Glycolytic Enzymes Can Modulate Cellular Life Span

Hiroshi Kondoh,1,2 Matilde E. Leoneart,1 Jesus Gil,1,2 Jing Wang,1 Paolo Degan,3 Gordon Peters,5 Dolores Martinez,1 Amancio Carnero,3 and David Beach1

1Wolfson Institute for Biomedical Research, University College London and Cancer Research UK, London Research Institute, London, United Kingdom; 2Mutagenesis Laboratory, National Cancer Research Institute-Genova, Genoa, Italy; and 3Experimental Therapeutics Program, Centro Nacional de Investigaciones Oncológicas, Madrid, Spain

Abstract

An unbiased screen for genes that can immortalize mouse embryonic fibroblasts identified the glycolytic enzyme phosphoglycerate mutase (PGM). A 2-fold increase in PGM activity enhances glycolytic flux, allows indefinite proliferation, and renders cells resistant to ras-induced arrest. Glucosephosphate isomerase, another glycolytic enzyme, displays similar activity and, conversely, depletion of PGM or glucosephosphate isomerase with short interfering RNA triggers premature senescence. Immortalized mouse embryonic fibroblasts and mouse embryonic stem cells display higher glycolytic flux and more resistance to oxidative damage than senescent cells. Because wild-type p53 down-regulates PGM, mutation of p53 can facilitate immortalization via effects on PGM levels and glycolysis. (Cancer Res 2005; 65(1): 177-85)

Introduction

The metabolism of cancer cells differs significantly from that of normal cells (1). Cancer cells are able to maintain high rates of aerobic glycolysis even under the high-oxygen (20%) conditions of normal tissue culture. This property, known as the "Warburg effect," has been recognized for over 70 years (2) and is exploited clinically in positron-emission tomography measurement of 2-[18F]fluoro-2-deoxy-D-glucose uptake (3). Moreover, several recent studies have shown that enhanced glycolysis is a distinctive marker of cancer progression (4, 5), and a correlation has been noted between the expression of oncogenes such as ras, myc, or src and enhanced aerobic glycolysis (1). However, despite the undisputed significance of the Warburg effect in cancer, there have been few mechanistic insights into its relationship with the well-characterized molecular mechanisms are also important (10). For example, primary mouse embryo fibroblasts (MEF) lose proliferative capacity after only 10 to 20 population doublings despite having exceptionally long telomeres. In this setting, the growth arrest is generally attributed to the stresses imposed by culture conditions (11), but the molecular details are poorly understood.

At least one of the stresses seems to be oxygen poisoning because it has been noted that the life span of mouse and human fibroblasts can be extended by culturing them under low-oxygen conditions (12, 13). Conversely, deliberate oxidative stress, for example, with low doses of hydrogen peroxide, can elicit a senescence-like arrest (14), and the production of reactive oxygen species seems to underlie the premature senescence phenotype induced by oncogenes such as ras (15). Mouse cells seem to be particularly sensitive to oxidative stress but also escape from the ensuing growth arrest at a relatively high frequency. Almost all of the immortal cell clones that emerge have defects in either p53 or one of its upstream regulators or downstream effectors (16, 17), in line with a direct involvement of p53 in the response to oxidative stress (18).

Using an unbiased genetic screen for cDNAs that confer immortality in MEFs, we identified the muscle form of phosphoglycerate mutase (PGM-M). Whereas overexpression of PGM or glucosephosphate isomerase (GPI), another glycolytic enzyme, can enhance glycolysis and bypass senescence, depletion of PGM or GPI was found to shorten cellular life span. PGM-immortalized MEFs show increased resistance to oxidative DNA damage and ras-induced arrest. Interestingly, mouse embryonic stem cells, which are highly resistant to oxidative stress despite an apparently normal p53 response, also show high levels of glycolysis. Our findings suggest that the Warburg effect may reflect a mechanism for escaping the restrictions to cellular life span caused by oxidative stress.

Materials and Methods

Cell Culture and Retroviral Infection. Primary MEFs were isolated from 13.5-day postcoitum embryos of CD1 or C57BL/6 mice as described previously (19). The cells were grown in DMEM (Life Technologies, Gaithersburg, MD) with 10% FCS (Sigma, St. Louis, MO). Mouse embryonic stem cells were cultured as described by Smith et al. (20). All retroviruses were produced by transfecting the relevant plasmid DNA into the LinXE packaging cell line (21) and infections were conducted in the presence of 8 μg/mL polybrene. Infected cells were selected in hygromycin (75 μg/mL), blasticidin (1 μg/mL), or G418 (400 μg/mL) as appropriate. After 8 to 10 days of selection, cultures were propagated according to a 3T3 protocol as described previously (19). Briefly, every
3 days were trypsinized and replated at 10^6 cells per 10-cm plate. Cells were plated onto 24-well plates for growth curves (19) or into 10-cm dishes for 2, 7, 12 dichlorodihydrofluorescein diacetate (DCF) staining. Soft agar assays were done as described (22).

**Plasmids and Retroviral cDNA Libraries.** A mouse embryonic cDNA library was constructed from pooled RNA prepared from total mouse embryos at days 7 and 15, mouse embryo heads at day 15, and adult mouse brain and testis. The cDNAs were cloned into the pHgyro MarX II retrovirus vector (21). Full-length cDNAs for the brain-specific form of PGM (PGM-B, accession no. BC005661), GPI (NM008155), PGK1 (NM008828) and ENO1 (XM09407) were isolated by reverse transcription–PCR (RT-PCR) using total RNA as described previously (23). Twenty-four hours after addition of 1 g/mL doxycycline to the medium, cells were arrested whose extract was subject to PAGE assay and Western blotting.

**Small Interfering RNA Experiments.** All small interfering RNAs (siRNA) were purchased from Dharmacon Research Inc. (Lafayette, CO) (Supplementary Table A). Mouse and human PGM siRNAs were designed to target both muscle and brain forms. Mutant PGM siRNA with a single mismatch was used for all assays. Non-targeting siRNA was used as a control. siRNA transfections were performed according to the manufacturer’s protocol. Transfection efficiency was calculated by fluorescence-activated cell-sorting analysis. Semi-quantitative RT-PCR was done as below. ATP levels were measured by ATP bioluminescent somatic cell assay kit (Sigma) as described previously (24).

**Glycolytic Enzyme Assay and Flux Measurement.** The activities of PGM, GPI, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), PGK, and enolase were measured spectrophotometrically at 37°C as described (25). For the PGM assay, 20 μg of cell lysate were incubated with NADH (20 mM), ADP (10 mM), and 2,3-diphosphoglycerate (10 μM) in 50 mM sodium phosphate buffer, pH 7.5. Enzyme activity is defined as the formation of ADP at 340 nm per minute. The method used to determine glycolytic flux was based on the metabolism of [3-3H]glucose into water after the triose phosphate isomerase reaction (23). Cells were plated at 0.7 × 10^6 cells per 10-cm dish the day before analysis. Feeder-independent mouse embryonic stem cells were replated on gelatin-coated dishes 1 day before the assay. Eleven hours after transfection, cells were extracted for Western blotting. For Western blotting, cell lysates were prepared as described (19) and resolved on polyacrylamide gels. Immunoblotting was done using antibodies against Mdm2 (SMP14 from Santa Cruz Biotechnology, Santa Cruz, CA), actin (A5316 from Sigma), GFP (Roche, Indianapolis, IN), and ubiquitin (clone FK2 from Affinity Research, Plymouth Meeting, PA, which can detect ubiquitinated proteins but not free ubiquitin).

**Results**

**PGM Can Bypass Replicative and ras-Induced Senescence in MEFs.** Wild-type CD1 MEFs were infected at passage 4 with a mouse embryonic cDNA library in the pHgyro MarX II retrovirus vector (21). Human p53<sup>175H</sup> (a dominant negative allele, designated p53DN) in the same vector was used as a positive control to validate the screen (19). A total of 24 immortal clones were obtained from over 10<sup>7</sup> infected cells. Because the pHgyro MarX II vector has a loxp site that becomes duplicated in the long terminal repeats of the provirus, candidate retroviruses were recovered by treating the genomic DNA of each surviving clone with Cre recombinase and retested for their ability to immortalize primary MEFs. Among the rescued cDNAs that allowed bypass of senescence in this assay we found a full-length clone encoding PGM-M.

As shown in Fig. 1A, a retrovirus encoding PGM-M conferred unlimited proliferation on primary MEFs with an efficiency comparable to that of p53DN. Superinfection with a retrovirus encoding Cre recombinase, which will excise the integrated proviral DNA, reversed the senescence bypass in cells immortalized by PGM-M or p53DN, but had no effect on spontaneously immortalized MEFs (3T3 cells) infected with the empty pHgyro MarX II vector (Fig. 1B). This confirms that PGM-M immortalization of MEFs is a single genetic event. We also confirmed that PGM-M can immortalize MEFs from C57BL6 mice, excluding some genetic predisposition in the CD1 strain (data not shown). Both PGM- and p53DN-immortalized MEFs underwent a senescence-like arrest upon ectopic expression of p16<sup>ink4a</sup> or p19<sup>arf</sup>, implying that they had not sustained mutations in the effector pathways required for senescence (data not shown).

We also examined the ability of PGM-M to protect cells against premature senescence induced by oncogenic ras (28). Wild-type MEFs (at passage 6) rapidly arrested upon infection with a retrovirus encoding ras-Val12, whereas MEFs expressing either PGM-M or p53DN (at passage 8) continued to proliferate at different passages.

The levels of 8-OHdG were expressed as the number of 8-OHdG adducts per 1 million deoxyguanosine bases in the samples.

**RNA and Protein Analysis.** Total RNA was extracted from MEts and from adult muscle and brain using the Trizol reagent. Northern blotting was done using standard protocols (27). Probes specific for the M or B isoforms of PGM were prepared by RT-PCR.

Semi-quantitative RT-PCR was done by using SuperScript one-step RT-PCR kit (Invitrogen) as described (19). After DNase treatment, indicated RNA extracts were used as template. All primers designed are shown in Supplementary Table B.

**TGFP3-4, p53<sup>−/−</sup> MEFs bearing tetracycline-inducible p53GF, was used as described previously (27). Twenty-four hours after addition of 1 g/mL doxycycline to the medium, cells were arrested whose extract was subject to PAGE assay and Western blotting.

**p53<sup>−/−</sup> MEFs were transfected with various plasmids by Lipofectamine 2000 (Invitrogen) as follows: PGM-green fluorescent protein (GFP) (0.5 μg) and GFP (0.5 μg) with increasing amounts of p53 (0.5, 1, or 2 μg) or vector, Mdm2, or p53GF (1 μg). Total DNA amounts were adjusted by vector DNA up to 4 μg. Twenty-four hours after transfection, cells were extracted for Western blotting. For Western blotting, cell lysates were prepared as described (19) and resolved on polyacrylamide gels. Immunoblotting was done using antibodies against Mdm2 (SMP14 from Santa Cruz Biotechnology, Santa Cruz, CA), actin (A5316 from Sigma), GFP (Roche, Indianapolis, IN), and ubiquitin (clone FK2 from Affinity Research, Plymouth Meeting, PA, which can detect ubiquitinated proteins but not free ubiquitin).
vigorously in the presence of ras-Val12 (Fig. 1C). However, whereas oncogenic ras caused neoplastic transformation of the p53DN-expressing cells, as expected (22, 29, 30), the PGM-M-expressing MEFs did not acquire the ability to form colonies in soft agar, and are thus not fully transformed (Fig. 1D). These data suggest that PGM-induced immortalization is not equivalent to that conferred by loss of p53.

**PGM Activity Is Required for Immortalization of MEF.** In glycolysis, PGM catalyses the transfer of phosphate from 3-phosphoglycerate to 2-phosphoglycerate (Fig. 2A). The enzyme functions as a dimer and has been highly conserved throughout evolution. Mammals express two isoforms, designated as PGM-M (muscle form) and PGM-B (brain form) based on their relative abundance in these tissues (31). Northern blotting using cDNA probes for the two mouse isoforms indicated that PGM-B is much more abundant than PGM-M in cultured MEFs (Fig. 2B).

MEFs transduced with ectopic PGM-M showed relatively physiologic levels of expression, but MEFs immortalized by p53DN showed no significant up-regulation of PGM-M or PGM-B mRNA (Fig. 2B).

Mouse PGM-M is 43% and 53% identical to its counterparts in fission and budding yeast, respectively, and all of the catalytically important residues identified in the crystal structure of yeast PGM are conserved in mouse PGM-M and PGM-B (refs. 32, 33; Fig. 2C). In MEFs infected with retroviruses encoding either PGM isoform, the total PGM activity was increased approximately 2-fold relative to the endogenous levels in uninfected cells (Fig. 2C and data not shown). Importantly, PGM-B was also able to immortalize MEFs, although the cells showed consistently slower growth rates than parallel cultures expressing either PGM-M or p53DN (data not shown). These results imply that the total PGM activity is important for immortalization, rather than a specific attribute of the PGM-M isoform.

As further confirmation, we mutated critical residues in PGM-M that are required for catalytic activity. Residue R90 is required for catalytic activity, whereas R90, R116, and R117 are involved in substrate binding (32). These residues were individually mutated by PCR-based mutagenesis, as shown in Fig. 2C, and each mutant allele was transferred into the pHygroMarX II vector and used to infect early-passage MEF cells. All of the mutations were shown to ablate or substantially reduce the enhancement of PGM activity conferred by wild-type PGM-M (Fig. 2C). When the infected cells were passaged according to a 3T3 protocol, only the wild-type PGM-M resulted in efficient bypass of senescence, as shown by both increasing cell numbers (Fig. 2D) and senescent-associated β-galactosidase staining (data not shown). The R90Q, R116Q, and R117Q mutations were completely inactive in these assays. Thus, we concluded that PGM activity is required for immortalization of MEFs. Interestingly, the R116Q and R117Q mutations seemed to promote rather than alleviate senescence, as compared to MEFs infected with the empty vector control (Fig. 2D and data not shown).

Figure 1. PGM overcomes replicative and ras-induced senescence in MEFs. A, growth curves showing the effect of PGM-M and p53DN expression on replicative life span of CD1 MEFs. B, MEFs immortalized spontaneously (left) or by expression of p53DN (middle) or PGM-M (right) were infected with either control retrovirus (top) or a virus encoding Cre recombinase (bottom). After drug selection, cells were replated (10^6 cells per 10-cm dish) and proliferation was monitored by crystal violet staining after 8 days. C, growth curves of the indicated MEFs after infection with control or ras-Val12–expressing retroviruses. D, ability of p53DN- or PGM-immortalized MEFs to form anchorage-independent colonies following expression of ras-Val12. WT, wild-type.
Immortalization Correlates with Effects on Glycolysis.

Because the most likely consequence of excess PGM activity would be enhanced glycolysis, we investigated whether other glycolytic enzymes were capable of immortalizing MEFs. Ectopic GPI showed a similar effect on cell proliferation as PGM-M (Fig. 3A), and PGK reproducibly showed an intermediate effect with sustained cell proliferation but at a reduced rate compared with PGM-M. The other glycolytic enzymes tested had little if any effect, as judged by population doublings and senescent-associated h-galactosidase staining (Fig. 3A and data not shown).

A potential explanation for these differences is that the introduction of exogenous PGM-M and GPI resulted in a significant increase in glycolytic flux.

The production of $^3$H$_2$O from [3-$^3$H]glucose is catalyzed by aldolase and triose phosphate isomerase, several steps upstream of PGM (see Fig. 2A), but at glucose concentrations of <20 mmol/L, the rate of detrilation of [3-$^3$H]glucose is similar to that of lactate production and can therefore be used as an indicator of glycolytic flux (34). In passage 8 MEFs, ectopic expression of GPI or PGM-M increased the rate of glucose detrilation approximately 2-fold compared with control cells infected with the empty vector (Fig. 3B, filled bar). An analogous effect was noted on the ratio of lactate production to glucose consumption (Fig. 3B, open bar), suggesting that these enzymes were indeed capable of stimulating glycolytic flux. In contrast, phosphofructokinase and enolase had no impact on these parameters. The results imply a correlation between the ability of glycolytic enzymes to enhance glycolysis and their ability to bypass senescence in MEFs.

Inhibition of Glycolytic Enzymes Can Induce Senescence. If glycolysis can facilitate the bypass of senescence, we reasoned that inhibition of glycolysis should provoke premature senescence. Short interfering RNA oligonucleotides (siRNA) against mouse PGM, GPI, GAPDH and PGK were therefore tested for
their effect on the proliferation of early-passage MEFs. In the case of PGM, the siRNA was designed to target both isoforms. However, the existence of multiple isoforms precluded efficient knockdown of phosphofructokinase and enolase. Transfection efficiencies were ~30% (data not shown). Under these conditions, each siRNA reduced the respective endogenous mRNA level and enzymatic activity by ~40% to ~50% (Fig. 3C, bottom and top, respectively). Importantly, siRNA against PGM had no effect on the activity of GPI and vice versa (data not shown). One nucleotide substitution in PGM siRNA, designated mutant PGM siRNA (Supplemental Table A), abolished this effect (Supplemental Fig. C). Both control siRNAs, scramble, and fluorescein-labeled GL2 siRNA, showed no significant effect on each enzyme activity and cell growth (data not shown). Despite the relatively modest effects, the siRNA against either PGM or GPI inhibited proliferation (Supplemental Fig. A), reduced the cellular ATP content by 34% and 19%, respectively (data not shown), and induced premature senescence as assessed by senescent-associated β-galactosidase activity (Fig. 3D). Even 5 days after transfection, cells transfected with PGM- or GPI-siRNA displayed a highly senescent morphology (Supplemental Fig. B). In contrast, the siRNA against GAPDH had no effect on cell behavior despite reducing the activity of the enzyme. The partial effects seen with PGK siRNA are reminiscent of the intermediate effect of ectopic expression of the cDNA recorded in Fig. 3A. These observations are in line with the idea that modulation of some but not all glycolytic enzymes can influence cell proliferation via effects on glycolysis.

Enhanced Glycolysis in Spontaneously Immortalized MEF. We next asked whether enhanced glycolysis is a general feature of immortal cells. Primary MEFs were passaged on a 3T3 protocol until immortal segregants grew out after passage 12 (Fig. 4A, top). Interestingly, glycolytic flux, as measured by glucose detection, declined as the culture senesced and then rose again coincident with spontaneous immortalization (Fig. 4A, top). Total PGM activity increased approximately 2-fold in the immortal segregants, analogous to the increase observed upon ectopic expression of PGM-M in Fig. 2C, and a similar trend was noted for PGK and enolase activity (Fig. 4A, bottom). In nine separate 3T3 MEF cultures allowed to undergo spontaneous immortalization, PGM activity was invariably elevated relative to the senescent population (data not shown).

To further investigate the apparent correlation between enhanced glycolysis and immortality, we measured glucose detritiation and total PGM activity in the CGR8 mouse embryonic stem cell line. These cells are feeder cell–independent and proliferate indefinitely without apparent genetic damage. As shown in Fig. 4B, CGR8 cells showed enhanced glycolytic flux and concomitant elevation in total PGM activity relative to early-passage MEFs. Embryonic stem cells also display an elevated lactate/glucose ratio. Similar results were obtained with the DE3 strain of mouse embryonic stem cells (data not shown).
Potential Links between PGM and p53 Function. Because spontaneous immortalization of MEFs generally involves disruption of the p53 pathway (17), and p53DN enables MEFs to escape senescence (Fig. 1A), we were interested in exploring the mechanisms that might connect p53 function with PGM activity. MEFs expressing p53DN showed a relatively modest but significant increase in PGM activity compared with vector-only controls (Fig. 5A). This was accompanied by similar increases in glucose detritiation and lactate production. In a reciprocal context, tetracycline regulated expression of wild-type p53 (GFP tagged) in the TGP53-4 mouse cell line (27) caused a significant decline in endogenous PGM activity (Fig. 5B). Immunoblotting confirmed the induction of p53-GFP and its ability to activate expression of Mdm2.

These results suggest that p53 is able to down-regulate PGM, but we did not observe a change in PGM mRNA levels following the introduction of p53DN (Fig. 2) or wild-type p53 (data not shown). To further investigate these effects, we cotransfected p53−/− MEFs with a GFP-tagged PGM (PGM-GFP) and increasing amounts of p53. Under these conditions, we observed a marked and dose-dependent reduction in the level of PGM-GFP (Fig. 5C) consistent with reduced translation or stability. In line with this idea, we noted that ectopic expression of PGM-GFP in wild-type MEFs resulted in the appearance of a higher molecular weight form of the protein (Fig. 5D, asterisk). This slower migrating form was detected more clearly after N-acetyl-L-leucinyl-L-leucinyl-norleucinal (a proteasome inhibitor) treatment of the cells (data not shown). When immunoprecipitated via the GFP epitope, the higher molecular weight form was detectable by immunoblotting with an antibody against ubiquitin (Fig. 5D). However, PGM levels were unaffected by either Mdm2 or p53RFP, two ubiquitin ligases that are known to be regulated by p53 (refs. 35, 36; data not shown).

Oxidative DNA Damage in Immortalized MEFs. It is now appreciated that under standard conditions of tissue culture (i.e., in 20% oxygen), oxidative stress has a major impact on cellular life span. Growing cells under reduced-oxygen conditions can delay senescence and it has recently been reported that MEFs are essentially immortal and show less oxidative DNA damage if propagated in 3% oxygen (13). We hypothesized that under normoxic conditions, enhanced glycolysis by ectopic PGM or GPI might protect MEFs from effects of oxidative damage.

To evaluate this possibility, we subjected MEFs infected with the empty vector control or with retroviruses encoding PGM-M, p53DN, and CRG8 (mouse embryonic stem cells) to stress induced by hydrogen peroxide. CRG8 cells proved to be remarkably resistant to oxidative stress, showing only marginal effects on viability at hydrogen peroxide concentrations as high as 100 μmol/L (Fig. 6A). In comparison, MEFs showed severe loss of viability even at low concentrations of hydrogen peroxide. MEFs expressing PGM-M or p53DN were significantly more resistant to hydrogen peroxide than the controls infected with the empty vector (Fig. 6A).

We also compared cytosolic reactive oxygen species production in these cells by staining with DCF. As previously reported (18), the p53DN-expressing cells showed reduced DCF staining (Fig. 6B). The PGM-M- or GPI-expressing MEFs also had significantly less DCF staining than vector control cells at passage 8 and passage 12 (Fig. 6B; Supplemental Fig. D and data not shown). These results indicate that PGM or GPI expression can reduce reactive oxygen species production in the cells during serial culture. Because oncogenic ras can also stimulate reactive oxygen species production (15), we examined the effect of oncogenic ras on reactive oxygen species production in PGM or wild-type MEFs. We found that oncogenic ras induced less DCF staining in PGM-M MEFs compared with wild-type MEFs (Fig. 6C), in line with our findings that PGM-immortalized MEFs are resistant to arrest by oncogenic ras (Fig. 1C). To directly assess whether these effects could protect the cells from oxidative damage, we compared the levels of 8-OHdG, which is one of the most abundant types of DNA lesions caused by oxidative damage (37), in the genomic DNA by HPLC (Fig. 6D). The vector control cells showed an ~2-fold
Figure 5. p53 regulates PGM activity and protein levels. A, MEFs infected with a retrovirus encoding p53DN show increased PGM activity (left) accompanied by increased glucose detritiation (right, filled bar) and lactate production (right, open bar) relative to the empty vector controls. B, TGP53-4 cells (containing a tetracycline-inducible p33GFP construct) were grown with or without 1 μg/mL doxycycline, and total cellular PGM activity was determined (left). Levels of p53GFP protein, Mdm2 (a p53 target gene), and actin (loading control) were assessed by immunoblotting (right). C, MEFs derived from p53−/− mice were transfected with a plasmid encoding PGM-GFP together with increasing amounts of similar vector encoding p53 or empty vector. GFP was included as a control for transfection and loading. Levels of PGM-GFP were assessed by immunoblotting with an antibody against GFP. D, ubiquitination of PGM protein. CD1 MEFs were infected with either control or PGM-GFP expressing retroviruses. Whole-cell extracts and GFP immunoprecipitates were analyzed by SDS-PAGE followed by immunoblotting with antibodies (ab.) against GFP, actin, or ubiquitin.* ubiquitinated forms of PGM-GFP (top or bottom), which are completely superimposable. WB, Western blotting; IP, immunoprecipitate.

increase in 8-OHdG levels between passage 8 and passage 12, consistent with the accumulation of oxidative DNA damage as cells approach senescence (Fig. 6D). Introduction of PGM or GPI did not prevent the accumulation of 8-OHdG but reduced the levels by about 2-fold at each passage. Taken together, these data are consistent with the notion that PGM or GPI can protect cells from the senescence effect of oxidative damage under normal oxygen culture conditions.

Discussion

Here we have established that early-passage MEFs have high glycolytic activity, which declines during replicative senescence and is reestablished during spontaneous immortalization. That this pattern is more than correlative is evidenced by the identification of PGM-M in a genetic screen for cDNAs that overcome senescence (Fig. 5). However, as PGM cannot overcome a cell cycle arrest induced by ectopic p53 (41), but we did not find evidence for transcriptional control of PGM-M or PGM-B in MEFs (Fig. 2B and data not shown). We did find a potential link between PGM protein level and p53a function (Fig. 5). However, as PGM cannot overcome a cell cycle arrest induced by ectopic p53 (data not shown), the role of p53 in senescence cannot be explained solely by effects on PGM down-regulation.

Our observations can be encompassed by a simple hypothesis that incorporates much other experimental support. A correlation between glycolysis, reactive oxygen species, and cell proliferation has been clearly established in human thymocytes (42). Similarly, it has been shown that MEFs in culture at 20% O2 are subject to oxygen poisoning and senescence can be avoided by growing most MEF strains in 3% O2 (13). Switching cells from 3% to 20% O2 conditions can promote senescence (13), and in human fibroblasts senescence can be mimicked experimentally by exposing cells to low doses of hydrogen peroxide (14, 43). We propose that the high glycolytic rate of early-passage MEFs partially protects against oxidative damage, but that oxidative stress eventually inhibits glycolysis. Immortalization of MEFs...
thus follows glycolytic activation, which can be mediated directly with PGM or GPI, indirectly with p53DN, or as a consequence of spontaneous abrogation of the p53 pathway. Glycolytic activation protects from oxidative damage, and we hypothesize that the constitutively active glycolytic pathway observed in embryonic stem cells at atmospheric O2 conditions contributes to their immortal characteristics even though they present a robust p53 response to DNA damage (44, 45).

Although it is widely held that tumors are highly glycolytic, even in 20% O2 conditions in vitro, because they have adapted to survive in relatively anoxic conditions in vivo, we suggest rather the contrary. Mitogenic stimulants and activated oncogenes, such as ras-Val12, produce reactive oxygen species that are thought to be functionally important for the downstream signaling mechanisms (46, 47). Glycolytic activation may therefore protect against oxidative damage caused by reactive oxygen species production of mitogenic bombardment in vivo. In effect, the tumor can resist damaging free radical production. Thus, in a small or well-vascularized tumor, glycolytic activation may be an early and essential step in tumorigenesis. A tumor that subsequently becomes anoxic by outgrowing its vasculature is also presumably well served by active glycolysis.

At present the Warburg effect is exploited clinically for tumor imaging by positron-emission tomography scanning of 2-[18F]fluoro-2-deoxy-D-glucose (5). Because tumors, whether anoxic or not, are likely to be highly glycolytically dependent (48), it may be worth exploring antiglycolytic agents that do not cross the blood-brain barrier as potential cancer therapeutics. Although it has been suggested that the activity of complex metabolic pathways cannot be modulated by the abundance of a single component enzyme (49), our studies reveal the unexpected ease with which the glycolytic flux of mammalian cells can be genetically manipulated, at least in MEFs. Genetic or pharmaceutical modulation of the glycolytic pathway might also have an effect on animal life span, as our data support the rate-of-living oxygen radical theory of cellular and organismal aging (50). Of more immediate practical impact is the potential to develop immortal mouse cell lines in which the p53 and pRb pathways have been functionally preserved and to further dissect the links between p53, PGM, and glycolysis.

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Figure 6. Enhanced glycolysis protects cells from oxidative damage. A, cell viability after exposure to H2O2 for 24 hours as measured by trypan blue exclusion. B, representative result of DCF staining in MEFs expressing vector, p53DN or PGM-M at passage 8. C, DCF staining in PGM expressing MEF is less affected by ras overexpression than in wild-type MEF. D, expression of PGM-M and GPI protects MEFs from oxidative DNA damage. The amount of 8-OHdG in the genomic DNA from the indicated MEFs was measured by HPLC at passage 8 and 12. The values are expressed as the number of 8-OHdG lesions per 1 million guanine residues.
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