Metabolic analysis of senescent human fibroblasts reveals a role for AMP in cellular senescence

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Cellular senescence is considered a major tumour-suppressor mechanism in mammals, and many oncogenic insults, such as the activation of the *ras* proto-oncogene, trigger initiation of the senescence programme. Although it was shown that activation of the senescence programme involves the up-regulation of cell-cycle regulators such as the inhibitors of cyclin-dependent kinases p16INK4A and p21CIP-1, the mechanisms underlying the senescence response remain to be resolved. In the case of stress-induced premature senescence, reactive oxygen species are considered important intermediates contributing to the phenotype. Moreover, distinct alterations of the cellular carbohydrate metabolism are known to contribute to oncogenic transformation, as is best documented for the phenomenon of aerobic glycolysis. These findings suggest that metabolic alterations are involved in tumourigenesis and tumour suppression; however, little is known about the metabolic pathways that contribute to these processes. Using the human fibroblast model of *in vitro* senescence, we analysed age-dependent changes in the cellular carbohydrate metabolism. Here we show that senescent fibroblasts enter into a metabolic imbalance, associated with a strong reduction in the levels of ribonucleotide triphosphates, including ATP, which are required for nucleotide biosynthesis and hence proliferation. ATP depletion in senescent fibroblasts is due to dysregulation of glycolytic enzymes, and finally leads to a drastic increase in cellular AMP, which is shown here to induce premature senescence. These results suggest that metabolic regulation plays an important role during cellular senescence and hence tumour suppression.

Key words: AMP, fibroblast, glycolysis, metabolism, senescence, tumour suppression.

INTRODUCTION

Cellular senescence of HDF (human diploid fibroblasts) is a current model [1], which has revealed many fundamental processes underlying cellular aging [2], and premature senescence in response to either oncogene expression or environmental stress is now considered a major tumour-suppressor mechanism [3]. To immortalize mammalian cells, a series of molecular events is required that co-operates to break the senescent phenotype (for review, see [4]). While these observations are the basis of our current knowledge of cellular senescence, a systematic analysis of senescence-associated changes in the cellular phenotype may reveal additional factors relevant for tumour suppression and immortalization.

Substantial evidence suggests that the metabolic rate influences processes of cellular aging and senescence [4], and it is also clear that metabolic parameters are critically involved in the ability of tumour cells to survive and proliferate *in vivo* (for review, see [5]). The fact that the lifespan of many species can be extended through caloric restriction (for review, see [6]) suggests a critical role for alterations of carbohydrate metabolism in the control of regulatory processes that influence proliferation and survival. This effect is also visible at the single-cell level, as shown by the observation that caloric restriction, mediated by a reduction of the available glucose in culture medium, extends lifespan in yeast, thereby delaying age-associated growth arrest and apoptosis [7]. These studies have established a new concept, according to which changes in the carbohydrate metabolism, and in particular regulation of glycolytic energy production, contribute to the control of cell proliferation and survival. Since lifespan extension through caloric restriction is conserved throughout evolution, it will be of interest to determine changes in the metabolism of human cells which contribute to cellular senescence, as was observed in yeast cells. Using the well-established *in vitro* senescence model of HDF, we have determined changes in the glycolytic pathways of HDF, as a first step to analyse senescence-associated changes in cellular carbohydrate metabolism. These experiments revealed a drastic de-regulation of the carbohydrate metabolism in senescent cells, characterized by an imbalance of glycolytic enzyme activities and the failure to maintain ATP (and other NTP) levels. This leads to an up-regulation of AK (adenylate kinase) activity and the levels of AMP, which is shown here to act as a growth-suppressive signal that induces premature senescence.

EXPERIMENTAL

Cell culture

Normal diploid fibroblasts were isolated from human foreskin [8] and cultured in Dulbecco's modified Eagle's medium (Gibco Life Technologies, Vienna, Austria), supplemented with

Abbreviations used: HDF, human diploid fibroblasts; LDH, lactate dehydrogenase; PK, pyruvate kinase; M2-PK, M2 subtype of PK; FBP, fructose 1,6-bisphosphate; PEP, phosphoenolpyruvate; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; MDH, malate dehydrogenase; AK, adenylate kinase; AMPK, AMP-activated protein kinase; PRPP synthetase, 5-phosphoribosyl-1-pyrophosphate synthetase; SA-β-gal, senescence-associated β-galactosidase; AICAR, 5-aminoimidazole-4-carboxamide 1-β-D-ribofuranoside; BrdU, 5-bromo-2′-deoxyuridine.

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penicillin/streptomycin solution (Gibco Life Technologies) and 10 % fetal calf serum (PAA, Linz, Austria). The cells were subcultured in an atmosphere of 5 % CO_2 (pH 7.7) at 37 °C by passaging them at a ratio of 1:5 in regular intervals. At later passages, the splitting ratio was reduced to 1:3 and then 1:2. Cells were passaged such that the monolayers never exceeded 70–80 % confluency. The number of population doublings were estimated using the following equation: $n = (\log_{10}F - \log_{10}I)/0.301$, where n is the number of population doublings, F is the number of cells at the end of one passage and I is number of cells that were seeded at the beginning of one passage. After roughly 55 population doublings, the cells reached growth arrest. The senescence status was verified by *in situ* staining for SA-β-gal (senescence-associated β-galactosidase) as described in [9]; 90–100 % of the cells at 55 population doublings stained positive for SA-β-gal.

Inhibition of cell proliferation by external nucleotides

Cells were seeded in cell culture medium as described above. After 5 h, the medium was replaced by medium containing 3 mM 5′-IMP, 5′-GMP, 5′-AMP and AICAR (5-aminoimidazole-4-carboxamide 1-β-D-ribofuranoside; all from Sigma). DNA synthesis was assessed using a BrdU (5-bromo-2′-deoxyuridine) Labelling and Detection Kit (Roche, Vienna, Austria) as described in [10]. To this end, 72 h post-treatment, cells were incubated with BrdU for 24 h. Subsequently BrdU-positive nuclei were counted (minimum 300 cells/assay).

Gel-permeation analysis of glycolytic enzymes

Cells were extracted in a lysis buffer containing 100 mM Na_2HPO_4/NaH_2PO_4, 1 mM dithiothreitol, 1 mM NaF, 1 mM 2-mercaptoethanol, 1 mM ε-aminocaproic acid, 0.2 mM PMSF and 10 % glycerol, pH 7.4. The extracts were passed over a gel-permeation column (Amersham Biosciences) and the activities of the different enzymes were determined in the fractions as described in [11].

Detection of PK (pyruvate kinase) isoforms by cellulose acetate electrophoresis

Cells were extracted in a lysis buffer (0.1 M potassium phosphate, pH 7.3, 50 mM NaCl, 1 mM dithiothreitol, 1 mM ε-aminocapronic acid, 1 mM 2-mercaptoethanol and 0.2 mM PMSF) and separated by cellulose acetate electrophoresis on a solid support, as first described in [12] and modified in [13]. To visualize PK isoforms, the cellulose acetate sheet was placed on top of an agarose gel, and PK activity detected by an in-gel coupled LDH (lactate dehydrogenase)/PK assay, using NADH as the substrate. The reaction product NAD was detected densitometrically at 366 nm.

Isoelectric focusing

Cells were extracted with homogenization buffer containing 10 mM Tris, 1 mM NaF and 1 mM 2-mercaptoethanol, pH 7.4. Isoelectric focusing was carried out with a linear gradient of glycerine (50–0 %, v/v) and ampholines (pI 3.5–10.5) as described previously [11,14].

Flux measurements

Metabolic flux rates depend strongly on cell density. To measure the flux rates at different cell densities, cells were grown to different cell densities (low, middle and high) before cell-culture supernatants were collected and the metabolites measured as described in [14]. The metabolic concentrations were determined at the beginning of the experiment and at regular time points after the change of medium. These raw data were used to calculate the consumption and production rates for individual metabolites.

Nucleotide and metabolite measurements

The cells were extracted with 0.6 M $HClO_4$. FBP (fructose 1,6-bisphosphate) and PEP (phosphoenolpyruvate) were determined enzymically as described in [14]. Nucleotides were measured via reversed-phase ion-pair liquid chromatography as described in [15]. In the experiment shown in Figure 4 (see below), AMP levels were determined enzymically [16].

Preparation of cellular extracts and Western blotting

To prepare whole-cell extracts, cells were trypsinized from culture flasks, washed with PBS and lysed for 30 min on ice in a buffer containing 50 mM Tris/HCl, pH 8, 300 mM NaCl, 1 % Nonidet P-40, 0.5 % desoxycholate, 0.1 % SDS, 1 mM NaF and one Complete Mini EDTA-free Protease Inhibitor Cocktail Tablet (Roche). The lysates were centrifuged at 20 000 ***g*** for 10 min at 4 °C, and the supernatants separated on 10 % SDS/polyacrylamide gels. The amounts of extract loaded corresponded to equal cell quantities. After electrophoresis, the proteins were transferred to PVDF membranes by wet electroblotting and processed as described in [10]. Immunoreactive proteins were detected using an enhanced chemiluminescence system (Amersham Life Science, Braunschweig, Germany). The following antibodies were used for Western blot analysis: polyclonal goat anti-LDH (Rockland Immunochemicals, Gilbertsville, PA, U.S.A.), monoclonal mouse anti-M2-PK (ScheBo Biotech AG, Giessen, Germany; M2-PK is the M2 subtype of PK) and polyclonal goat anti-hexokinase II (Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.); the following secondary antibodies were used: horseradish peroxidase-conjugated anti-mouse IgG (Promega, Vienna, Austria) and horseradish peroxidase-conjugated anti-goat IgG (Dako, Glostrup, Denmark).

Statistical analysis

For the glycolytic, glutaminolytic and serinolytic flux measurements statistical analyses were performed using the statistical program package BMDP. For the comparison of the different cell groups two-way analysis of covariance with cell density was performed. In all other cases Student's t test was employed.

RESULTS

Aerobic glycolysis is strongly decreased in senescent fibroblasts

To study changes in cellular energy metabolism associated with cellular senescence, we determined the consumption of the key nutrients and production of various metabolites in young and senescent HDF (Table 1). We found a significant increase in the consumption of glucose, pyruvate and serine as well as an increased production of lactate, alanine and glutamate in senescent cells, while no significant change was observed for glutamine consumption. These findings would suggest that glycolysis and serine consumption are considerably up-regulated in senescent cells, whereas glutaminolysis is not affected during cellular senescence. Since young cells are actively proliferating and senescent

Table 1 Metabolic fluxes in young and senescent HDF

The production and consumption of nutrients and metabolic end products was determined for young (15 population doublings) and senescent (52 population doublings) HDF through a dynamic analysis of the respective concentrations in cellular supernatants, as described in [21]. Means ± S.D. are shown; $n_{young} = 5$, $n_{senescent} = 10$; n.s., not significant.

	Flux (nmol/h × 10^5 cells)		
	Young HDF	Senescent HDF	Significance
Glucose consumption	41 ± 3	104 ± 43	$P < 0.05$
Lactate production	94 ± 22	240 ± 81	$P < 0.01$
Pyruvate consumption	15 ± 2	31 ± 4	$P < 0.001$
Alanine production	1.6 ± 1.4	5.6 ± 1.6	$P < 0.001$
Serine consumption	10.0 ± 7.6	18 ± 5.2	$P < 0.05$
Glutamine consumption	1.2 ± 58	25 ± 181	n.s.
Glutamate production	3.9 ± 1.9	8.9 ± 4.1	$P < 0.05$

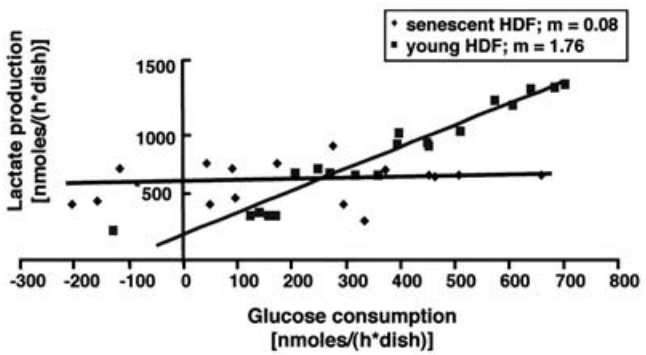


Figure 1 Conversion of glucose and pyruvate to lactate in young and senescent HDF

For young and senescent HDF, the consumption of glucose and production of lactate was determined, as described in [28]. Cells were seeded at different densities. Negative values for glucose consumption indicate the occurrence of gluconeogenesis in fibroblasts, in accordance with the findings in [55]. Negative values for lactate production suggest that under certain conditions cells consume rather than produce lactate.

cells are growth-arrested, these findings are surprising, because proliferating cells generally exhibit a highly active glycolytic metabolism as compared with arrested cells [17].

To elucidate further whether the high amounts of glucose used in old cells are in fact channelled into glycolysis or used for other pathways, the actual metabolic flux rates were reconstructed from a dynamic analysis of the consumption and production of the key nutrients and metabolites, respectively. The slopes of the regression lines were used to determine the relative amount of lactate that is produced from glucose in each case. As shown in Figure 1, the production of lactate depends strongly on the consumption of glucose in young cells (slope, 1.8), whereas in senescent cells lactate production is only marginally dependent on glucose consumption (slope, 0.08). As deduced from the data in Figure 1, about 90 % of the consumed glucose is converted into lactate in young cells, a process that yields a net gain of 2 mol of ATP/mol of glucose. Thus aerobic glycolysis, characterized by a high rate of lactate production from glucose in the presence of oxygen [18], prevails in young HDF. The remaining 10 % of input glucose may be used for ATP production in the mitochondria or for biosynthetic processes, such as nucleic acid synthesis or the production of NADPH. In marked contrast to young fibroblasts, only a minor proportion of the glucose is converted into lactate in senescent cells (Figure 1), suggesting that most of the input glucose is used for other purposes in senescent HDF.

Table 2 Activities of glycolytic enzymes in young and senescent fibroblasts

Extracts were prepared from young and senescent cells as indicated. In the extracts, the activities of various glycolytic enzymes were determined as described in [21]. Means ± S.D. ($n = 3$) are shown. The ratio of enzyme activities between senescent and young cells is also given. n.s., not significant; G6PDH, glucose-6-phosphate dehydrogenase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PGK, phosphoglycerate kinase; PGM, phosphoglycerate mutase.

Enzyme (m-units/10^7 cells)	Young HDF	Senescent HDF	Senescent/young	Significance
Hexokinase	50 ± 11	315 ± 143	6.3	$P < 0.05$
G6PDH	248 ± 83	1011 ± 249	4.1	$P < 0.01$
GAPDH	405 ± 101	349 ± 132	0.86	n.s.
PGK	398 ± 18	1379 ± 298	3.5	$P < 0.01$
PGM	1042 ± 41	3817 ± 755	3.7	$P < 0.01$
Enolase	1203 ± 126	1630 ± 350	1.4	n.s.
LDH	11160 ± 1278	44344 ± 8318	4.0	$P < 0.01$
Adenylate kinase	358 ± 44	2027 ± 532	5.7	$P < 0.01$

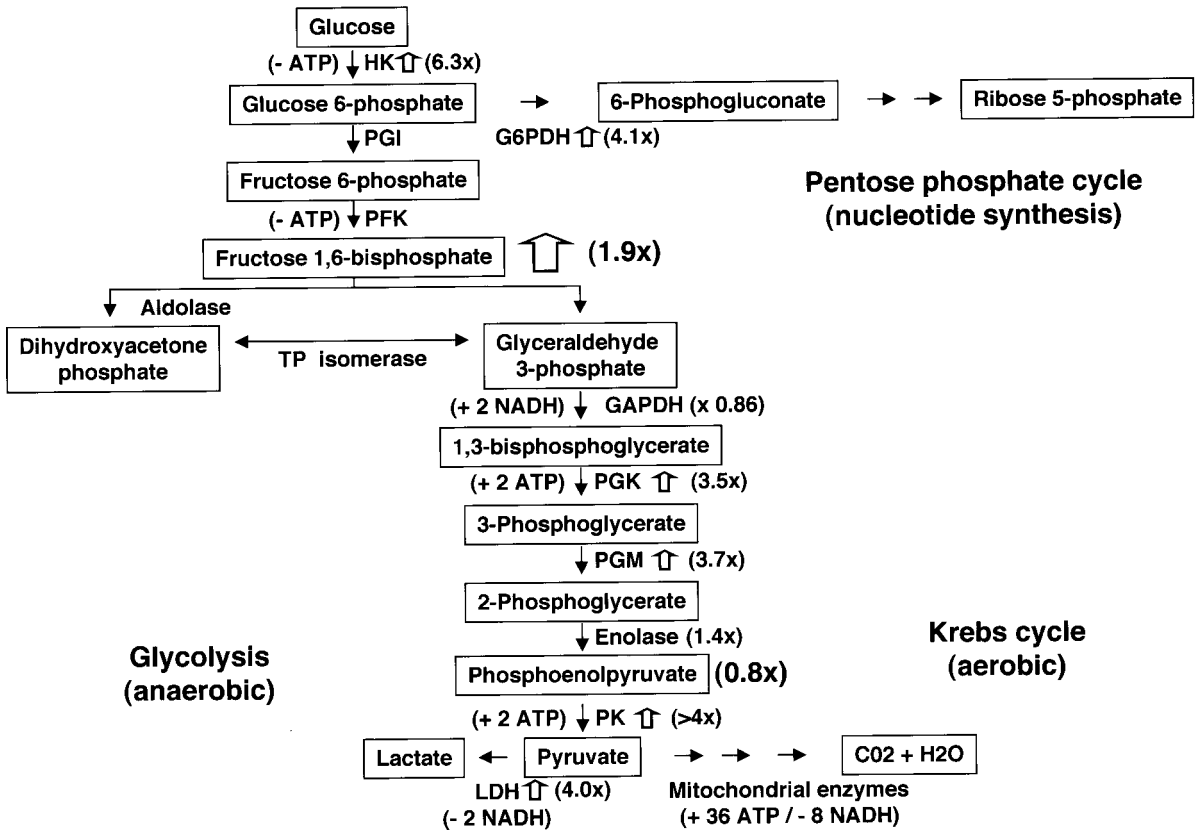
The data indicate that, while young fibroblasts display substantial aerobic glycolysis, the flux of metabolites from glucose into lactate is interrupted in senescent fibroblasts at a point which remains to be defined. The reduced glycolytic conversion of glucose into lactate, as observed in senescent human fibroblasts, is reminiscent of similar changes observed in aging yeast cells, which were shown to switch metabolism from glycolysis to increased energy storage and gluconeogenesis [19]. In muscle, a substantial amount of pyruvate generated by glycolysis is released into the blood as alanine, along with lactate. Our finding, that alanine production is significantly increased by senescent fibroblasts (Table 1), suggests that age-dependent changes in the metabolism include a channelling of glucose into alanine production.

Enzyme activities and glycolytic metabolites

To determine the molecular basis for the observed changes of glycolysis during cellular senescence, we analysed the activity of the glycolytic enzymes in extracts obtained from young and senescent human fibroblasts. When selected glycolytic enzymes were measured in young and senescent HDF, we observed a significant increase in the specific activity of hexokinase, phosphoglycerate kinase, phosphoglycerate mutase (Table 2; see also Scheme 1) and PK.

While it was shown that overexpression of oncogenic Ras in normal cells induces premature senescence [20], we found previously that Ras-induced growth arrest leads to an up-regulation in the activity of several glycolytic enzymes, most notably a conversion of M2-PK from its dimeric form into tetramers, with much higher substrate affinity [21]. Several isoforms of PK are known in human tissue, and human skin fibroblasts express two isoforms derived from the M-type gene, referred to as the M1 and M2 isoenzymes, respectively (reviewed in [22]). Previous work has established a critical role of M2-PK during cell proliferation. Specifically, M2-PK is the embryonic form, expressed in all growing cells and, in particular, in tumour cells [23,24]. M2-PK can be allosterically regulated and occurs in two conformations: a tetrameric enzyme with high affinity for its substrate PEP, and a dimeric enzyme with strongly reduced substrate affinity. In tumour and normal growing cells, the dimeric form of M2-PK is predominant [23].

To investigate changes in PK isoforms during replicative senescence, cells were lysed and PK isoforms characterized. As shown by cellulose acetate electrophoresis, both young and



Scheme 1 Alterations of glycolytic pathways in senescent fibroblasts

Metabolization of glucose through various metabolic intermediates is shown. Depending on the cellular enzyme apparatus, glucose can be used for energy production by glycolysis, energy production by mitochondrial respiration or for the production of cellular constituents, such as nucleotides by the pentose phosphate cycle (in growing cells). Glycolytic enzymes catalysing each conversion are indicated. Changes in the activity of selected glycolytic enzymes and in the intracellular concentration of selected metabolites, which were observed in senescent cells, are indicated by arrows and numbers. HK, hexokinase; PGI, phosphoglucose isomerase; PFK, phosphofructokinase; G6PDH, glucose-6-phosphate dehydrogenase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PGK, phosphoglycerate kinase; PGM, phosphoglycerate mutase.

senescent cells express predominantly (>80% of total PK protein) the M2 isoform of PK with a small contribution from the M1 isoform. When the status of M2-PK was analysed by gel filtration, we found that in asynchronously growing young cells M2-PK is distributed evenly between the dimeric form and the tetrameric form. In marked contrast, M2-PK isolated from senescent cells is predominantly in the tetrameric form (Figure 2a), and this is accompanied by a strong increase in its catalytic activity. To determine whether increased catalytic activities are associated with increased protein expression, the abundance of glycolytic enzymes was analysed by immunoblotting. In these experiments we found a considerable up-regulation in the abundance of M2-PK, hexokinase and LDH (Figure 2b). The up-regulation of glycolytic enzymes, and in particular M2-PK, can be expected to considerably increase the flux from glucose to lactate in senescent cells, given the well-established regulatory function of M2-PK [23]. In contrast to this assumption, we even observed a drastic reduction of the glycolytic flux in senescent fibroblasts (Figure 1), suggesting that additional changes in the glycolytic pathway may have occurred.

Strikingly, the activity of GAPDH (glyceraldehyde-3-phosphate dehydrogenase) was not increased (Table 2). Similarly, we observed no up-regulation of enolase activity in senescent cells (Table 2). These findings raise the possibility that the failure of senescent cells to maintain aerobic glycolysis (Figure 1) may be due to their inability to co-ordinately regulate the activity of the glycolytic enzymes (Scheme 1). However, the molecular mechanisms underlying deregulation of glycolytic enzyme activities in senescent HDF remain obscure. In order to address the nature of the changes in glycolytic pathways, we decided to determine the steady-state levels of some key metabolites in the cellular aging model used here. To analyse if altered enzyme activities, as determined in cellular extracts, are reflected by corresponding changes within the cells, the intracellular concentrations of FBP and PEP were determined. We found a 2-fold increase in the steady-state level of fructose 1,6-bisphosphate in senescent cells (Table 3), which may result from both the increased activity of hexokinase (and possibly phosphofructokinase) and the decreased activity of GAPDH. The level of PEP was not up-regulated in senescent cells, probably reflecting the decreased activity of GAPDH and the increased activity of M2-PK. These results confirm that the reduced activity of GAPDH (and probably enolase) leads to a selective increase in the C_6-phosphometabolite pools, as shown here for FBP.

During glycolysis, 2 mol of ATP are used by hexokinase and 6-phosphofructo-1-kinase to produce FBP from glucose, and 4 mol of ATP (or GTP) are generated in subsequent steps by phosphoglycerate kinase and PK (Scheme 1). The energy-consuming and energy-producing steps of glycolysis are connected by GAPDH, which catalyses the oxidation of glyceraldehyde 3-phosphate to 1,3-bisphosphoglycerate. We found that in senescent cells a general up-regulation of glycolytic enzymes is

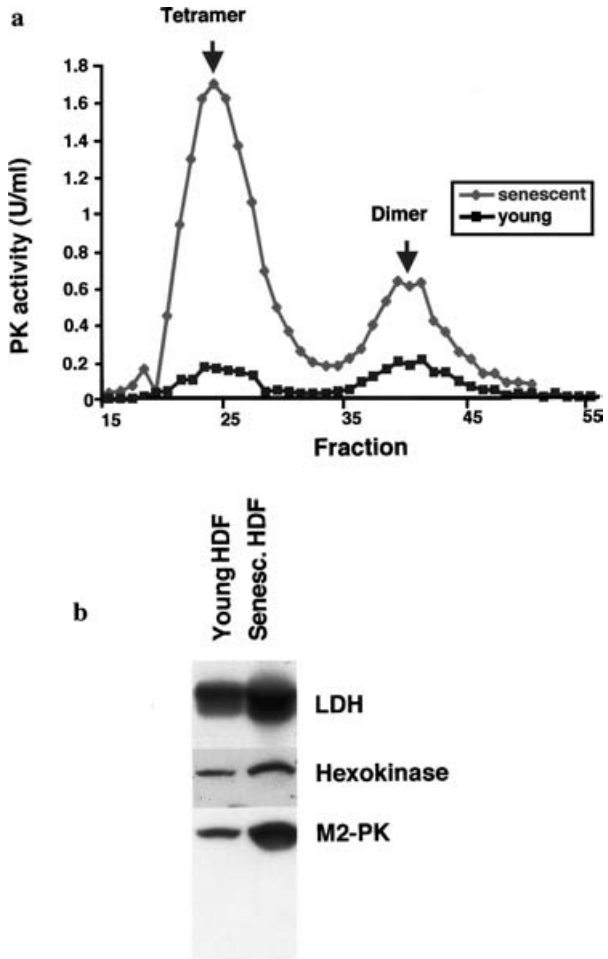


Figure 2 Up-regulation of glycolytic enzymes in senescent fibroblasts

(**a**) Accumulation of tetrameric M2-PK in senescent HDF. Extracts were prepared from young and senescent HDF and separated by gel filtration, as described in [21]. M2-PK activity was determined in individual fractions at various PEP concentrations. Here we show the activity of M2-PK at 2 mM PEP. The positions of the M2-PK tetramer and M2-PK dimer are indicated. (**b**) Immunoblot analysis. Extracts were prepared from young and senescent HDF, as indicated. Extracts were analysed for the abundance of M2-PK, LDH and hexokinase by Western blotting. PFF, primary foreskin fibroblasts. In both panels the same number of cells was used to prepare each extract.

accompanied by the failure to up-regulate GAPDH and enolase activity. This observation raises the possibility that this constellation may result in difficulties in maintaining the ATP/GTP levels. To test this prediction, we analysed the intracellular levels of GTP and ATP in young and senescent fibroblasts, revealing a strong down-regulation of both the ATP and GTP levels in senescent cells (Table 3). Taken together, the data suggest that a significant amount of the available ATP is used for the upper branch of glycolysis (by hexokinase and 6-phosphofructo-1-kinase) to generate high levels of FBP. The use of FBP for subsequent energy production is hampered by the decreased activity of GAPDH, and the energy derived from ATP is probably trapped in the form of phosphometabolites, such as FBP, as was described in other systems [25,26]. However, the ATP levels decreased by nearly 100 nmol/mg of protein and the increase in FBP is only 0.8 nmol/mg, suggesting that other effects contribute to ATP depletion. Indeed, others have reported an increase in adenosine and inosine release in aging human fibroblasts [27].

Table 3 Levels of intracellular metabolites in young and senescent HDF

The levels of the various metabolites were determined enzymically in extracts from young and senescent HDF, as described in [21]. Nucleotides were measured via reversed-phase ion-pair liquid chromatography; means ± S.D. ($n = 5$) are shown. For each metabolite, the ratio observed between senescent and young cells is also given. n.s., not significant.

Metabolite (nmol/mg of protein)	Young HDF	Senescent HDF	Senescent/young	Significance
FBP	0.80 ± 0.59	1.56 ± 0.17	2.0	$P < 0.05$
PEP	1.56 ± 0.78	1.23 ± 0.17	0.8	n.s.
ATP	152 ± 70	50 ± 21	0.33	$P < 0.05$
GTP	26 ± 10.6	9.1 ± 3.7	0.35	$P < 0.01$
NAD	7.9 ± 0.5	9.1 ± 1.0	1.15	$P < 0.05$
NADH	0.037 ± 0.006	0.079 ± 0.056	2.1	n.s.
UTP	84 ± 23	34.0 ± 0.1	0.4	$P < 0.01$
CTP	32 ± 11	16.2 ± 2.8	0.51	$P < 0.05$
IMP	0.15 ± 0.21	0.24 ± 0.26	1.6	n.s.
AMP	0.3 ± 0.7	2.9 ± 1.0	9.7	$P < 0.05$
ADP	21 ± 0.3	14 ± 2	–	n.s.
ATP/ADP	8.2 ± 5.0	3.6 ± 1.6	–	n.s.

Characterization of hydrogen shuttles

During glycolysis, energy is also stored in the form of $NADH/H^+$, which could be recycled to NAD^+ by cellular hydrogen shuttles, known as the malate–aspartate shuttle and glycerol 3-phosphate shuttle, respectively. Thereby, hydrogen derived from cytosolic $NADH/H^+$ is transported into the mitochondria, resulting in the production of ATP or GTP in subsequent enzymic reactions. Given the depletion of ATP/GTP stores in senescent fibroblasts, we determined whether the ability of these cells to channel cytosolic $NADH/H^+$ into mitochondria is altered with respect to young cells. The activity of enzymes involved in the glycerol 3-phosphate shuttle was quite low in both young and senescent cells (results not shown), indicating that this pathway has no major role in young and senescent human fibroblasts. However, we found evidence for age-related changes in the malate–aspartate shuttle. In young cells, MDH (malate dehydrogenase) appears to be associated with p36 (Figure 3a), a regulator of MDH activity, which allows cytosolic MDH to function as a shuttle enzyme [11]. In contrast, MDH is apparently dissociated from p36 in senescent cells, which would disable the enzyme to channel the flux of hydrogen from the cytosol into the mitochondria. The other MDH peaks in Figure 3(a) correspond to the cytosolic and mitochondrial isoenzymes, respectively [14]. These results suggest that the ability to transport cytosolic hydrogen into the mitochondria via the malate–aspartate shuttle may be compromised in senescent cells.

In the absence of functional hydrogen shuttle systems, the reduction of pyruvate to lactate can be used for the regeneration of NAD^+ from $NADH/H^+$, a reaction carried out by LDH. Lactate is secreted and the cells are capable of continuing glycolytic ATP production without the requirement for mitochondrial function, although under these conditions the energy contained within $NADH/H^+$ is not used for ATP production. In line with our assumption that down-regulation of the malate–aspartate shuttle prevents regeneration of NAD^+ from cytosolic $NADH/H^+$ in the mitochondria, we found an increased activity of LDH in senescent cells (Table 2), accompanied by an increased consumption of pyruvate and increased production of lactate (Table 1). This is also confirmed by the close association between pyruvate consumption and lactate production (results not shown). However, the level of NADH was not increased in senescent cells (Table 3), suggesting that the failure of the cells to get rid of cytosolic hydrogen is compensated by increased LDH activity, given the

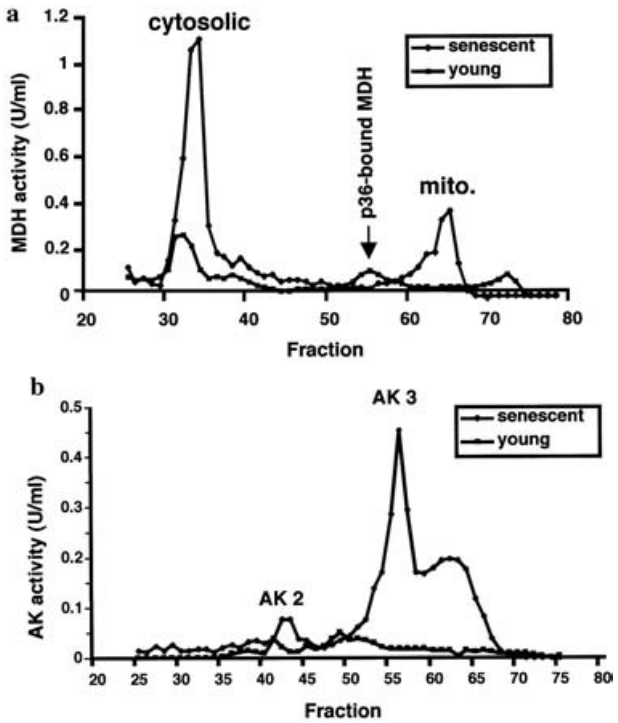


Figure 3 Isoelectric focusing analysis of senescent HDF

Extracts were prepared from senescent and young HDF and separated by isoelectric focusing, as described in [11]. (**a**) The activity of MDH was determined in individual fractions; the position of p36-bound MDH is depicted by an arrow. (**b**) AK activity was determined in individual fractions. The activity in senescent HDF is identified as AK-2 and AK-3 activities by isoelectric point. The same number of cells was used to prepare each extract.

apparent reduction in the activity of the malate–aspartate shuttle. Since GAPDH activity is not up-regulated in senescent cells, it is to be expected that endogenous pyruvate may become limiting in these cells, in agreement with our finding that pyruvate consumption is markedly increased in senescent cells (Table 1).

Block of cell proliferation by exogenous AMP

The status of the glycolytic apparatus within the senescent cells, as described above, can be expected to produce high levels of ribose via the pentose phosphate pathway for nucleotide biosynthesis [17] (Scheme 1). Thus the concentration of FBP is elevated, and both hexokinase and glucose-6-phosphate dehydrogenase are highly active in senescent cells (Table 2). However, the levels of ATP and GTP dropped dramatically. Therefore we additionally determined the UTP and CTP levels. We found a significant down-regulation of both metabolites in senescent cells (Table 3), suggesting that the pentose phosphate pathway may be inhibited in senescent cells, possibly at a point downstream of ribose 5-phosphate synthesis. It is known that the activity of PRPP synthetase (5-phosphoribosyl-1-pyrophosphate synthetase) is under allosteric control by various metabolites, including AMP [28]. To determine if the lack of UTP/CTP may be due to inhibition of PRPP synthetase, the concentration of AMP was determined in extracts from young and senescent cells. Indeed, we found a roughly 10-fold increase in AMP in senescent cells (Table 3), which may explain the decreased levels of UTP and CTP.

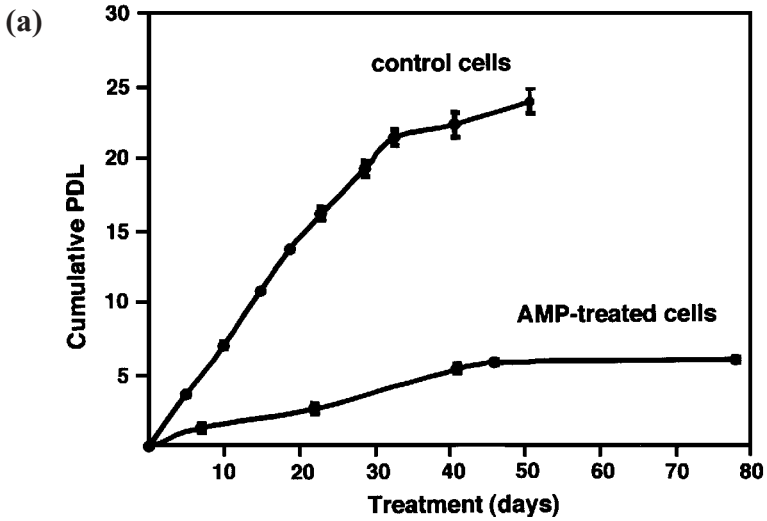
AMP is produced from ADP in a reaction catalysed by AK, comprising five distinct isoforms in humans ([29]; for review see [30]). The strong up-regulation of AMP levels in senescent cells prompted us to determine the activity of AK in young and senescent fibroblasts. Overall activity of AK was strongly increased in senescent cells (results not shown). Cellular extracts were subjected to isoelectric focusing and the activity of AK isoforms was determined. As shown in Figure 3(b), there is a significant increase in the activities of both AK2 and AK3 in extracts from senescent cells, whereas enzyme activity is barely detectable in extracts from young cells. Although the equilibrium constant of the AK reaction is near one, the low ATP level facilitates the reaction in the direction to ATP and AMP. Activation of AK in senescent cells may reflect a cellular response to ATP depletion, as was described in other systems [31]. It is worth noting that the age-dependent increase in AK3 activity was also observed in aging rats [32]. However, activation of AKs is apparently not sufficient to overcome the loss of ATP in senescent fibroblasts.

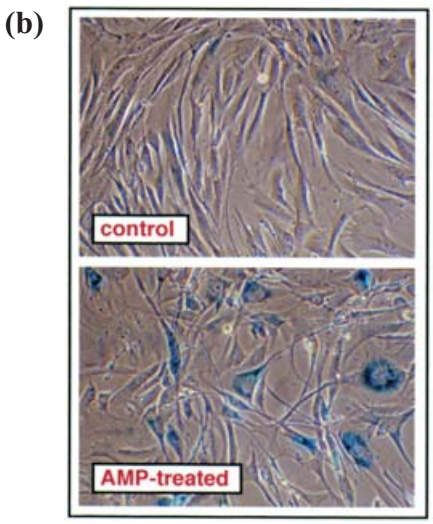
It has been shown before that intracellular AMP levels of many cell types can be manipulated by addition of exogenous AMP to the culture medium. Apparently, AMP is first dephosphorylated, transported through the cell membrane and rephosphorylated to AMP inside the cell, the net effect being an up-regulation of intracellular AMP levels [14,28,33–36]. We made use of this pathway to determine whether an increase in the intracellular AMP concentration would influence the proliferation rate of normal human fibroblasts. To this end, young diploid fibroblasts were incubated with AMP, and AMP levels determined in extracts from untreated and AMP-treated cells; 24 h after AMP addition, intracellular AMP levels were increased by 60 %. Subsequently, the effect of AMP on the proliferation rate was determined. As shown in Figure 4(a), addition of AMP led to a significant and reproducible inhibition of cell proliferation. To determine whether the AMP-dependent growth arrest may be related to the senescence phenotype, we first analysed the morphology of the AMP-treated cells. Microscopic inspection revealed a rapid change in morphology, and within 3 days of treatment AMP-treated cells acquired the typical morphology of senescent cells. To investigate this further, we also tested the expression of SA-β-gal, a molecular marker for replicative senescence [9]. As is shown in Figure 4(b), AMP treatment resulted in the appearance of SA-β-gal-positive cells and the time-course analysis revealed the appearance of 60 % SA-β-gal-positive cells in AMP-treated cultures, when control cultures were still completely negative (Figure 4c). This result suggests that elevation of the intracellular AMP level, as was shown to occur during replicative senescence, is sufficient to induce both growth arrest and properties of the senescent phenotype.

It is known that AMPK (AMP-activated protein kinase), the human homologue of yeast Snf 1p [19], is activated in AMP-treated cells, and this may contribute to the growth-arrest phenotype observed here. To test this prediction, cells were also treated with AICAR, an adenosine analogue and cell-permeable activator of AMPK [37]. Similar to the results obtained with AMP addition, addition of AICAR to young fibroblasts induced complete cell-cycle arrest (Table 4), suggesting that activation of AMPK may be involved. However, cell cycle arrest was also obtained when GMP was added to the cells, whereas addition of IMP had no effect (Table 4). We conclude from these findings that AMP-induced cell cycle arrest of human fibroblasts is probably linked to both activation of AMPK (also exerted by AICAR) and inhibition of PRPP (also exerted by GMP but not IMP [33]).

DISCUSSION

Cellular senescence is a major mechanism of tumour suppression, and senescence-associated growth arrest is believed to contribute





(c)

SA-ß-gal positive cells

time	untreated	3 mM AMP
day 1	< 1 %	< 1 %
day 9	< 1 %	1 %
day 18	< 1 %	50 %
day 30	1-2 %	35 %
day 46	15 %	40 %

Figure 4 Induction of premature senescence in HDF by AMP

Young HDF were incubated with 3 mM AMP, which was added to the culture medium. (**a**) Cells were counted at every passage and growth curves established for AMP-treated and control cells. (**b**) AMP-treated and control cells were stained for SA-β-gal activity [9] and pictures were taken at identical magnifications. (**c**) Percentage of SA-β-gal-positive cells during the course of the experiment. PDL, population doubling.

Table 4 Inhibition of cell proliferation by nucleotides

Young primary foreskin fibroblasts (25 population doublings) were incubated with nucleotides/nucleotide analogues as indicated. The percentage of proliferating cells (cells in S phase) was determined by BrdU-incorporation studies. 72 h post-treatment, cells were incubated with BrdU for 24 h, and BrdU-positive nuclei were counted. 300 cells were counted in each case; the results from two independent experiments are shown.

		Proliferating cells (% in S phase)				
Experiment no.	Nucleotide added (3 mM)...	None	AMP	GMP	IMP	AICAR
1		67	2.9	0.1	72	0.3
2		83	2.8	1.7	82	2.3

to this phenotype. While it was reported that up-regulation of several cdk inhibitors mediates growth arrest in senescent fibroblasts [38–40], the signals leading to cdk inhibitor up-regulation were not identified. In previous work, it was shown that immortalization of primary rodent cells by an activated *ras* oncogene in combination with the E7 oncogene of HPV-16 [41] involves a reprogramming of the cellular carbohydrate metabolism [21], whereby Ras-induced up-regulation of glycolysis leads to proliferation arrest in the G_1 phase of the cell cycle [42], similar to the expression of oncogenic ras in primary human cells which induces premature senescence [20]. Ras-induced senescence is overcome by E7 which leads to a reprogramming of both cell-cycle control [42] and carbohydrate metabolism [21], allowing the cells to exit from G_1 arrest. These findings suggest that spontaneous senescence may also involve metabolic changes that influence the cellular capacity for DNA synthesis and hence S phase entry. In the present study, we show that this is indeed the case and identify a metabolic imbalance in senescent HDF which precludes further DNA synthesis. Moreover, it is shown that metabolic imbalances lead to the up-regulation of AK in senescent cells, leading to increased AMP levels. In additional experiments, AMP is identified as an anti-proliferative signal for HDF.

Genetic experiments in lower eukaryotes, such as yeasts and worms, have suggested that regulation of the carbohydrate metabolism is tightly linked with developmental signals that determine cell proliferation and survival, key factors governing the aging process at the cellular level [43]. If this holds true for mammalian cells, a thorough analysis of glycolysis, as the most central pathway in cellular carbohydrate metabolism, should reveal first clues concerning the flux of major carbohydrate nutrients (e.g. glucose) in senescent cells. Whereas control of the metabolic flux into anabolic and catabolic pathways branching off from glycolysis is crucial for a proliferating cell, the data presented here suggest the occurrence of significant metabolic imbalances in senescent human fibroblasts. The activities of several glycolytic enzymes are strongly up-regulated; however,

no such increase is observed for GAPDH. Moreover, the function of the malate–aspartate shuttle is reduced in senescent cells, preventing the transport of hydrogen into the mitochondria, where it could be used for ATP production. Instead, senescent cells activate LDH and take up pyruvate to get rid of hydrogen. Thus ATP-consuming steps of glycolysis are enhanced, whereas the ATP-producing steps are inhibited, and this leads to a severe reduction of the intracellular concentration of both ATP and GTP. ATP depletion is known to elicit cellular adaptation responses, in particular, an up-regulation of glycolytic enzymes [44,45] and AK [31]. Indeed, we observed a strong up-regulation of several glycolytic enzymes and AK activity in senescent fibroblasts. Of the five AK isoenzymes, AK2 and AK3 were detectably up-regulated in senescent cells. Whereas AK3 primarily acts as a GMP-ATP exchanger and is located in the mitochondrial membrane, it was also described that AK3 actively contributes to the maintenance of cytoplasmic AMP pools [46]. AK2, on the other hand, which is located in the cytosol, is considered a major regulator of AMP homoeostasis. While AK activation apparently is not sufficient to compensate for ATP loss, it leads to an increase in levels of intracellular AMP, which imposes a strong anti-proliferative signal on the cells.

The present findings suggest a metabolic 'vicious cycle', where depletion of ATP leads to an adaptive response that increases the metabolic imbalance to a point where nucleotide biosynthesis is impossible. In that model, metabolic events override changes in gene expression in human fibroblasts, as was shown before in model experiments in lower eukaryotes [47]. While it remains to be shown how metabolic failure is translated into cell cycle arrest, the data shown in Figure 4 suggest that intracellular AMP plays a critical role. The increase in intracellular AMP levels can be caused either by dephosphorylation of ATP or by uptake of extracellular AMP. The extracellular AMP is dephosphorylated to adenosine by 5-ecto-nucleotidases and imported into the cells. Intracellular adenosine is phosphorylated to AMP by adenosine kinase [33]. Whereas our current model would suggest that, by analogy to previous findings [14,28], AMP inhibits PRPP to block the synthesis of UTP (and subsequently CTP), it is also possible that AMPK, the human homologue of yeast Snf 1p [19] is activated in AMP-treated cells. Thus it was shown that AMPK mediates the stimulation of glycolysis in response to hypoxia [48], and data presented in Table 4 suggest that activation of AMPK by addition of AICAR can induce cell-cycle arrest, similar to the effects observed with AMP. When this work was in progress, others have reported that AMPK is indeed activated in senescent HDF, and addition of AICAR to HDF induces cell-cycle arrest [49], similar to the findings reported here. However, data presented in Table 4 indicate that GMP can also efficiently induce cell-cycle arrest in human fibroblasts. Since GMP is known to inhibit PRPP synthetase activity, but is unable to activate AMPK, this leaves open the possibility that part of the anti-proliferative action of AMP is indeed due to PRPP synthetase inhibition [33]. More work will be required to elucidate fully the mechanism by which AMP induces growth arrest and premature senescence in young HDF.

The activation of glycolytic enzymes upstream of GAPDH in conjunction with low GAPDH activity would provide favourable conditions for active nucleotide precursor synthesis through the pentose phosphate cycle (Scheme 1). However, this pathway is blocked in senescent cells, apparently due to the up-regulation of AMP levels, and the actual end products of glucose metabolism in senescent cells remain to be determined. Given the morphological changes observed with replicative senescence ([9]; see also Figure 4), it appears possible that in senescent fibroblasts glucose is used in other biosynthetic pathways which are less well controlled than the nucleotide synthesis pathway.

A key finding of the present report is the inability of senescent fibroblasts to up-regulate GAPDH activity. It appears possible that the GAPDH enzyme, which is known to be sensitive to oxidative stress [50], is partly inactivated in senescent cells, e.g. by reactive oxygen species, which are known to contribute to cellular aging in yeast [51]; similarly, reactive oxygen species levels are significantly increased in senescent fibroblasts (E. Hütter and P. Jansen-Dürr, unpublished work). Increased reactive oxygen species production was also shown to activate poly(ADP-ribose) polymerase-1, a major component of DNA-repair signalling that is involved in cellular aging processes (for review, see [52]). It was reported that poly(ADP-ribose) polymerase activation, via depletion of cellular NAD stores, induces apoptosis [53]. However, the unaltered intracellular pool of NAD/NADH, as observed here for senescent fibroblasts, suggests that this pathway is not activated in senescent fibroblasts. Recent evidence suggests a key role for GAPDH in several regulatory pathways, including cell proliferation and survival [54], and the data presented here suggest that the failure to up-regulate GAPDH activity in senescent cells may contribute to the chain of events which lead to the AMP-induced growth arrest of senescent fibroblasts.

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REFERENCES

1 Hayflick, L. (1992) Aging, longevity, and immortality *in vitro*. Exp. Gerontol. **27**, 363–368
2 Harley, C. B. (1997) Human ageing and telomeres. CIBA Found. Symp. **211**, 129–139
3 Serrano, M. and Blasco, M. A. (2001) Putting the stress on senescence. Curr. Opin. Cell Biol. **13**, 748–753
4 Jazwinski, S. M. (2000) Metabolic control and ageing. Trends Genet. **16**, 506–511
5 Hockel, M. and Vaupel, P. (2001) Biological consequences of tumor hypoxia. Semin. Oncol. **28**, 36–41
6 Lane, M. A., Black, A., Handy, A., Tilmont, E. M., Ingram, D. K. and Roth, G. S. (2001) Caloric restriction in primates. Ann. N.Y. Acad. Sci. **928**, 287–295
7 Lin, S. J., Defossez, P. A. and Guarente, L. (2000) Requirement of NAD and SIR2 for life-span extension by calorie restriction in *Saccharomyces cerevisiae*. Science **289**, 2126–2128
8 Dürst, M., Dzarlieva-Petrusevska, R. T., Boukamp, P., Fusenig, N. E. and Gissmann, L. (1987) Molecular and cytogenetic analysis of immortalized primary human keratinocytes obtained after transfection with human papillomavirus type 16 DNA. Oncogene **1**, 251–256
9 Dimri, G. P., Lee, X., Basile, G., Acosta, M., Scott, G., Roskelley, C., Medrano, E. E., Linskens, M., Rubelj, I., Pereira-Smith, O. and Smith, J. (1995) A biomarker that identifies senescent human cells in culture and in aging skin *in vivo*. Proc. Natl. Acad. Sci. U.S.A. **92**, 9363–9367
10 Wagner, M., Hampel, B., Bernhard, D., Hala, M., Zwerschke, W. and Jansen-Durr, P. (2001) Replicative senescence of human endothelial cells *in vitro* involves G1 arrest, polyploidization and senescence-associated apoptosis. Exp. Gerontol. **36**, 1327–1347
11 Mazurek, S., Hugo, F., Failing, K. and Eigenbrodt, E. (1996) Studies on associations of glycolytic and glutaminolytic enzymes in MCF-7 cells: role of p36. J. Cell. Physiol. **167**, 238–250
12 Susor, W. A. and Rutter, W. J. (1971) Method for the detection of pyruvate kinase, aldolase, and other pyridine nucleotide linked enzyme activities after electrophoresis. Anal. Biochem. **43**, 147–155
13 Domingo, M., Einig, C., Eigenbrodt, E. and Reinacher, M. (1992) Immunohistological demonstration of pyruvate kinase isoenzyme type L in rat with monoclonal antibodies. J. Histochem. Cytochem. **40**, 665–673
14 Mazurek, S., Michel, A. and Eigenbrodt, E. (1997) Effect of extracellular AMP on cell proliferation and metabolism of breast cancer cell lines with high and low glycolytic rates. J. Biol. Chem. **272**, 4941–4952
15 Mazurek, S., Weisse, G., Wust, G., Schafer-Schwebel, A., Eigenbrodt, E. and Friis, R. R. (1999) Energy metabolism in the involuting mammary gland. *In Vivo* **13**, 467–477
16 Bergmeyer, H. U. (ed.) (1974) Methoden der enzymatischen Analyse, 3rd edn, vols I and II, Verlag Chemie, Weinheim (Bergstrasse)

17 Boros, L. G., Lee, W. N. and Go, V. L. (2002) A metabolic hypothesis of cell growth and death in pancreatic cancer. Pancreas **24**, 26–33
18 Mathupala, S., Rempel, A. and Pedersen, P. L. (1997) Abberant glycolytic metabolism of cancer cells: a remarkable coordination of genetic, transcriptional, post-translational, and mutational events that lead to a critical role for type II hexokinase. J. Bioenerg. Biomembr. **29**, 339–343
19 Lin, S. S., Manchester, J. K. and Gordon, J. I. (2001) Enhanced gluconeogenesis and increased energy storage as hallmarks of aging in *Saccharomyces cerevisiae*. J. Biol. Chem. **276**, 36000–36007
20 Serrano, M., Lin, A. W., McCurrach, M. E., Beach, D. and Lowe, S. W. (1997) Oncogenic ras provokes premature cell senescence associated with accumulation of p53 and p16(INK4a). Cell **88**, 593–602
21 Mazurek, S., Zwerschke, W., Jansen-Durr, P. and Eigenbrodt, E. (2001) Metabolic cooperation between different oncogenes during cell transformation: interaction between activated ras and HPV-16 E7. Oncogene **20**, 6891–6898
22 Yamada, K. and Noguchi, T. (1999) Regulation of pyruvate kinase M gene expression. Biochem. Biophys. Res. Commun. **256**, 257–262
23 Eigenbrodt, E., Basenau, D., Holthusen, S., Mazurek, S. and Fischer, G. (1997) Quantification of tumor type M2 pyruvate kinase (Tu M2-PK) in human carcinomas. Anticancer Res. **17**, 3153–3156
24 Zwerschke, W., Mazurek, S., Massimi, P., Banks, L., Eigenbrodt, E. and Jansen-Dürr, P. (1999) Modulation of type M2 pyruvate kinase activity by the human papillomavirus type 16 E7 oncoprotein. Proc. Natl. Acad. Sci. U.S.A. **96**, 1291–1296
25 Glaser, G., Giloh, H., Kasir, J., Gross, M. and Mager, J. (1980) On the mechanism of the glucose-induced ATP catabolism in ascites tumour cells and its reversal by pyruvate. Biochem. J. **192**, 793–800
26 Ronen, S. M., DiStefano, F., McCoy, C. L., Robertson, D., Smith, T. A., Al-Saffar, N. M., Titley, J., Cunningham, D. C., Griffiths, J. R., Leach, M. O. and Clarke, P. A. (1999) Magnetic resonance detects metabolic changes associated with chemotherapy-induced apoptosis. Br. J. Cancer **80**, 1035–1041
27 Ethier, M. F., Hickler, R. B. and Dobson, Jr, J. G. (1989) Aging increases adenosine and inosine release by human fibroblast cultures. Mech. Ageing Dev. **50**, 159–168
28 Hugo, F., Mazurek, S., Zander, U. and Eigenbrodt, E. (1992) *In vitro* effect of extracellular AMP on MCF-7 breast cancer cells: inhibition of glycolysis and cell proliferation. J. Cell. Physiol. **153**, 539–549
29 Inouye, S., Yamada, Y., Miura, K., Suzuki, H., Kawata, K., Shinoda, K. and Nakazawa, A. (1999) Distribution and developmental changes of adenylate kinase isozymes in the rat brain: localization of adenylate kinase 1 in the olfactory bulb. Biochem. Biophys. Res. Commun. **254**, 618–622
30 Van Rompay, A. R., Johansson, M. and Karlsson, A. (2000) Phosphorylation of nucleosides and nucleoside analogs by mammalian nucleoside monophosphate kinases. Pharmacol. Ther. **87**, 189–198
31 Gutierrez, G. (1991) Cellular energy metabolism during hypoxia. Crit. Care Med. **19**, 619–626
32 Lee, H. M., Greeley, Jr, G. H. and Englander, E. W. (2001) Age-associated changes in gene expression patterns in the duodenum and colon of rats. Mech. Ageing Dev. **122**, 355–371
33 Henderson, J. F. and Scott, F. W. (1980) Inhibition of animal and invertebrate cell growth by naturally occurring purine bases and ribonucleosides. Pharmacol. Ther. **8**, 539–571
34 Rapaport, E. (1988) Experimental cancer therapy in mice by adenine nucleotides. Eur. J. Cancer Clin. Oncol. **24**, 1491–1497
35 Collavin, L., Lazarevic, D., Utrera, R., Marzinotto, S., Monte, M. and Schneider, C. (1999) wt p53 dependent expression of a membrane-associated isoform of adenylate kinase. Oncogene **18**, 5879–5888
36 Weisman, G. A., Lustig, K. D., Lane, E., Huang, N. N., Belzer, I. and Friedberg, I. (1988) Growth inhibition of transformed mouse fibroblasts by adenine nucleotides occurs via generation of extracellular adenosine. J. Biol. Chem. **263**, 12367–12372

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37 Longnus, S. L., Wambolt, R. B., Parsons, H. L., Brownsey, R. W. and Allard, M. F. (2003) 5-Aminoimidazole-4-carboxamide 1-beta-D-ribofuranoside (AICAR) stimulates myocardial glycogenolysis by allosteric mechanisms. Am. J. Physiol. Regul. Integr. Comp. Physiol. **284**, R936–R944
38 Hara, E., Smith, R., Parry, D., Tahara, H., Stone, S. and Peters, G. (1996) Regulation of p16CDKN2 expression and its implications for cell immortalization and senescence. Mol. Cell. Biol. **16**, 859–867
39 Alcorta, D. A., Xiong, Y., Phelps, D., Hannon, G., Beach, D. and Barrett, J. C. (1996) Involvement of the cyclin-dependent kinase inhibitor p16 (INK4a) in replicative senescence of normal human fibroblasts. Proc. Natl. Acad. Sci. U.S.A. **93**, 13742–13747
40 Wagner, M., Hampel, B., Hutter, E., Pfister, G., Krek, W., Zwerschke, W. and Jansen-Durr, P. (2001) Metabolic stabilization of p27 in senescent fibroblasts correlates with reduced expression of the F-box protein Skp2. Exp. Gerontol. **37**, 41–55
41 Crook, T., Morgenstern, J. P., Crawford, L. and Banks, L. (1989) Continued expression of HPV-16 E7 protein is required for maintenance of the transformed phenotype of cells co-transformed by HPV-16 plus EJ-ras. EMBO J. **8**, 513–519
42 Zerfass, K., Schulze, A., Spitkovsky, D., Friedman, V., Henglein, B. and Jansen-Dürr, P. (1995) Sequential activation of cyclin E and cyclin A gene expression by HPV-16 E7 through sequences necessary for transformation. J. Virol. **69**, 6389–6399
43 Tissenbaum, H. A. and Guarente, L. (2002) Model organisms as a guide to mammalian aging. Dev. Cell **2**, 9–19
44 Schoonen, W. G., Wanamarta, A. H., van der Klei-van Moorsel, J. M., Jakobs, C. and Joenje, H. (1990) Respiratory failure and stimulation of glycolysis in Chinese hamster ovary cells exposed to normobaric hyperoxia. J. Biol. Chem. **265**, 1118–1124
45 Schoonen, W. G., Wanamarta, A. H., van der Klei-van Moorsel, J. M., Jakobs, C. and Joenje, H. (1991) Characterization of oxygen-resistant Chinese hamster ovary cells. III. Relative resistance of succinate and alpha-ketoglutarate dehydrogenases to hyperoxic inactivation. Free Radicals Biol. Med. **10**, 111–118
46 Heldt, H. W. and Schwalbach, K. (1967) The participation of GTP-AMP-P transferase in substrate level phosphate transfer of rat liver mitochondria. Eur. J. Biochem. **1**, 199–206
47 ter Kuile, B. H. and Westerhoff, H. V. (2001) Transcriptome meets metabolome: hierarchical and metabolic regulation of the glycolytic pathway. FEBS Lett. **500**, 169–171
48 Marsin, A. S., Bouzin, C., Bertrand, L. and Hue, L. (2002) The stimulation of glycolysis by hypoxia in activated monocytes is mediated by AMP-activated protein kinase and inducible 6-phosphofructo-2-kinase. J. Biol. Chem. **277**, 30778–30783
49 Wang, W., Yang, X., Lopez De Silanes, I., Carling, D. and Gorospe, M. (2003) Increased AMP:ATP ratio and AMP-activated kinase activity during cellular senescence linked to reduced HuR function. J. Biol. Chem. **278**, 27016–27023
50 Rivera-Nieves, J., Thompson, W. C., Levine, R. L. and Moss, J. (1999) Thiols mediate superoxide-dependent NADH modification of glyceraldehyde- 3-phosphate dehydrogenase. J. Biol. Chem. **274**, 19525–19531
51 Nestelbacher, R., Laun, P., Vondrakova, D., Pichova, A., Schuller, C. and Breitenbach, M. (2000) The influence of oxygen toxicity on yeast mother cell-specific aging. Exp. Gerontol. **35**, 63–70
52 Burkle, A. (2000) Poly(ADP-ribosyl)ation, genomic instability, and longevity. Ann. N.Y. Acad. Sci. **908**, 126–132
53 Yu, S. W., Wang, H., Poitras, M. F., Coombs, C., Bowers, W. J., Federoff, H. J., Poirier, G. G., Dawson, T. M. and Dawson, V. L. (2002) Mediation of poly(ADP-ribose) polymerase-1-dependent cell death by apoptosis-inducing factor. Science **297**, 259–263
54 Sirover, M. A. (1999) New insights into an old protein: the functional diversity of mammalian glyceraldehyde-3-phosphate dehydrogenase. Biochim. Biophys. Acta **1432**, 159–184
55 Raghunathan, R., Russell, J. D. and Arinze, I. J. (1977) Pyruvate carboxylase and phosphoenopyruvate carboxykinase in cultured human fibroblasts. J. Cell. Physiol. **92**, 285–292