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REVIEW

A Common Metabolic Profile Shared Between Murine ES Cells and Primary Cells Bypassing Senescence

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Abstract. Enhanced glycolysis even under 20% oxygen culture condition is the characteristic property of most cancer cells and it is known as the Warburg effect. The Warburg effect is well exploited in the clinic, what suggest its undisputed significance in cancer. However, there have been limited mechanistic insights into its relationship with the well-characterized molecular and genetic events associated with cellular immortalization. We recently identified that overexpression of the glycolytic enzyme phosphoglycerate mutase (PGM) immortalized primary mouse embryonic fibroblasts (MEFs). Immortalized MEFs and mouse embryonic stem cells (ES cells) display higher glycolytic flux with reduced oxygen consumption, and present more resistance to oxidative damage than senescent cells. We discovered an unexpected aspect of the Warburg effect, protecting cells from senescence effect of oxidative damage. These metabolic properties might contribute to the proliferative potential of ES cells, which are immortal primary cells without any genetic alteration.

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INTRODUCTION

There is considerable hope in ES cells being promising donor sources for treating several human diseases. They are now facing several critical problems to be overcome; immune rejection after transplantation, ethical issues regarding the usage of human embryos, quite complicated culture process and so on. One possible resolution for these concerns is to establish pluripotent stem cells directly from patients' somatic cells. In recent works, several groups reported that murine stem-like cells could be induced from mouse fibroblasts after retrovirus-mediated introduction of four transcription factors (Oct3/4, Sox2, c-Myc, and Klf4)[1, 2]. Although these induced pluripotent stem cells, called iPS cells, can be good tools for cell transplantation therapy, they still need to be better characterized and improved. One big problem of iPS cells is their observed tumourigenicity in recipient, implicating that genetic manipulation of oncogenes or tumour suppressor genes to derive stem cells are not favourable for these purpose [3].

Unlike other primary cells, both murine and human ES cells are immortal under standard tissue culture conditions. Stem cells are characterized by their immortality and indefinite self-renewal potential while retaining their ability to differentiate. Moreover, ES cells are genome-protected as primary cells, while the immortalization requires several genetic alterations in any other primary cells. This ability to self-renew indefinitely in ES cells is lost upon differentiation, suggesting that the immortality in ES cells is strictly regulated by unknown intrinsic mechanisms. Now rising question is how ES cells can maintain their immortality without any tumorigenic genetic alteration. To understand why ES cells are immortal, it would be useful to explore and compare the immortalization of other primary cells and cancer cells with that of ES cells (Fig. 1).

Our recent findings suggest that enhanced glycolysis critically contributes to the proliferation of immortalized primary cells and ES cells. In this review, we spotlight the metabolic characteristics of ES cells and primary cells, which support their immortality.

ES CELLS ARE IMMORTAL AS PRIMARY CELLS

Most somatic cells have a limited replicative capacity under standard tissue culture conditions and suffer a permanent cell cycle arrest, called replicative senescence. Replicative senescence is induced by telomere erosion upon reaching replicative exhaustion, which can be bypassed by the ectopic expression of telomerase in human fibroblasts. Now it is well established that senescence can be also induced in a telomere-independent manner, called stress-induced senescence (SIS). Cells suffering either replicative or stress-induced senescence (SIS) are phenotypically similar; they adopt an enlarged and flattened appearance, deposit increased amounts of extracellular matrix, express elevated levels of PAI-1, develop lipofuscin granules, single prominent nuclei, senescence-associated heterochromatin, senescence-associated β-galactosidase activity and show negligible DNA synthesis. However the underlying mechanisms initiating them are quite different between replicative senescence and SIS. SIS can be triggered in cells of virtually any age by the expression of active oncogenes, CDK inhibitors, mild oxidative stress, and other stimuli [4-6].

Murine and human embryonic stem cells show intrinsic telomerase activity [7]. ES cells continue to express high levels of telomerase even after serial passage for more than a year in tissue culture. However, the immortality of ES cells cannot be explained solely by their telomerase activity.

Normal laboratory mice have exceptionally long telomeres, yet mouse embryo fibroblasts (MEFs) with intact telomerase activity generally have a lifespan of only 10-20 population doublings under normal culture conditions [8, 9]. This senescence response observed in MEFs has been attributed to 'culture shock' [8-10] and is not observed in ES cells, which argues for additional mechanisms besides telomere regulation involved in ES cell immortality. Although the details remain poorly defined, possible factors contributing to this 'culture shock' are, amongst

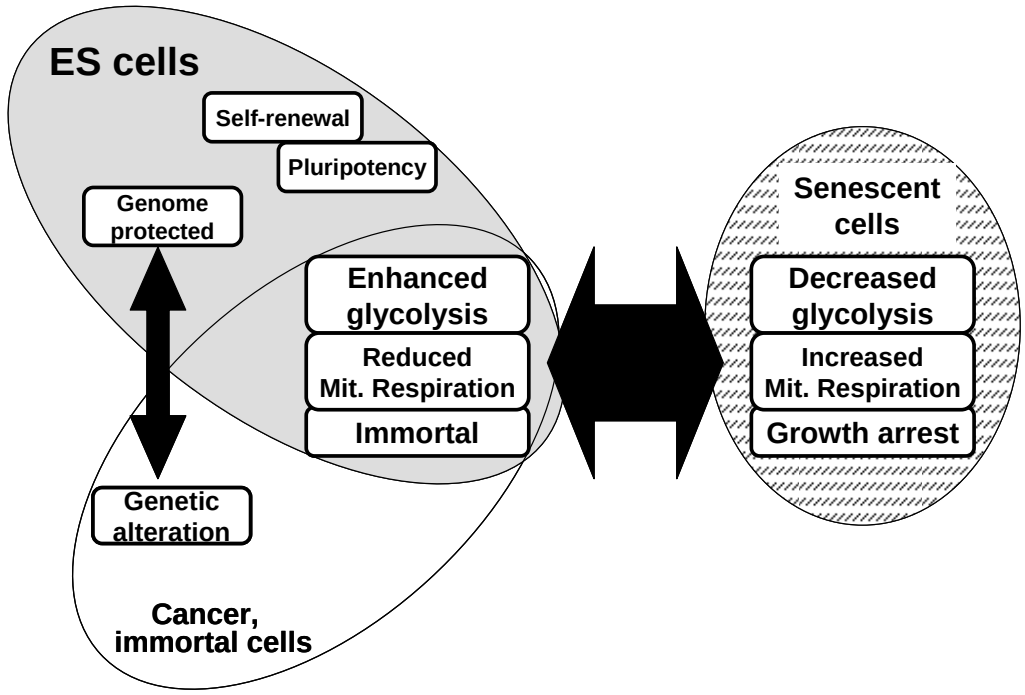

Figure 1 Common metabolic features shared between ES and immortal cells. Immortalized cells show common metabolic features such as, enhanced glycolysis and lower mitochondrial O_2 consumption than senescent cells. See details in the text.

others, growth on plastic, constant exposure to serum or high oxygen levels. The modification of tissue culture conditions correcting these stresses such as the use of serum-free medium or culture in low oxygen conditions can avoid this premature senescence [11-13]. ES cells are also significantly resistant to culture-stress induced senescence by mechanisms not understood (Fig. 2).

The most likely explanation to why telomere erosion, sub-optimal culture conditions, and other insults can elicit a similar phenotype in primary cells is that the same signalling pathways are engaged to implement the cell cycle arrest. An arising notion from recent studies suggests that the accumulation of reactive oxygen species (ROS) and oxidative damage [14], are among the common pathways involved in senescence.

IMMORTALITY AND RESISTANCE TO OXIDATIVE STRESS

In a normal environment, approximately 90% of the oxygen that enters into the cell is used to produce energy via the mitochondrial respiratory chain. During this process, four electrons are added to each O_2 molecule and together with 2 H^+, resulting in the liberation of two molecules of water. It is estimated that 1-4% of the O_2 up taken into cells forms partially reduced oxygen species or ROS (reactive oxygen species); such as superoxide anion, hydroxyl radical or hydrogen peroxide [15]. Since the initial hypothesis of the free radical theory of aging by Harman [16], a link between organism aging and oxidative stress has been suggested [17-20]. Recent studies suggest that oxidative stress can also have a great impact on cellular life span in tissue culture. Mild oxidative stress (for example, treatment with a low concentration of hydrogen peroxide) is enough to induce senescence [21, 22]. Also culture-stress or oncogene-induced stress seems to cause oxidative damage in cells, in line with the notion that mouse cells are particularly prone to oxidative stress [14, 23] and hereby premature senescence.

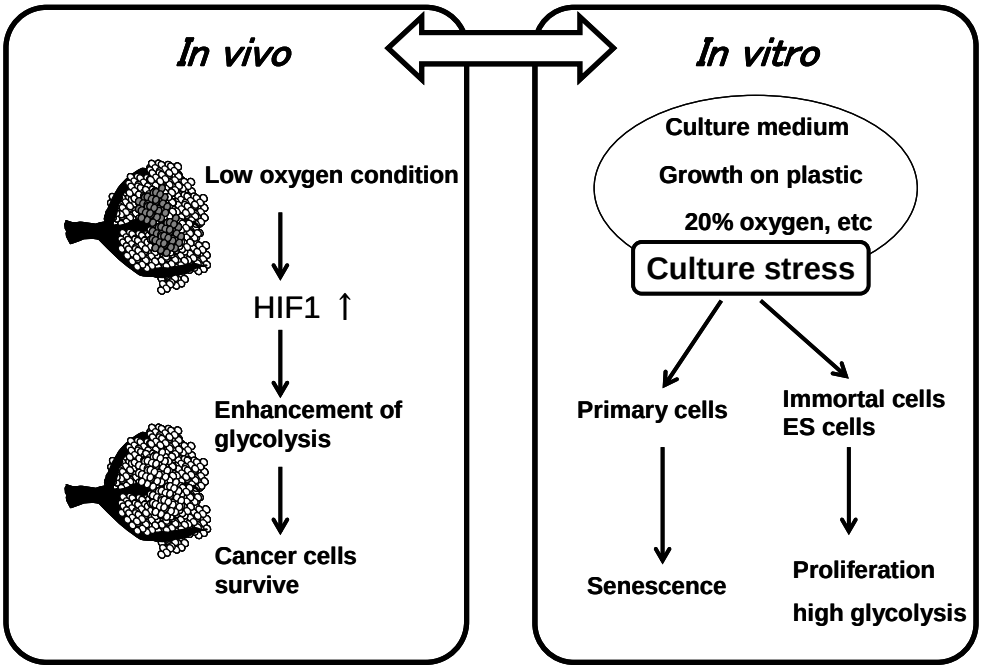

Figure 2 Cancer cells display enhanced glycolysis *in vivo* and *in vitro*. Cancer cells exhibit enhanced glycolysis both *in vivo* and *in vitro*. In tissue, enhanced glycolysis has been considered as an effect of transcription factor HIF-1. *In vitro*, however, it cannot be explained solely by HIF-1.

Noteworthy, increasing ROS accumulation is observed during replicative senescence. The replicative potential of both murine and human fibroblasts are significantly extended under low oxygen, correlating with less oxidative damage inflicted than under normoxia (O_2 20%) [12, 24]. Murine ES cells suffer less oxidative damage than primary fibroblast when cultured under 20% O_2 [25]. Moreover murine ES cells are much more resistant to the deleterious effects of hydrogen peroxide than MEFs [26, 27]. These findings suggest that the ability of ES cells to buffer oxidative stress might be key for explaining their immortality. To elucidate how ES cells are protected from oxidative stress, it is important to understand how normal cells try to adapt to the oxidative stress that they are subjected to during senescence.

ENHANCED GLYCOLYSIS PROTECT CELLS FROM THE SENESCENCE EFFECT OF OXIDATIVE STRESS

We found recently that expression of glycolytic enzymes can modulate cellular life span in MEFs [26]. In a senescence bypassing screening in MEFs using a retroviral cDNA library, we isolated the glycolytic enzyme phosphoglycerate mutase (PGM). PGM catalyzes the conversion of 3-phosphoglycerate to 2-phosphoglycerate during glycolysis (Fig. 3). Analysis of the impact of other glycolytic enzymes over senescence in MEFs showed that glucophosphate isomerase (GPI) could also drive proliferation of MEFs. Ectopic expression of PGM or GPI increases glycolytic flux diminishing the oxidative damage that MEFs are exposed to, and extend the life span of primary MEFs. Conversely, knockdown of PGM or GPI via specific siRNA induces premature senescence. Moreover, others and we found that the glycolytic flux declines during senescence both in murine and human fibroblasts [28].

How an increase in glycolysis can immortalize primary cells? From the data discussed above it seems that enhanced glycolysis can protect cells from oxidative stress and as a consequence avoid senescence triggered by oxidative stress [26]. MEFs immortalized by PGM or GPI suffer less oxidative damage than control cells as estimated by cytosolic ROS staining, or quantifica-

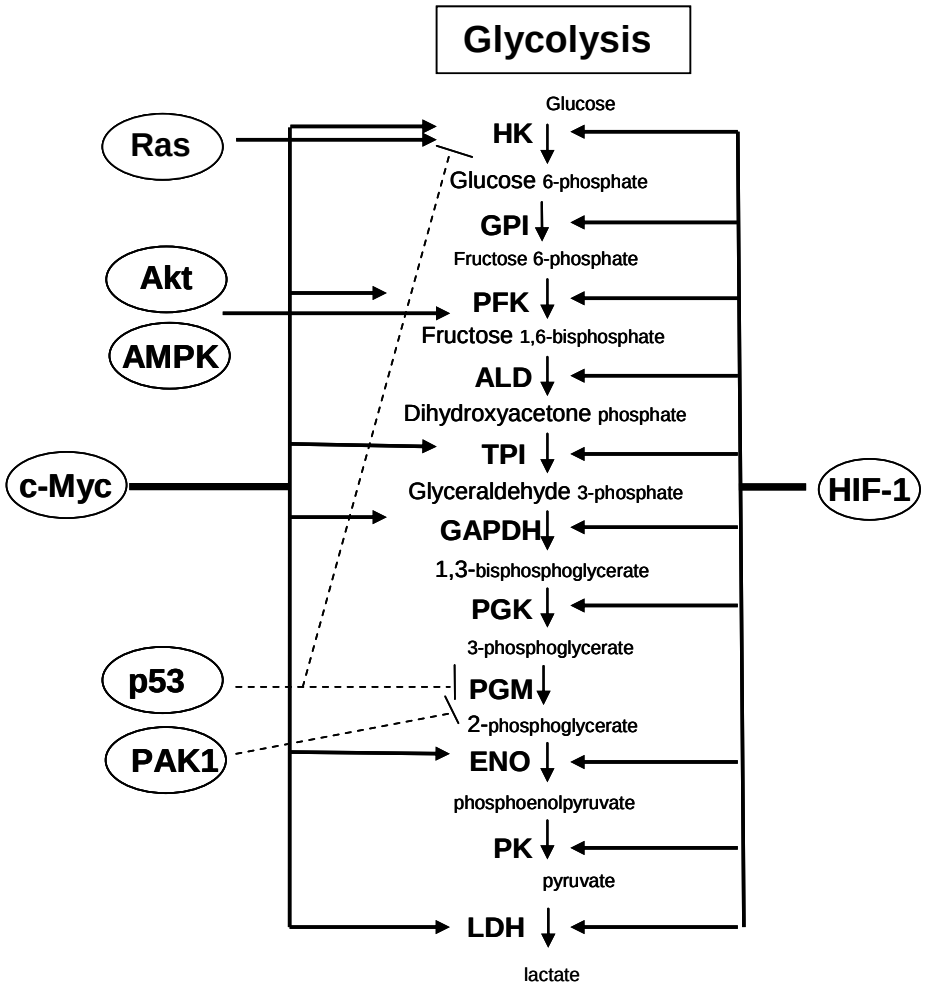

Figure 3 Regulation of the glycolytic pathway. Glycolysis is regulated by different transcription factors (HIF-1, c-Myc, p53) and signal transduction components such as Ras, Akt, AMPK or Pak1.

tion of 8-hydroxydeoxyguanosine (8-OHdG) genomic lesions, that is one of the most common types of oxidative DNA damage.

Several other reports support our notions. The impact of glucose-6-phosphate dehydrogenase (G6PD) activity on cell proliferation is well established [29]. G6PD catalyzes the rate-limiting step in the pentose phosphate pathway (PPP), which is responsible for the recycling of NAPDH and maintenance of the redox balance as described above. G6PD deficient human fibroblasts have a reduced lifespan that is attributable to oxidative stress and can be corrected by the ectopic expression of this enzyme [30, 31]. Both G6PD activity and the NADPH pool decline during continued culture passage, presumably as a consequence of the accumulation of oxidative damage. Importantly ES cells ablated from G6PD expression are extremely sensitive to oxidative damage, showing massive apoptosis at low concentration of oxidants, not lethal for wild type ES cells [32]. It would, therefore, be worthy to explore whether enhanced glycolysis can promote increased NADPH production via the PPP and exert its anti-senescence function.

ENHANCED GLYCOLYSIS IN ES CELLS

Interestingly, mouse ES cells present a surprisingly high glycolytic rate [26]. Mouse ES cells

exhibit more enhanced glycolytic flux and more PGM activity compared with well proliferating primary or immortalized MEFs, and that other glycolytic enzymes are also activated. Moreover, we found that the mitochondrial O_2 consumption in murine ES cells is unexpectedly reduced compared with that of proliferating MEFs [25]. Interestingly, immortalized MEFs also exhibited much less mitochondrial O_2 consumption than senescent MEFs. These results showed that a very unique metabolic pattern; enhanced glycolysis and decreased mitochondrial O_2 consumption, is a common feature shared between mouse ES cells and immortalized MEFs (Fig. 1). Noteworthy, while primary MEFs acquire these metabolic properties after immortalization mainly via inactivation of tumour suppressor genes, mouse ES cells are highly glycolytic without need for genetic modification.

Several lines of evidence show a close correlation between the glycolytic flux and proliferative potential of murine ES cells. First, ES cells posses a higher replicative potential and a higher glycolytic flux than immortalized MEFs or wild type MEFs at early passages. Second, ES cells are more sensitive to glycolytic inhibition than MEFs. When glycolysis was inhibited by adding a low-dose of 2-deoxyglucose (2DG), that did not affect MEFs, most ES cells died [25]. Third, upon ES cells differentiation, the glycolytic flux of ES cells drops dramatically. Once differentiated by the removal of cytokine LIF from culture medium, mouse ES cells stopped proliferation and their glycolytic flux reduced to half. These findings suggest that enhanced glycolysis could play a very important role in the proliferative potential of ES cells.

As a result of these findings in ES cells and primary MEFs, we hypothesized that a factor playing a role in murine ES cells immortality is their enhanced glycolysis that can protect cells from the senescence effects of oxidative stress. The metabolic protection might contribute to preserve the genome integrity of ES cells avoiding genetic alterations and allowing them to maintain their self-renewal capacity. Also these metabolic properties of ES cells are reverted via the induction of differentiation, what might explain why differentiated ES cells do not present enhanced glycolysis.

THE WARBURG EFFECT; COMMON METABOLIC CHARACTERISTIC OF ES, IMMORTALIZED AND CANCER CELLS

As SIS is partly thought to be a barrier against oncogenic stimuli, bypass of senescence might be an essential early step in tumorigenesis *in vivo*. Besides immortalization, there are several hallmarks that distinguish cancer cells from their normal counterparts, such as growth factor independence, evasion of apoptosis, sustained angiogenesis, tissue invasion and metastasis [33].

Interestingly an increase in glycolysis is a hallmark of most cancers, as first noted by Otto Warburg over seven decades ago. This property of tumours is used in clinical practice to detect primary or metastatic tumours in patients by positron-emission scanning of 2-[^{18}F] fluoro-2-deoxy-D-glucose [34]. Thus, the fact that tumours are highly glycolytic in hypoxic conditions is well established *in vivo* and called the Warburg effect (Fig. 2). The concomitant induction of glycolysis in hypoxic condition is mediated partly by activating hypoxia-inducible transcriptional factor (HIF-1). Hypoxia increases HIF-1α levels in most cell types and HIF-1 mediates adaptative responses to change tissue oxygenation. Thus, HIF-1 can directly upregulate the expression of a set of genes involved in both local and global reaction to hypoxia, including angiogenesis, erythropoiesis, breathing and most of the glycolytic enzymes: HK1, HK2, AMF/GPI, ENO1, GLUT1, GADPH, LDHA, PFKBF3, PFKL, PGK1, PKM, TPI [35]. Interestingly, both HIF-1 and glycolytic enzymes are overexpressed in many tumours and cancer cells [34]. Altogether these data support a functional link between enhanced glycolysis and cellular adaptation during tumor formation and expansion in hypoxic condition *in vivo*.

REGULATION OF GLYCOLYSIS UNDER STANDARD CULTURE CONDITION

However, the Warburg effect cannot be simply explained by cellular adaptation to hypoxic condition in the core of solid tumours that

overgrow the feeding vasculature, as cancerous cells maintain enhanced glycolysis even under standard 20% O_2 tissue culture conditions. This fact suggests that stable alterations at the genetic or epigenetic level may be the explanation for the enhanced glycolysis that cancer cells present *in vitro* [36]. The rising question now is how high glycolytic flux is maintained in immortalized primary cells, cancer cells and ES cells under standard condition, which could not be explained exclusively by HIF-1 (Fig. 2).

Recent studies identified several novel glycolytic regulators, which might explain why cancer cells and immortalized primary cells display enhanced glycolysis *in vitro*. They are signalling molecules, oncogenes, and tumour suppressors (Fig. 3). But these cannot be the case in ES cells for following reasons. First, unlike cancer cells, oncogenic alteration could not explain enhanced glycolysis in ES cells, because ES cells do not present genetic alterations. Second, while the several signalling molecules can increase glycolysis, they are reported so far to have the opposing effect on the proliferative potential in primary cells.

Finally, it would be interesting to explore the molecular links between the glycolytic metabolism and tumour suppressor pathways such as p53, RB and others. Indeed, recent work indicates that p53 is involved in the concerted regulation of glycolytic metabolism and mitochondrial respiration. One possible interpretation would be that one of the major targets of p53 involved in senescence induction impacts on metabolic regulation, which render cells sensitive to oxidative stress. Identifying this factor could provide further links between oxidative stress and cellular life span with potential implications on both cancer and stem cell biology.

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