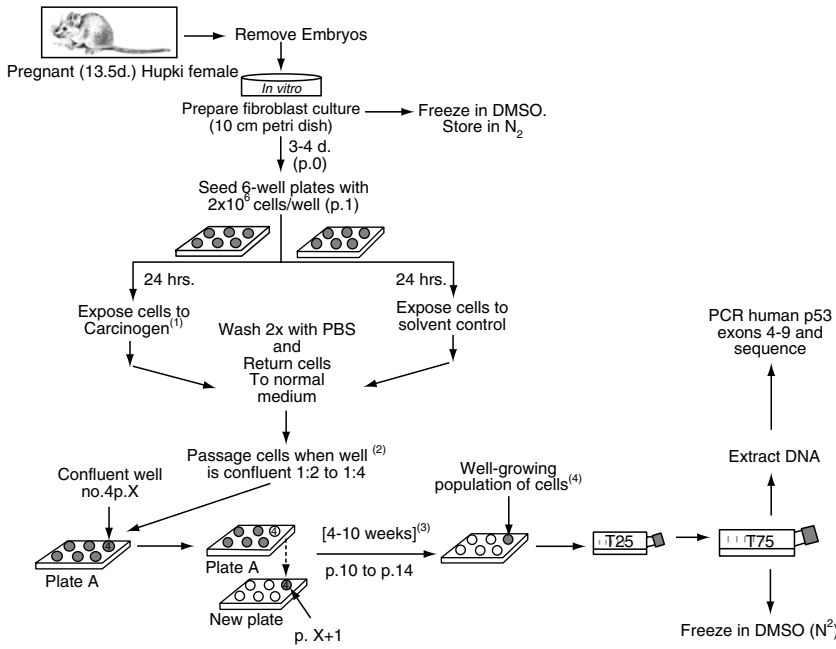

Figure 2. P53 mutation assay in HUFs: Flow Diagram. Notes: (1) Various treatment times are possible, e.g., from hours to days and can be repeated during early passages. (2) When the well is fully confluent, even at the periphery of the well, we split 1:4. When the confluent area is concentrated in the center of the dish, we split 1:2. (Early passage cells tend to migrate towards the center of the well over time.) The passaging conditions are critical because prior to immortalization, sparse cultures may not survive. (3) At passage 5-6, cells become senescent and the cultures can remain subconfluent for several weeks.

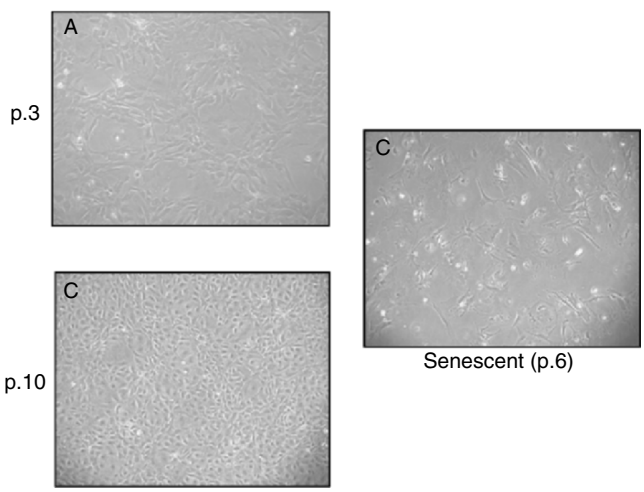

Figure 3. Photomicrographs of HUF cells (Courtesy of J. vom Brocke). A. Primary cells at passage 3. B. A senescent culture at passage 6. C. An immortalized cell culture, passage 10.

P53 MUTAGENESIS ASSAY: SHORT PROTOCOL

Note: For guidance on isolation of primary embryonic fibroblasts refer to the procedure for preparation of MEF feeder layers, Chapter 13 of Torres & Kuhn 1997 (33):

1. Sacrifice pregnant females at day 13.5 of pregnancy. Using sterile technique, dissect out the embryos, place in sterile PBS, and remove head, spleen and liver of each embryo.
2. Mince the embryo with fine scissors.
3. Add 1-2 ml of sterile medium, and pass tissue through a 20G needle 10X, then through a 25G needle 5X.
4. Transfer cell suspensions to a 10-cm dish containing 10 ml medium (cells from 1-2 embryos per dish). Medium: DMEM with 10% FCS, supplemented with penicillin and streptomycin, L-glutamine, and sodium pyruvate. See Torres & Kuhn 1997 (33).
5. Incubate the cells in a tissue culture incubator (37 °C, 5% CO_2), changing the medium daily to discard floating cells and debris. When cells are confluent, trypsinize and transfer cells to two 15-cm dishes containing 25 ml fresh medium.
6. When confluent: a) freeze cells in DMSO and store in liquid nitrogen for future experiments, and/or b) proceed with setup of mutagenesis experiment.
7. Seed 6-well plates with (1 to) 2×10^5 cells per well*. Label each well and plate. Prepare plates destined for treatment, and for solvent control. (*For >24 hr treatment protocols, we seed a lower number of cells).
8. On the following day, remove medium and replace with carcinogen- or solvent-containing medium. Incubate the cells for the treatment time chosen.
9. Remove medium, wash cells 2 X with sterile PBS, then pipet fresh medium into all the wells.
10. On the following day, or when a well becomes confluent, trypsinize and passage cells at 1:2 or 1:4 depending on the degree of confluency. Evaluate and handle each well separately.
11. By passage 5 the cells usually have stopped growing, the monolayer becomes sparse, and the cells acquire irregular and often enlarged morphologies. In this senescent stage, which can last for several weeks, the cultures are not passaged. Medium is changed 1X per week.
12. As growth resumes, and a well acquires large areas of confluency or is confluent, resume serial passaging of the cells. Each well is an independent separate culture.
13. When a culture in a well again becomes confluent within several days following a passaging at 1:4, all the cells in the well can be transferred to a T25 culture flask.
14. When the T25 flask is confluent, transfer all the cells to a T75 flask. When this flask is confluent, freeze half the cells in DMSO medium for safe-keeping and store in liquid nitrogen. Continue passaging the remaining cells until passage number reaches at least 15. These cultures then are considered immortalized and we refer to them as immortalized HUF cell lines.
15. Extract DNA from an aliquot of cells from each immortalized cell line. Amplify p53 exons individually (4-9) using the primers in Table 1A. Purify

Table 1A. PCR primers for p53 sequencing from genomic DNA.

Amplicon	Primer	Sequence	Product size
Exon 4	GCEx4F	GTCCTCTGACTGCTCTTTTCACCCATCTAC	368 bp
	GCEx4R	GGGATACGGCCAGGCATTGAAGTCTC	
Exon 5	GCEx5F	CTTGTGCCCTGACTTTCAACTCTGTCTC	272 bp
	GCEx5R	TGGGCAACCAGCCCTGTCGTCTCTCCA	
Exon 6	GCEx6F	CCAGGCCTCTGATTCCTCACTGATTGCTC	204 bp
	GCEx6R	GCCACTGACAACCACCCTTAACCCCTC	
Exon 7	GCEx7F	GCCTCATCTTGGGCCTGTGTTATCTCC	175 bp
	GCEx7R	GGCCAGTGTGCAGGGTGGCAAGTGGCTC	
Exon 8	GCEx8F	GTAGGACCTGATTTCCTTACTGCCTCTTGC	241 bp
	GCEx8R	ATAACTGCACCCTTGGTCTCCTCCACCGC	
Exon9	GCEx9F	CACTTTTATCACCTTTCCTTGCCTCTTTCC	146 bp
	GCEx9R	AACTTTCCACTTGATAAGAGGTCCCAAGAC	

the PCR products with Microcon filters and perform dideoxy sequencing of each fragment. (Alternatively, multiplex PCR can be performed, and PCR products fragmented, labeled, and analyzed with Affymetrix p53GeneChip microarrays as described by Liu et al., 2004, ref. 30).

INDICATORS OF LOSS OF p53 FUNCTION IN HUF CELL LINES

p53 generally is kept at low levels in normal cells and tissues. Under certain kinds of stress, such as DNA damage, p53 is stabilized and accumulates in the nucleus. The level and kinetics of p53 accumulation are carefully controlled by a series of positive and negative feedback loops (34-36). Basal p53 protein level can also change if the *p53* gene becomes mutated. p53 structure and function are highly sensitive to a myriad of single amino acid changes in protein sequence that destroy p53 function and disturb the control of p53 stability (37, 38). In the case of a missense mutation, the dysfunctional p53 protein typically accumulates to abnormally high levels. However, in the case of nonsense mutations and most of the splicing mutations, p53 protein is usually absent due to nonsense-mediated RNA decay or the instability of truncated p53 proteins.

Under normal (unstressed) conditions, primary HUFs (pHUFs) and HUF cell lines with wild-type unmutated (WT) *p53* display weak nuclear staining when incubated with antiserum CM1 against human p53, just as their wild-type (WT) MEF counterparts do when treated with mouse anti p53 antiserum. However, when cells are treated with UVC (30 J/m^2, 12 hrs after irradiation), or with adriamycin (1 microM for 12 hrs), and subsequently stained, an intense signal is observed in the nuclei. This is in keeping with the known wild-type p53 response to DNA damage. Immunocytochemical staining of treated or untreated pHUFs with normal rabbit IgG is used as a negative control for specificity of staining. Cellular p53 protein levels can also be detected conveniently by immunoblot analysis.

Immortalized HUF cell lines harboring missense mutant p53, but not HUF cell lines with unmutated (i.e., WT) p53, stain intensely without prior exposure to a DNA damaging agent. HUF cell lines that do not produce p53 protein due to a nonsense or frameshift mutation do not show this staining; instead, they

stain similarly to cells with wild-type p53, as expected. However, they can be distinguished in their staining pattern from nonmutant (WT) cell lines by treating the cells with damaging agent first: WT cells will show strong staining because p53 is induced, whereas p53 null cells remain immunohistochemically negative. A first rapid appraisal of *p53* gene status in HUFs thus can be obtained by examining p53 protein basal level and/or lack of the typical wild-type response to DNA damage (i.e., p53 nuclear accumulation).

Another preliminary means to identify HUF cell lines with missense mutations is to extract RNA from the culture, amplify the p53 transcript from cDNA with specific p53 primers in a single PCR reaction (Table 1B), and sequence. A screen for coding region inactivating mutations in p19/ARF exons can be performed in similar fashion (ARF-specific primers in Table 1B).

PERSPECTIVES

One of the reasons for establishing the Hupki mouse strain was to provide an experimental tool for investigating mutagenic activity of carcinogens that employs the human *p53* gene DBD as target sequence. In this chapter we describe an assay utilizing Hupki embryonic mouse fibroblasts (HUFs) to select for mutations in human p53 sequences induced by carcinogens *in vitro*. Characterization of mutations that have arisen due to pro-mutagenic conditions such as oxidative stress or as a consequence of DNA repair defects may also be feasible with this assay. Frequent detection of HUF cell lines with *p53* mutations derived from primary cultures exposed to mutagenic carcinogens confirms that *p53* mutation is a key component of *in vitro* immortalization of HUFs.

Generation of mutation spectra in HUF cells could be facilitated by further development and experimentation with the assay. For example, novel sequencing methods sensitive enough to detect a small population of mutated cells among large numbers of wild-type cells could be applied a few weeks after mutagen treatment to accelerate discovery of mutations. This would reduce the waiting time to mutation screening (typically 3 months), otherwise needed to allow the number of mutant cells to expand, take over the culture, and become a cell line. A second variant would be to determine the protocol conditions that maximize chances for recovery of immortalized cells harboring defects in p53 rather than defective p19ARF (19, 26). Further studies of genetic mechanisms that lead to HUF immortalization also could lead to discovery of strategies that favor selection of cells with mutant p53. Subcloning of cell populations, in which mutant p53 cells may reside as a small subpopulation, although too labor-intensive to be

Table 1B. PCR primers for p53 and p19ARF sequencing from cDNA.

Amplicon	Primer	Sequence	Product size
p53	mp53e2F	ATGACTGCCATGGAGGAGTC	1.2 kb
Exon 2-11	mp53e11R	TCAGGCCCCACTTTCTTGAC	
p19ARF	p19-F1	CTTGGTCACTGTGAGGATTC	568 bp
Exon 1-2	p19ex2-R1	TGAGGCCGGATTTAGCTCTGCTC	

practical as a mutation assay protocol, would undoubtedly increase the number of independent mutations identified per experiment. With the current protocol at least, it is already clear that mutations recovered do correspond remarkably well to the common p53 mutations found in human tumors (11, 30, 31).

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