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LOH-proficient embryonic stem cells: a model of cancer progenitor cells?

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Cancers are thought to originate in stem cells through the accumulation of multiple mutations. Some of these mutations result in a loss of heterozygosity (LOH). A recent report demonstrates that exposure of mouse embryonic stem cells to nontoxic amounts of mutagens triggers a marked increase in the frequency of LOH. Thus, mutagen induction of LOH in embryonic stem cells suggests a new pathway to account for the multiple homozygous mutations in human tumors. This induction could mimic early mutagenic events that generate cancers in human tissue stem cells.

Mutations generate human cancers

Cancers are thought to arise in pluripotential stem cells, and, when clinically detected, these stem cells contain numerous mutations. Cancer cell genomes are frequently aneuploid (see Glossary), epigenetically altered and 'peppered' with mutations [1–3]. This raises the following important questions: (i) how are the mutations generated?; (ii) how are the mutations selected?; and (iii) what is the biological significance of the various mutations for tumorigenesis? The origins of mutations in cancer cells are unknown but include random events that damage DNA, such as attack by environmental carcinogens and reactive cellular metabolites. Mutations in oncogenes (e.g. *K-ras* and *myc*) and tumor-suppressor genes (e.g. *RB* and *APC*) can impart growth advantages to malignant cells, resulting in clonal selection [4–6]. Other, non-clonal mutations occur randomly throughout the genome and contribute to the characteristic heterogeneity of malignant cells within a tumor [7]. Various methods have been established to detect clonal mutations, including loss of heterozygosity (LOH) in

Glossary

Aneuploid: having a chromosome number that is not an exact multiple of the normal diploid number, with either fewer or more than the normal number of chromosomes in the cell.
Loss of heterozygosity (LOH): loss of the contribution of one parent to the genome of the cell.
Mutator phenotype hypothesis: that normal rates of mutation in somatic cells are insufficient to account for the multiple mutations observed in cancer cells; therefore, an increase in the mutation frequency is necessary to account for the large number of genetic changes observed in human tumors.

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Box 1. Monitoring spontaneous and induced LOH

Donahue and coworkers generated a collection of reporter ES cell clones by randomly integrating gene-trap retroviral vectors (GTR1.3) into the genome [14]. GTR1.3 contains a neomycin-resistance (*Neo*) cassette and a splice acceptor site, and it disrupts genes by fusing exons to the *Neo* cassette (Figure Ib). After retroviral integration and selection, ten ES clones were tested and found to maintain their pluripotentiality; they produced germline chimeras and viable offspring. Using the *Neo*-coding sequences, the precise integration sites were mapped. Fifty-three of the ES clones, each of which contained a single *Neo* cassette on different chromosomes, were incubated with a variety of carcinogens to screen for LOH induction at the *Neo* loci (Figure Ia). To score LOH events, they used the protocol previously implemented by Mortensen and Seidman [17], in which a heterozygous locus containing a selection cassette can be transformed to a homozygous state simply by the selection of cells in a higher concentration of the antibiotic G418. LOH resulted in a doubling of the number of *Neo* cassettes and therefore enabled growth at the increased drug concentration. The spontaneous frequency of LOH was similar to that reported previously [22,23]. Transient addition of mutagens to the cultures markedly increased the frequency of LOH. On the basis of known mechanisms of DNA damage by mutagens and carcinogens, it can be surmised that the increase in LOH in ES cells can occur by a variety of mechanisms. The mutagens used by Donahue *et al.* in their selection system [14] and the possible mechanism of action of these agents are as follows: methylnitrosourea is a methylating agent; hydroxyurea alters nucleotide pools; mitomycin C crosslinks DNA; and ultraviolet light generates intrastrand and interstrand pyrimidine dimers [24].

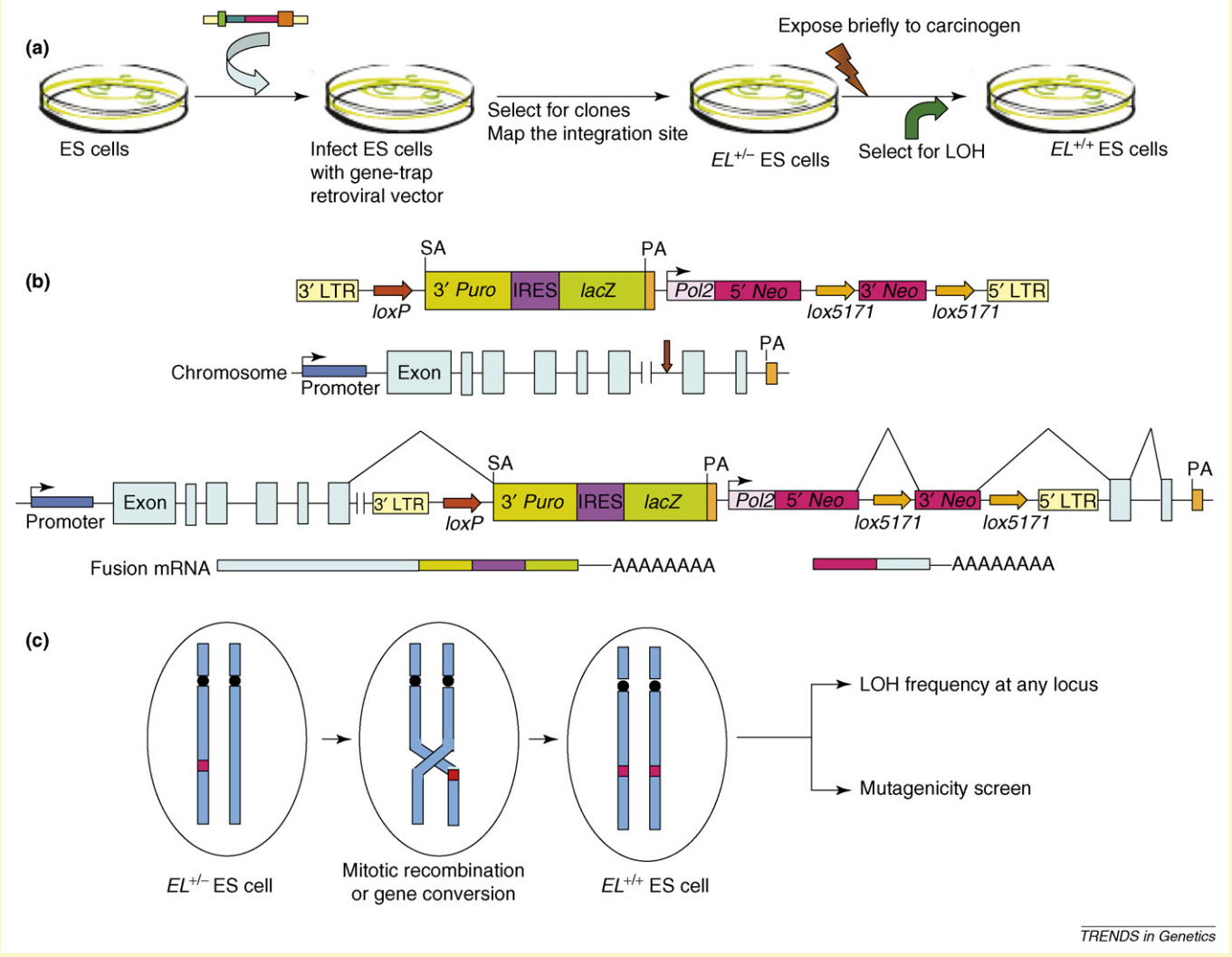

Figure I. Gene-trap mutagenesis. **(a)** Strategy of gene-trap mutagenesis and selection. ES cells are infected with packaged virus that contains gene-trap vector. This process is followed by selection and purification of clones, and expansion of the transduced ES cells. Integration sites are mapped by inverse PCR, and fusion mRNA is detected by RT–PCR. Entrapment locus (*EL*)-mapped ES cells are treated transiently with carcinogen and are selected in 2 mg/ml of the antibiotic G418 for LOH-type events. **(b)** Mechanism of insertion into genome locus (entrapment locus) by poly(A)$^+$-trapping gene-trap vectors (GTRs). This is a simplified model adapted from Donahue *et al.* [14]. The GTR inserts into an intron proximal to the 3′ end of an open reading frame (ORF). The upstream ORF exon splices to a splice acceptor (SA) site on the 3′ *Puro* (3′ end of the puromycin-resistance gene)–IRES (internal ribosomal entry sequence)–*lacZ* cassette, disrupting the ORF and producing truncated ORF-*Puro* fusion protein. Full-length LacZ is translated with the help of the IRES sequence. The RNA polymerase II (*Pol2*) gene promoter drives expression of the *Neo* cassette, which splices to the 3′ end of the ORF. **(c)** LOH-type event. To survive in 2mg/ml G418, ES cells are thought to duplicate the *EL* locus by three possible mechanisms: gene conversion, mitotic recombination or amplification of the *EL* locus. The propensity of ES cells to undergo LOH at high frequency in the presence of a low dose of carcinogen holds immense potential for biotechnology applications. Homozygous ES cells and derived differentiated cells at any *EL* locus can be obtained easily without generation of homozygous knockout mice. This is especially useful when the *EL* locus of interest causes early embryonic lethality in the homozygous condition in mice. This technique can also be used to screen for carcinogenicity or mutagenicity of compounds. Abbreviations: *loxP* and *lox5171*, wild-type and mutant recombination elements recognized by the recombinase Cre; LTR, long terminal repeat; PA, polyadenylation signal sequence.

tumors. By contrast, the detection of random mutations requires an analysis of single cells or single DNA molecules [8].

Spontaneous mutations in normal human somatic cells are mainly single-base substitutions that occur at low frequencies (between 1 in 10^8 and 1 in 10^{10} substitutions per division) [9]. Furthermore, in mouse embryonic stem (ES) cells, the frequencies of spontaneous and induced mutations are reported to be tenfold lower than in their somatic counterparts [10]. These low frequencies are in accord with the hypothesis that the normal somatic mutation rate is insufficient to account for the large numbers of mutations in tumors and therefore that cancer cells must express a mutator phenotype at some point during tumorigenesis [11] (i.e. the mutator phenotype hypothesis). However, others have argued that a mutator phenotype might not be required for the development of cancers [12], and some mathematic models indicate that – at least in exceptionally rapidly dividing tissues – the normal mutation rates might be sufficient to explain the increased frequency of mutations in cancer cells [13].

LOH can generate large numbers of mutations and cancer

A recent paper by Donahue *et al.* presents a model system that could explain the origin of cancer-causing mutations [14] (see Figure I in Box 1). The researchers used a mouse ES cell line to establish a panel of 53 clones, each containing a neomycin-resistance cassette inserted at a different chromosomal locus. Brief exposure of each of the clones to a variety of carcinogens resulted in LOH at frequencies as high as 8×10^{-3} (i.e. 1 in 8000 cells). LOH can result from deletions, recombination, chromosomal rearrangements or even point mutations [15]. In the mouse ES cells analyzed, LOH was scored only if it resulted in duplication of the neomycin marker. The results suggest that noninherited cancers could arise from prior exposure to genotoxic agents and that the high incidence of LOH might obviate the requirement for a mutator phenotype. Alternatively, the high incidence of LOH might result from an experimentally induced mutator phenotype. However, there is convincing evidence against this possibility, because the frequency of LOH at a second reporter gene, encoding thymidine kinase, was not increased in cells that had previously undergone spontaneous or carcinogen-induced LOH. Moreover, 90% of all observed LOH events arose within 24 h of treatment, demonstrating that the high frequency of induced LOH is a transient, isolated event. Thus, if mouse ES cells can be equated with cancer stem cells, the findings of Donahue *et al.* suggest that LOH might occur in the absence of mutations in genes that promote genetic instability [14].

It has been demonstrated that tumors show a mutator phenotype at the chromosomal level [16] and the nucleotide level [7]. However, it is not known when this mutator phenotype is expressed during tumorigenesis. Is it an early initial event, or is it continuously expressed? Many researchers think that, given the extremely low rate of spontaneous mutation in normal stem cells and the large number of mutations required for transformation, a mutator phenotype initiates carcinogenesis. If, however, the same high frequency of LOH (3–4 orders of magnitude higher than the frequency of spontaneous nucleotide mutation) occurs in cancer stem cells, then generating the large number of mutations for cancer initiation is feasible in the absence of a mutator phenotype.

Resolving the mechanism of LOH

LOH can result from a variety of mechanisms that mediate DNA exchange. The results of Donahue *et al.* indicate that LOH mediated by recombination or gene-conversion-type duplication in mouse ES cells can be quantitated at an inserted neomycin-resistance cassette [14]. These results cannot distinguish the mechanism of the LOH event because of the inbred genetic background of the mice from which the ES cells were derived [17]. Other types of mutation that produce LOH (see the previous section) were not observed in these experiments. Nevertheless, the high level of damage-induced LOH measured at retrovirally introduced loci might be misleading, because retroviral vector recombination might not accurately recapitulate LOH-type events at endogenous loci.

Given the wide variation in methylnitrosourea-induced mutation frequency among different genetic backgrounds, after different treatment regimens and at different mutational target sites (Table 1), it is reasonable to expect that other types of mutation, including point mutations, might occur at similar or higher frequencies in the same cells. Given that the technology now exists to track simul-

Table 1. Methylnitrosourea-induced mutations[a]

Cell type	Methylnitrosourea treatment[b]	Gene	Mutation frequency ($\times 10^{-4}$ per gene)	Fold increase[c]	Refs
Point mutations					
Big Blue rat embryo cell line	1 mM for 0.5 h	*lacI*	9.3	23	[18]
Big Blue B6C3F1 mouse splenic lymphocytes	20 mg/kg ip	*Hprt*	1.6	78	[19]
SWR × Muta mouse small intestine cells	50 mg/kg ip	*lacZ*	10.6	24	[20]
SWR × Muta mouse small intestine cells	50 mg/kg ip	*cII*	6.2	16	[20]
LOH					
SWR × Muta mouse small intestine cells	50 mg/kg ip	*Dlb1*	20.0	74	[20]
C57BL/6 inbred mouse splenic lymphocytes	60 mg/kg ip	*Aprt*	1.9	21.5	[21]
B6C3F1 hybrid mouse splenic lymphocytes	60 mg/kg ip	*Aprt*	3.1	49.0	[21]
129/SvJ mouse AC1 ES cell line	0.5 mM for 4 h	*Phgdhl1*	94.9	88	[14]
129/SvJ mouse AC1 ES cell line	0.5 mM for 4 h	ND	7.7	39	[14]

[a]Abbreviations: *Aprt*, adenine phosphoribosyl transferase; *Dlb1*, dolichos lectin binding 1 gene (also known as *B4galnt2*); *Hprt*, hypoxanthine guanine phosphoribosyl transferase gene; ip, intraperitoneal; ND, not determined; *Phgdhl1*, phosphoglycerate dehydrogenase like 1 gene.
[b]The relative methylnitrosourea-induced increase in mutation frequency over the spontaneous mutation frequency.
[c]Single dose treatment either *in vivo* by ip injection or *in vitro* at specific dosage.

taneously the frequency of mutagen-induced LOH and point mutations in the same cells and at the same loci [8,14], it should be possible to resolve the relative contribution of each class of mutational event to the carcinogenic potential of a mutagen.

Future analyses and directions

The high frequency of LOH induced by chemical carcinogens throughout the mouse ES genome and the simple method used for mutant selection affords an important protocol for the creation of double-knockouts in cultured cells. Homozygous mutants can be recovered directly from genetic screens of mouse ES cells. More importantly, nonviable homozygous mutations can be induced in heterozygous mouse ES cells by chemical carcinogens (see Figure I in Box 1). It is now important to determine whether the high frequency of induction of LOH at retrovirally induced loci in mouse ES cells is also observed at endogenous loci in human ES cells and whether tissue stem cells – the precursors for malignant transformation – also show a high frequency of LOH in response to chemical carcinogens.

Concluding remarks

LOH-type events can unmask numerous recessive cancer-initiating point mutations in a single event. If the frequency of LOH observed in mouse ES cells (at integrating gene-trap retroviral vector sites) is similarly high at endogenous sites throughout the genome of human tissue stem cells and similarly prone to stimulation with low doses of carcinogens, then the work of Donahue *et al.* [14] provides strong evidence against the requirement for a mutator phenotype in carcinogenesis.

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