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Fanconi Anemia Mutation Causes Cellular Susceptibility to Ambient Oxygen

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Summary

The gene defect causing the Fanconi anemia (FA) phenotype appears to be expressed at the cellular level, since FA fibroblasts show a protracted course of explant outgrowth, a diminished in vitro life span, and very poor cloning. We show that exposure of FA fibroblasts to hypoxic (5% v/v oxygen) culture conditions restores their growth in vitro to near normal. Exposure to elevated oxygen tension (35% v/v) causes accumulations of FA cells in the S and G2/M phases of the cell cycle that are in significant excess of those seen in heterozygote and control strains. In the absence of evidence for defective cytoplasmatic radical scavenging systems, these observations suggest increased nuclear susceptibility to ambient oxygen as cause of the FA cellular phenotype.

Introduction

Skin fibroblast–like cells cultured from patients with Fanconi anemia (FA) express the genetic defect with poor proliferation, both as clones and as mass cultures (Elmore and Swift 1976; Weksberg et al. 1979). The in vitro growth deficit of FA lymphocytes results from a specific cell cycle lesion, i.e., prolongation of and arrest in the G2 phase segment of the cell cycle (Dutrillaux et al. 1982; Kubbies et al. 1985). The latter observation suggests difficulties with maintaining the integrity of the genetic material during replication and preparation for mitosis.

Other lines of evidence also point toward the cell nucleus as a site rendered vulnerable by the FA mutation. Cells of FA genotype are more sensitive to chromosome-damaging agents than are normal cells (Latt et al. 1975; Sasaki and Tonomura 1973; Dean and Fox 1983); in addition, FA cells may be defective in removing interstrand DNA cross-links (Sasaki 1975; Remsen and Cerutti 1976) and are deficient in metabolic systems thought to be instrumental in DNA repair, such as NAD, endonuclease, and ligase activity (Hirsch-Kaufman et al. 1979; Berger et al. 1982). Despite these various inferential lines of evidence, the question whether FA represents a DNA repair mutation has not been settled.

Experimentally elevated oxygen tension affects the G2 phase transit in normal cells (Balin et al. 1978), as well as enhancing chromatid-type aberrations in FA cells (Joenje et al. 1981). The similarities between oxygen toxicity in normal cells and the cell kinetic behavior of cultured FA cells (defective G2 phase transit; Kubbies et al. 1985) caused us to postulate that FA cells are hypersensitive toward the effects of ambient oxygen (Hoehn et al., in press). If the poor growth performance of FA cells reflects oxygen toxicity, their growth might be improved under hyypoxic culture conditions. The present paper examines the effects of reduced (and, in one experiment, elevated) oxygen tension on the growth performance of fibroblast cultures from normal individuals, FA patients, and obligate heterozygotes.

Material and Methods

Primary fibroblast cultures were established from three FA patients (aged 2, 16.5, and 17.5 years), three obligate heterozygotes for FA (the parents of the 2- and 17.5-year-old FA patients, aged 20, 22.5, and 52.5 years), and three individuals without known chromosomal or metabolic disease (19, 21.5, and 55.5 years of age).

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Skin specimens were obtained by punch biopsy from the mesial aspect of the mid forearm. Each biopsy was cut into 20 pieces, and each ten were sandwiched into Leighton tubes (size 19 × 105 mm). The cultures were fed twice weekly with freshly prepared Eagle's minimum essential medium (MEM) supplemented with 16% pretested and heat-inactivated FCS. When outgrowth covered approximately half of the area of a Leighton tube, the cultures were trypsinized and passaged to 25-cm^2 tissue culture flasks. At confluency, these cultures were again trypsinized and counted. The serial growth and cloning experiments to be described below were begun at passage 2.

To assess growth velocities (population doublings per day) and total growth capacity (cumulative population doublings), 5×10^4 cells were inoculated into 25-cm^2 flasks and passaged at weekly intervals (less frequently toward the end of replicative life spans). When a culture failed to yield 5×10^4 cells at the end of 3 wk, it was terminated. Cells of terminal cultures were negative for mycoplasma. Population-doubling numbers were based on weekly cell counts of the cultures after trypsinization, the assumption being that all cells participated in the population doublings.

In parallel with these serially propagated mass cultures, 200 cells each were dilute plated at weekly intervals into triplicate 60-mm Corning culture dishes in order to determine cloning rates and colony diameters. After 12 days, the cloning dishes were fixed and stained in situ with crystal violet. Colonies with 20 or more cells were scored as clones. Colony diameters were determined from 10-fold-enlarged projections of the stained cloning dishes.

All experiments were carried out in incubators with sensor-regulated gas supply (Hereaus, Hanau, West Germany) in a humidified 5% (v/v) CO_2 atmosphere. Oxygen concentrations of 5% or 35% (v/v) were obtained by either replacing air by nitrogen or supplementing air with additional oxygen.

For flow cytometric determinations of cell cycle distributions, cells from early passage cultures were rendered quiescent by serum starvation (0.1% serum) for 3 days, after which 2,500 cells/cm^2 were plated into 80-cm^2 tissue culture flasks and stimulated by medium supplemented with 16% FBS. At 27 h and 72 h after stimulation, culture aliquots were harvested by trypsinization, pelleted, and frozen in 1 ml MEM with 10% FCS and 10% dimethyl sulfoxide at −30 C.

Prior to flow cytometric analysis, the stored frozen aliquots were thawed, pelleted, and adjusted to cell densities of 5×10^5/ml in staining buffer containing 10 mM Tris, pH 7.4, 154 mM NaCl, 1 mM $CaCl_2$, 0.5 mM $MgCl_2$, 0.1% nonidet P40, 0.2% BSA, and 4 μg DAPI/ml. Minimum staining time was 30 min at 4 C. Flow cytometric measurements were carried out with a positive-pressure, epi-illumination ICP mercury arc-lamp system (Phywe, Göttingen; now Ortho Diagnostic Systems, Raritan, NJ) equipped with KG1, BG38, UG1, and K45 glass filters (Schott, West Germany). Pulse-height analysis and data acquisition were performed by an Ortho 2103 multichannel analyzer. The resulting signals were transmitted to a PDP 11/23 microcomputer (Digital Equipment, Maynard, MA) for storage on floppy disks and further analysis of histograms by automated curve fitting (Rabinovitch 1983).

Results

The photographs shown in figure 1 are of culture dishes containing fibroblasts from FA, heterozygote, and normal individuals cultured at ambient (20% [v/v]) and reduced (5% [v/v]) oxygen tension. These pictures provide a qualitative impression of the number and size of colonies at day 12 after dilute plating of passage 6 cultures exposed to different oxygen concentrations. Regardless of genotype, cloning is improved at reduced compared with the customary atmospheric oxygen tension. However, improved growth at low oxygen is most pronounced for the FA cells, which show almost no growth at 20% oxygen. Heterozygote and control cells show very similar cloning patterns at either ambient or reduced oxygen.

Figure 2 depicts the long-term growth performance of three independent cultures each of FA, heterozygote, and normal fibroblasts. In each panel the difference between growth at 20% and 5% oxygen is represented by the shaded area. All cultures show improved growth under reduced oxygen culture conditions, whether that growth is measured as cumulative population doublings (upper-left panel), population doublings per day (upper-right panel), cloning efficiency (lower-left panel), or colony diameter (lower-right panel). However, the difference between 5% and 20% oxygen is consistently larger for FA than for either heterozygote or control fibroblasts.

Table 1 compares the average growth performance of the three types of strains at ambient relative to reduced oxygen conditions. From these data it is evident that the heterozygote and control strains show closely comparable growth deficits when grown at ambient oxygen; in contrast, the FA strains lag severely behind in all three of the growth parameters listed in table 1. The disproportionate growth-promoting effect of low oxy-

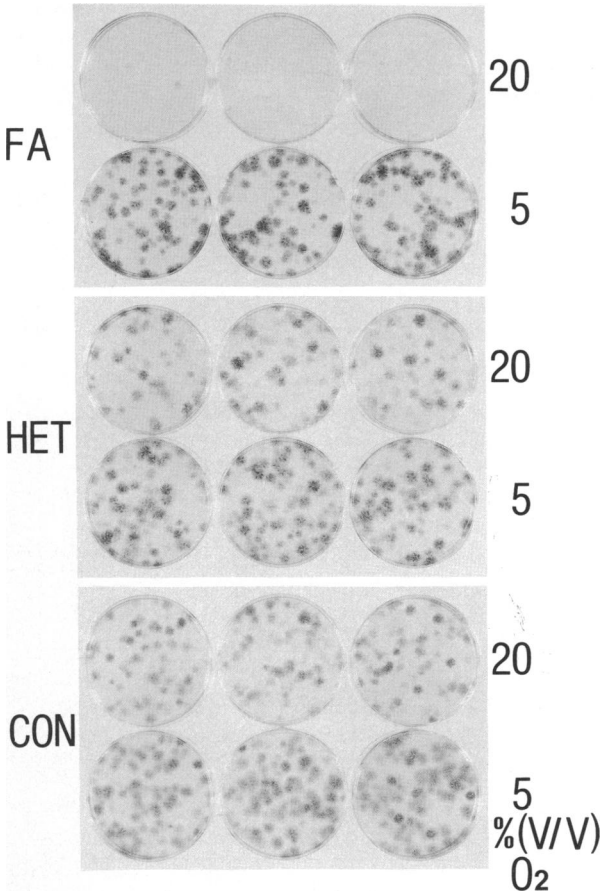


Figure 1 Photographs of 60-mm culture dishes showing passage 6 FA, heterozygote (HET), and control (CON) fibroblasts inoculated at 200 cells/dish and incubated for 12 days at 20% and 5% oxygen. Cells were fixed and stained with Crystal Violet in situ. Dishes are shown 1/8 actual size.

Table 1

Percentages of Growth Deficit Observed in Three Strains Each (Capital Letters; cf. Fig. 2) of Different Genotypes Exposed to 20% Relative to 5% (v/v) Oxygen Tension During Serial Passage and Cloning

	Fa		Heterozygotes		Controls
			Cumulative Population Doublings		
A,D,B	71.9		87.8		87.6
B,E,H	76.9		84.4		87.9
C,F,I	72.6		84.3		82.9
M ± SEM . .	73.8 ± 1.6	(.004)	85.5 ± 1.2	(.75)	86.1 ± 1.6
			Cloning Efficiency		
A,D,G	21.5		61.2		65.6
B,E,H	16.5		71.6		74.0
C,F,I	26.6		71.3		69.3
M ± SEM . .	21.5 ± 2.9	(.0005)	68.0 ± 3.4	(.70)	69.6 ± 2.4
			Colony Diameter		
A,D,G	44.3		82.1		79.1
B,E,H	42.3		82.8		78.5
C,F,I	39.8		81.9		75.3
M ± SEM . .	42.1 ± 1.3	(.00001)	82.3 ± 0.3	(.02)	77.6 ± 1.2

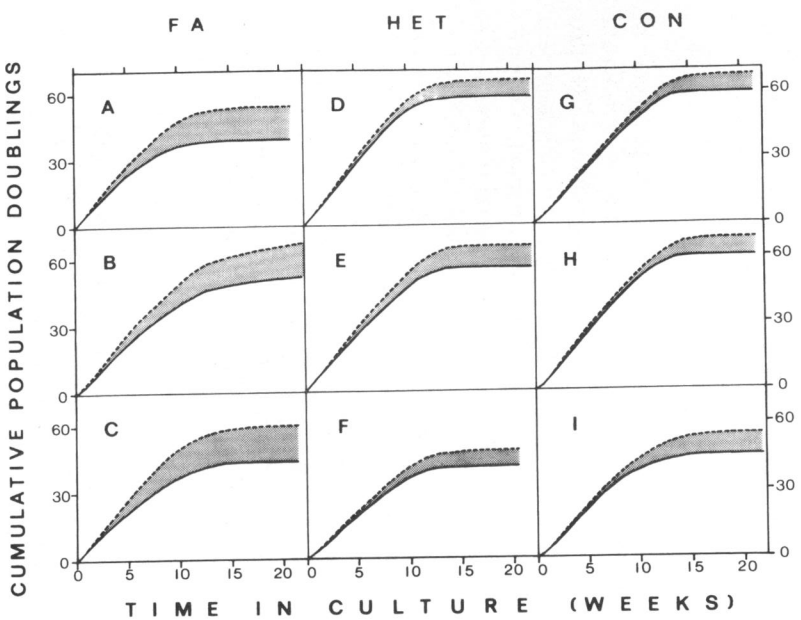
NOTE.—The cloning data are cumulative data of 8–13 determinations during the in vitro life history of each strain. Cumulative growth and cloning performance at 5% oxygen tension equals 100%. Numbers in parentheses are *P* values for differences between the genotypes mean (M) flanking them (Student *t*-test).

gen on homozygous FA fibroblasts relative to the other genotypes is most distinctive for the cloning parameters: the cumulative cloning efficiencies improve approximately fivefold, and the cumulative colony diameters increase by more than twofold by lowering the O_2 of the gas phase from atmospheric to 5%.

To compare the effects of oxygen tension on activation and progression of FA and control fibroblasts through the cell cycle, flow cytometric analysis was done on cells from passage 6 cultures at 27 h and 72 h after stimulation of previously serum-deprived cells. The results of these stimulation experiments are summarized in figure 3. Early in stimulation (at 27 h) more cells of both FA and control cultures remain in G1 and fewer have entered G2 when grown at ambient or elevated (20% and 35%) oxygen tension than at 5% oxygen. During this early phase of the mitogenic response, however, there is no difference between FA and control fibroblasts. The situation changes at 72 h after stimulation, when cells are cycling in greater numbers; at that time, the effects of the higher oxygen tensions (compared with 5%) are much more pronounced in FA than in control cells. The inhibitory effects of atmospheric and elevated oxygen culture conditions on the growth of FA cells are manifested by a relative deficit of S phase cells, as well as by excessive accumulation of cells in the G2 segment of the cell cycle (fig. 3). Both the S phase and the G2 phase differences in the 72-h cell cycle distributions between FA and control cells are highly significant ($P < .001$, Fisher's exact test).

Discussion

It is evident from the data presented here that growth of FA fibroblasts is more sensitive to ambient and elevated oxygen tension than are normal cells, although all genotypes tested grow better at less than ambient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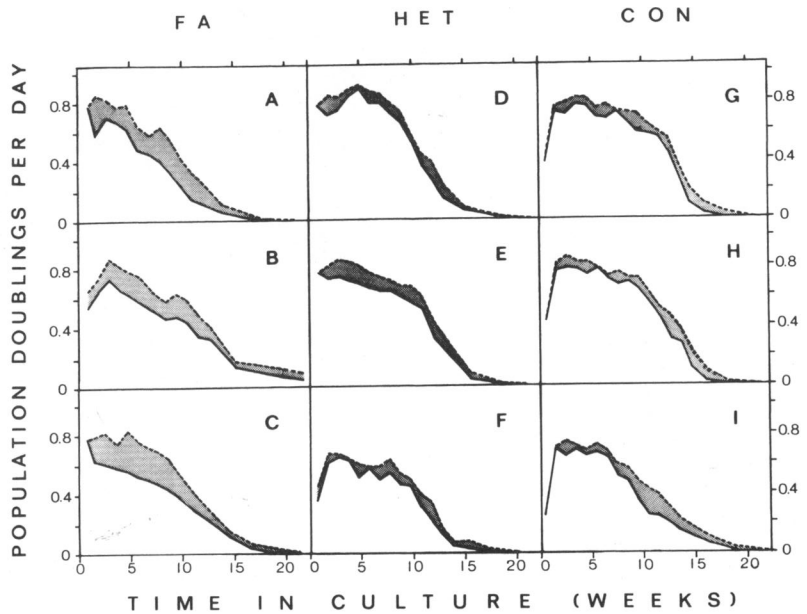
CUMULATIVE POPULATION DOUBLINGS
TIME IN CULTURE (WEEKS)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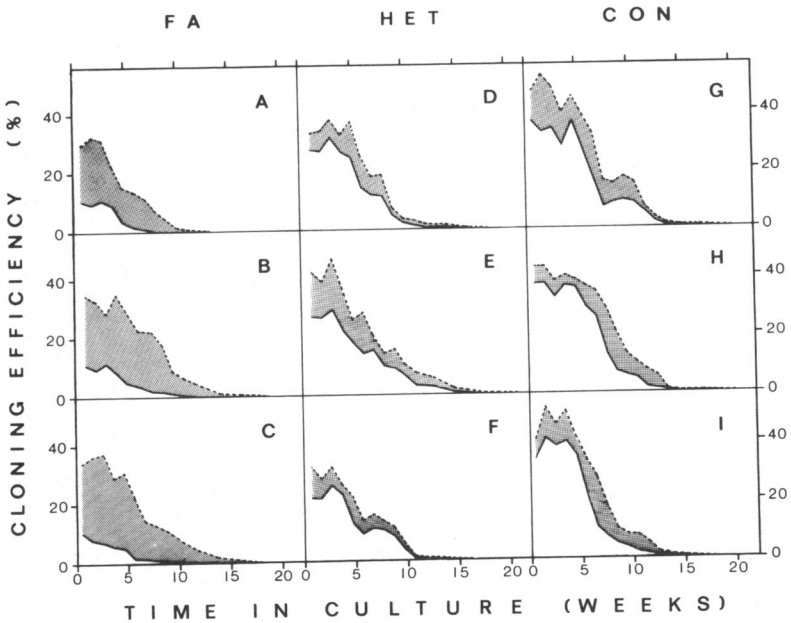
POPULATION DOUBLINGS PER DAY
TIME IN CULTURE (WEEKS)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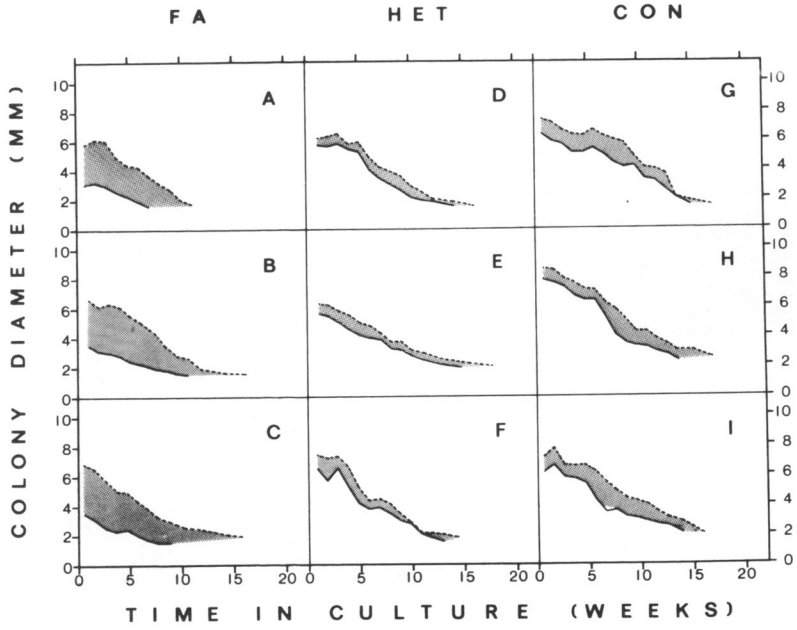
CLONING EFFICIENCY (%)
TIME IN CULTURE (WEEKS)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COLONY DIAMETER (MM)
TIME IN CULTURE (WEEKS)

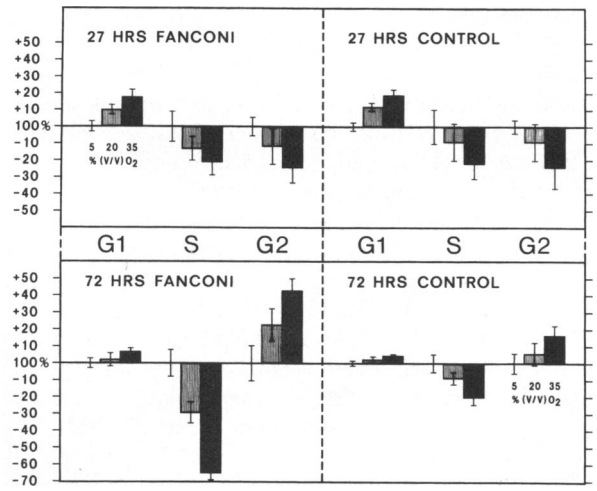

Figure 3 Percentage changes in numbers of FA and control fibroblasts in G1, S, and G2 phases of the cell cycle after 27 and 72 h of growth stimulation in three different oxygen concentrations, as determined by flow cytometry. The results for 20% oxygen (shaded bars) and 35% oxygen (solid bars) are percentage changes relative to the result at 5% oxygen, which is set as 100%. The bars denote means ± SEMs of three FA and three control strains.

oxygen tension. A growth-promoting effect of reduced oxygen tension is well established for a number of mammalian cell types, including human diploid fibroblasts (Taylor et al. 1974; Packer and Fuehr 1977; Balin et al. 1978). This effect appears to be most pronounced at low cell-plating densities (Taylor et al. 1978), such as were employed in our serial cloning experiments. The important new finding of our study is that FA fibroblasts benefit from hypoxic culture conditions to a much greater extent than do heterozygote or normal cells. For example, the data presented in figure 2 and table 1 show that FA cells exposed to 5% oxygen attain 94% of the average cloning efficiencies of the heterozygote strains (the cloning performance of the latter being not significantly different from that of the normal control strains). Whereas there is good evidence that the poor growth of FA lymphocytes is a result of impaired progression through the G2 phase of the cell cycle (Dutrillaux et al. 1982; Kubbies et al. 1985), no such evidence is available to date for FA fibroblasts. Our experiments summarized in figure 3 confirm that elevated oxygen tension causes perturbations of the cell cycle distribution of normal fibroblasts (Balin et al. 1978). However, these perturbations are significantly greater in FA than in control fibroblast strains. Together, these observations suggest that oxygen toxicity might be responsible for the poor growth performance of FA fibroblasts under atmospheric oxygen cell culture conditions.

Our finding of reversion of FA fibroblasts to near-normal growth and cloning under hypoxic conditions supports the view that FA cells are hypersensitive to pro-oxidant states (Cerutti 1985). The strongly oxygen-dependent rate of spontaneous chromosomal breakage (Joenje et al. 1981) and the reported protective effect of superoxide dismutase (Nordenson 1977; Sudharsan and Heddle 1980; Nagasawa and Little 1983; Dallapiccola et al. 1985) are consistent with the idea of hypersensitivity to oxidative damage as a hallmark defect in FA cells. In vivo, it is the monocytic cell lineage that is most prone to malignant conversion in FA patients (German 1983) and this cell lineage possesses an active superoxide-generating system (Chaudhry et al. 1982). One might therefore suspect that the genetic lesion in FA is expressed by a failure to properly protect the cell nucleus from oxidative damage. Entirely consistent with this hypothesis is the hypersensitivity of FA cells to free radical–producing antibiotics such as mitomycin C (Cachur et al. 1982). Paradoxically, FA fibroblasts show either normal or distinctly elevated levels of their primary enzymatic defenses against oxygen toxicity (superoxide dismutase, catalase, and glutathione peroxidase; Poot et al. 1986; Gille et al. 1987). The reported hyperactivity of antioxidant defenses in FA fibroblasts could, however, be reconciled with our data if one assumes that the only FA fibroblasts able to survive under standard (i.e., atmospheric-oxygen) cell culture conditions are those that have succeeded in coping with their intrinsic oxygen sensitivity by reactive increases of their antioxidant defense mechanisms. Our data show that such ambient oxygen-adapted FA cultures nevertheless grow and clone poorly. This suggests that even the stepped-up primary defense systems do not suffice to provide complete protection against oxidative damage, at least not within the realm of the cell nucleus. It is thus conceivable that the apparent nuclear hypersensi-

Figure 2 Growth characteristics of three strains each of FA fibroblasts (A–C), heterozygote (HET) fibroblasts (D–F), and control (CON) fibroblasts (G–I) with time (in weeks) in culture. In each panel, the solid line represents growth at ambient (20% v/v) oxygen tension and dotted lines represent growth at reduced (5% v/v) oxygen; the shaded area is meant to illustrate the difference in growth performance between ambient and reduced-oxygen conditions. The upper set of panels depicts cumulative population doublings (left) and population doublings per day (right); the bottom set depicts cloning efficiency (left) and colony diameter (right).

tivity toward oxygen might reflect a defective repair of oxidized DNA. There are now strategies and assays available that could be used for a direct test of the oxidative-damage hypothesis (Cathcart et al. 1984).

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