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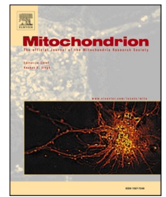

Review

Circumventing the Crabtree effect in cell culture: A systematic review

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ABSTRACT

Metabolic reprogramming and mitochondrial dysfunction are central elements in a broad variety of physiological and pathological processes. While cell culture established itself as a versatile technique for the elaboration of physiology and disease, studying metabolism using standard cell culture protocols is profoundly interfered by the Crabtree effect. This phenomenon refers to the adaptation of cultured cells to a glycolytic phenotype, away from oxidative phosphorylation in glucose-containing medium, and questions the applicability of cell culture in certain fields of research. In this systematic review we aim to provide a comprehensive overview and critical appraisal of strategies reported to circumvent the Crabtree effect.

1. Introduction

Metabolic flexibility is the ability to respond or adapt to changes in energy supply and demand under a variety of conditions, and is a prerequisite for optimal cellular function, proliferation, differentiation, and survival (Goodpaster and Sparks, 2017; Smith et al., 2018). While this adaptive capacity (e.g. increasing the reliance on glycolysis) is a critical aspect in many physiological processes, ranging from exercise metabolism to cell fate determination and immunometabolism, metabolic inflexibility is increasingly linked to a wide range of chronic diseases such as type 2 diabetes, obesity and Alzheimer's disease (Baik et al., 2019; Goodpaster and Sparks, 2017; Michaeloudes et al., 2020; Priolo and Henske, 2013; Storlien et al., 2004).

The most well-known example of metabolic reprogramming is the Warburg phenomenon, a condition characterized by a deviation of the metabolic profile in which cancer cells essentially rely on glycolysis instead of oxidative phosphorylation (OXHPOS) (Warburg, 1956). A parallel metabolic phenomenon, referred to as the Crabtree effect (Crabtree, 1929), has been described in cultured cells. The Crabtree effect describes the phenomenon that cells cultured in glucose-containing culture medium adapt to a glycolytic phenotype, despite the presence of oxygen and functional mitochondria (Crabtree, 1929; Mot et al., 2016). This phenomenon has major consequences for cell culture-based studies that aim at addressing aspects of the physiologic metabolic flexibility and/or studying mitochondrial processes. In fact, failure to address this point may lead to misinterpretation of conclusions such as 'dysfunctional' energy metabolism (metabolic inflexibility) or illusory cellular resistance to mitochondrial toxicants and drugs which diminishes the prediction of potential drug toxicity in vivo (Marroquin et al., 2007).

Consequently, in order to adequately study the aspects of metabolic flexibility and/or mitochondrial processes in cell culture, it is crucial to restore the cellular physiologic and metabolic state by reverting the cellular metabolism from glycolysis-dominated back to OXPHOS-dominated. Therefore, the aim of this systematic review was to provide a systematic, comprehensive overview and critical appraisal of reported strategies that circumvent the Crabtree effect. Molecular aspects underlying the Crabtree effect were considered beyond the scope of this review.

2. Material and methods

In order to identify relevant studies reporting on strategies circumventing the Crabtree effect in cell culture, two complementary search strategies were designed: a systematic literature search and a citation search. The systematic literature search was conducted in PubMed,

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EMBASE and Web of Science. The following keywords, or synonyms thereof, were included: Crabtree effect, cell culture, glycolysis, OXPHOS, metabolic switch. This systematic literature search was supplemented with a citation search in Web of Science. Herein, all articles were identified that referred to the original article of H.G. Crabtree (Crabtree, 1929), and specifically focused on cultured cells. Both search strategies were conducted on June, 2nd, 2020 and were performed following the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines (Shamseer et al., 2015). Details of both search strategies are provided in the Supplemental Data: https://figshare.com/s/195511957a78bfbfee4f.

All titles and abstracts were screened by two authors (MDK and AS) to identify relevant studies (Fig. 1). Discrepancies in article eligibility were resolved by joint review and consensus. Subsequently, full text articles were assessed for eligibility. Studies that were not published in English or Dutch, and studies that were not conducted in an in-vitro setting were excluded. Also articles focusing on yeast, embryonic or cancer cells were excluded.

From the included articles, the following data were extracted: year of publication, cell type, initial cell culture medium, intervention strategy to circumvent the Crabtree effect, whether the intervention is induced or performed under continuous control, duration of intervention, and author's key findings.

3. Results

3.1. Strategies circumventing the Crabtree effect

The systematic literature and citation search identified 950 unique references. Of these articles, 25 articles aimed at restoring OXPHOS dominance for adenosine triphosphate (ATP) synthesis under cell culture conditions (Fig. 1). The described intervention strategies reverting the Crabtree effect can be broadly classified into four categories: (1) adjustment of the cell culture medium, (2) the use of glycolytic inhibitors, (3) strategies that target mitochondria, and (4) miscellaneous interventions. The respectively characteristics and key findings of each article are summarized in Tables 1–4. A graphical summary of reported strategies aimed at reverting the Crabtree phenomenon is provided in Fig. 2.

3.2. Culture medium adjustments

The most extensively reported strategy to circumvent the Crabtree effect is replacement of the standard, high glucose (10–25 mM) cell culture medium, by a medium with (sub)physiologic glucose concentrations or no glucose at all (Table 1).

3.2.1. Lowering glucose concentrations

Several authors explored whether a reduction in glucose concentrations promoted a metabolic shift from glycolysis to OXPHOS (Arend et al., 2019; Griner and Schnellmann, 1994; Heywood et al., 2010, 2014; Krützfeldt et al., 1990; Mot et al., 2016; Nowak and Schnellmann, 1996;

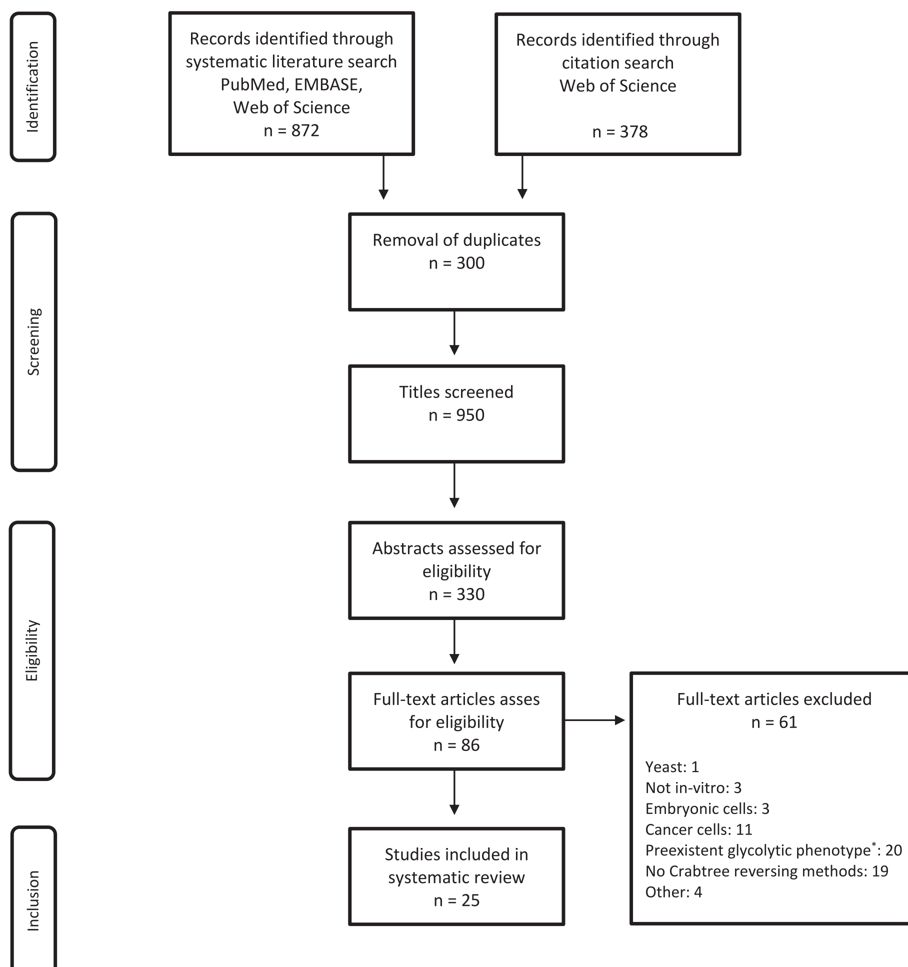

Fig. 1. PRISMA flow diagram of the systematic literature search. *The 20 articles that fall into this category concern articles in which the glycolytic phenotype is already part of the physiological response. For instance, the switch from oxidative phosphorylation to glycolysis is a hallmark of several cell types, including mesenchymal stem cells undergoing osteogenic differentiation, IL-33 activated mast cells, proliferation T cells, nucleus pulposus cells, and dendritic cells during acute activation.

Table 1
Summary of studies aimed at circumventing the Crabtree effect by adjustment of the cell culture medium.

	Cells	Initial cell culture medium	Intervention(-medium)	Incubated or continued	Duration of intervention	Author's key findings	Author - Year
Cell culture medium interventions	Non-transformed human fibroblasts	Glucose medium (conc. glucose not specified), 2 mM glutamine, 10% dialyzed FCS.	Medium **without hexose,** with glutamine and 10% dialyzed FCS Galactose medium (**5.5 mM galactose**), with glutamine and 10% dialyzed FCS	Incubated Incubated	1 day 1 day	○ "When **deprived of sufficient glucose** in growth medium, cells have been found to increase the supply of energy generated by respiratory metabolism, which can be measured as increased rates of glutamine oxidation." ○ "We have observed **that galactose grown** human cells activate respiratory metabolism to the same degree as cells incubated in **hexose-free medium**."	(Kuchka et al., 1981) (33)
	Coronary endothelial cells	Medium 199 (5.6 mM glucose), Earle's salts, 20% FCS.	Saline buffer solution, **<1mM glucose**	Incubated	1 h	○ "Coronary endothelial cells utilize **glucose,** at physiological concentrations, predominantly for glycolytic energy production. The metabolic pattern is characteristic for the **Crabtree** effect." ○ "Glucose oxidation in the Krebs cycle was increased at **glucose** concentrations **lower than 1 mM.**" ○ "Below 1 mM glucose, formation of lactate from glucose decreased."	(Krützfeldt et al., 1990) (32)
	Rabbit renal proximal tubular cells	DMEM/F-12 (no glucose or pyruvate), bovine insulin, human transferrin, selenium, hydrocortisone, heptanoate and: **5 mM glucose** or 17.5 mM glucose or **5 mM galactose**. Culture dishes were constantly shaken (SHAKE) or held stationary (STILL).	–	Continued	7 days	○ "All **SHAKE-treated cells** had equivalent lactate levels on day 1, and these levels were<50% of their STILL counterparts." ○ "**Lowering** the concentration of **glucose** to a physiologic level (**5 mM**) had **no effect** on lactate levels or LDH activity in **SHAKE or STILL** cells." ○ "**Substituting 5 mM galactose for 5 mM glucose** in the culture medium significantly reduced the lactate content of both SHAKE and STILL RPTEC but had no impact on LDH activity." ○ "**Substitution of glucose with galactose** produced an even greater increase in sensitivity to antimycin A in **SHAKE** cells."	Griner et al. 1994 (23)
	Rabbit renal proximal tubular cells	DMEM/F-12 without phenol red and pyruvate, **0 or 5 mM glucose**, 0.44 mM L-alanine, **5 mM lactate**, 15 mM $NaHCO_3$	–	Continued	12 days	○ "RPTC grown in the presence of **5 mM glucose** exhibited net lactate consumption during the first 8 days of culture. However, after 10 and 12 days of culture, lactate metabolism was shifted from net consumption to net production, which suggested the reversion of RPTC metabolism to glycolysis." ○ "In RPTC cultured in the **absence of glucose**, rates of net lactate consumption were equivalent during 12 days of culture."	(Nowak et al., 1996) (44) *(Also in Table 4)*
	Human skin fibroblasts	DMEM (25 mM glucose), 4 mM glutamine, 1 mM pyruvate, 10% FBS.	**10 mM galactose**, 1 mM pyruvate, 4 mM glutamine .	Incubated	3 days	○ "Cells grown in **galactose** exhibit a five- to sixfold decrease in ECAR, reflecting decreased glycolysis, and a twofold increase in the OCR, consistent with a switch to glutamine oxidation." ○ "Cells grown in **galactose-containing medium** maximize mitochondrial ATP production by using a larger fraction of mitochondrial respiration for ATP synthesis."	(Gohil et al., 2010) (20)
	Deep and superficial chondrocyte cells	Isolated chondrocytes, DMEM, 1.6 mM L-glutamine, 16% FCS.	DMEM, **deprived of** or supplemented with **glucose** (0.5–22 mM)	Incubated	Not specified	○ "The OCR in **19 mM glucose** was not significantly different to baseline values of **5 mM glucose.** The upregulation of oxygen consumption compared to values in **5 mM** was significant at glucose concentrations of **below 3 mM.**" ○ "The oxygen consumption by the superficial and deep cell subpopulations increased progressively with	(Heywood et al., 2010) (26) *(Also in Table 2)*

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Table 1 (*continued*)

Cells	Initial cell culture medium	Intervention(-medium)	Incubated or continued	Duration of intervention	Author's key findings	Author - Year
					glucose deprivation, rising 2.5-fold as the media glucose was **reduced from 5 to 0.5 mM**." "Over 90% of the increase in oxygen consumption with glucose depletion appears to be accounted for by the **oligomycin-sensitive compartment,** that is **oxidative phosphorylation**." ○ "Lactate release was progressively reduced by **increasing glucose deprivation**, indicating that the glycolytic rate of the cells is restricted by limited glucose availability."	
L6 cells	DMEM without glucose, **10 mM galactose,** 6 mM glutamine, 1 mM sodium pyruvate, 10% FBS.	–	Continued	Min. 7 days	○ "Investigation into cellular bioenergetics showed that **galactose cultured L6 cells** have a significantly increased OXPHOS capacity compared to glucose cultured cells." ○ "Importantly, cells in glucose were able to up-regulate glycolysis, while **galactose** cells were not." ○ "**Galactose cultured L6 cells** were significantly more sensitive to classical mitochondrial toxicants than glucose cultured cells." ○ "Seahorse extracellular flux analyser demonstrated that OCR was significantly increased whereas ECAR, a measure of glycolysis, was decreased in cells grown in **galactose**."	(Dott et al., 2014) (15)
Bovine chondrocytes	Isolated chondrocytes, DMEM (deprived of glucose), 2 mM L-glutamine, 10% FBS and: 10 mM glucose (high) or; **1 mM glucose (low) + 9 mM galactose.**	–	Continued	4 population doublings	○ "Chondrocytes exhibited significantly greater oxidative phosphorylation in **1 mM glucose** compared with **10 mM glucose**, both at day 0 and after 4 population doublings." ○ "Cells expanded in **low glucose** derived 57% of their ATP from aerobic metabolism, compared with 23% in high glucose." ○ "If, after 4 population doublings in **low glucose**, chondrocytes were switched to high glucose conditions and vice versa, the metabolic differences observed between the expansion conditions were mostly reversible."	(Heywood et al., 2014) (27)
LLC-PK1 cells	DMEM or DMEM/F-12 (conc. glucose not specified), 10% FBS.	**10 mM galactose** medium	Incubated	1 day	dfs ○ "Culturing cells with galactose as an energy source forces kidney tubular epithelial cells to rely on mitochondrial oxidative respiration rather than glycolysis."	(Kishi et al., 2015) (31)
Cardiac progenitor cells	DMEM/F12 (17.5 mM glucose), 2.5 mM glutamine, 0.5 mM pyruvate, 10% embryonic stem cell FBS and supplements.	DMEM without substrates and **0–5 − 25 mM glucose**.	Incubated	4 days	exposure of cells to 25 mM Glc resulted in a 40% increase in glycolytic flux com-	(Salabei et al., 2015) (52)
		DMEM without substrates for 1 h, followed by addition of **5 mM glucose, 1 mM pyruvate, 4 mM glutamine or 100 μM BSA-palmitate**	Incubated	± 40 min	pared with cells in 5 mM Glc ($p < 0.01$), and this was inhibited by the GAPDH inhibitor, KA (Fig. 2B) ○ "Exposure of cells to 25 mM glucose resulted in a 40% increase in glycolytic flux compared with cells in 5 mM glucose." ○ "Glucose, provided as the sole substrate, decreased OCR by up to 50% with maximal responses near the normal plasma levels of glucose. Such glucose-induced decreases in respiration are suggestive of the Crabtree effect."	*(Also in Table 2)*

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Table 1 (*continued*)

Cells	Initial cell culture medium	Intervention(-medium)	Incubated or continued	Duration of intervention	Author's key findings	Author - Year
					○ "**Glutamine** increase ATP demand and supported uncoupled respiration." ○ "Although **BSA-palmitate**, by itself, did not increase mitochondrial OCR, the presence of **glutamine** increased OCR by 66% when fatty acids were present, and this stimulation of respiration significantly exceeded that when **glutamine** was provided as the sole substrate." ○ The increase in glycolysis due to glucose treatment was almost completely inhibited by **koningic acid**.	
1) Primary adult human fibroblasts 2) Primary mouse brain astrocytes	1) DMEM (25 mM glucose), 1 mM L-glutamine, 10% FBS.* 2) DMEM (25 mM glucose), 10% FBS.**	1. DMEM (3.5 mM glucose), 1 mM L-glutamine, 9.7 mM mannitol, 10% FBS 2. DMEM (5.0 mM glucose), 10% FBS	Incubated Incubated	1) 13 days 2) 5 days	○ "**Autonomous depletion** of medium glucose induces a lactate-consuming phase." ○ "**Complete autonomous depletion** of medium glucose forces cells to utilise lactate via mitochondrial OXPHOS to supply their energy needs." ○ "**Autonomous depletion** of medium glucose induces increased sensitivity to the OXPHOS inhibitor rotenone."	(Mot et al., 2016) (38) Methods: (Garfield, 2010) (19) (Hare et al., 2013) (25)
C2C12 mouse myoblasts	DMEM (25 mM glucose), 10% FBS.	DMEM-A (**absence of serum, glucose, pyruvate and glutamine**)	Incubated	1 h	○ "Our findings suggest that the Crabtree effect is decreased in cells subjected to **short-term starvation**, which is possibly associated with the inhibition of the mitochondrial oxidation of glutamine**."**	(Zeidler et al., 2017) (64)
Primary and BV-2 microglial cells	BV-2: DMEM (25 mM glucose), 4 mM glutamine, 10% FCS. Primary cells: MEM (5.6 mM glucose), 4 mM glutamine, 10% FCS.	ACSF assay medium, **5 mM pyruvate or 2.5 mM glutamine**	Incubated	2 h	○ **Pyruvate**: "when glucose was replaced by **pyruvate**, ATP could not be produced in glycolysis; therefore, to produce ATP, pyruvate should enter into the TCA cycle." "Pyruvate-supported oxidation significantly enhanced the cellular ATP levels in primary cells." ○ **Glutamine**: "under starving conditions, the basal respiration is increased by the addition of **glutamine** as a single metabolic fuel, and the glutamine-induced oxygen-consumption is associated with ATP synthesis in primary and BV-2 microglial cells."	Nagy et al. 2018 (40) *(Also in Table 2)*
Human Pluripotent Stem Cell-Derived Cardiomyocytes (hPSC-CMs)	Glucose medium: RPMI medium (conc. glucose not specified), GlutaMAX, Gem21. Glucose fatty acids medium: RPMI medium (conc. glucose not specified), GlutaMAX, Gem 21, **50 µM Palmitic Acid** and **100 µM Oleic Acid**	Fatty acids only medium: RPMI medium **without glucose**, GlutaMAX, **50 µM Palmitic Acid** and **100 µM Oleic Acid**	Incubated	7 days	○ "**Glucose deprivation** results in the inhibition of HIF1α and LDH-A activity, and repression of aerobic glycolysis." ○ "Glucose rich medium promotes glycolytic metabolism, even in the presence of fatty acids whereas media containing **fatty acids** as the only energy source allowed for normal physiological metabolic substrate utilization."	(Hu et al., 2018) (29) *(Also in Table 4)*
Rat astrocytes	DMEM (25 mM glucose), 1 mM pyruvate, 44.6 mM sodium bicarbonate, 10% FCS.	Glucose-free **DMEM**, supplemented with **2 mM glucose**, 44.6 mM sodium bicarbonate	Incubated	0–15 days	○ "The cells rapidly consumed the available glucose and the culture medium was already within 2 days completely deprived of glucose. This cell-dependent metabolic glucose depletion was accompanied by a rapid increase in the extracellular concentration of lactate." ○ "The **lactate consumption phase** was characterized by a steady decrease in extracellular lactate concentration." ○ "In conclusion, glucose depletion experiments revealed that astrocytes in culture efficiently metabolize **glucose to lactate** via glycolysis and	(Arend et al., 2019) (2)

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Table 1 (*continued*)

Cells	Initial cell culture medium	Intervention(-medium)	Incubated or continued	Duration of intervention	Author's key findings	Author - Year
					subsequently **utilize the lactate** released for mitochondrial energy production."	
LLC-PK1 cells	DMEM/F12 without glucose, supplemented with 5 mM glucose, 3% FBS.	DMEM/F12 without glucose, supplemented **with 5 mM glucose**, 3% FBS and **5 mM acetoacetate.**	Incubated	48 h	○ "Basal respiration, maximal respiration, spare respiratory capacity and ATP-linked respiration were significantly increased in cells grown in growth medium supplemented with **5 mM acetoacetate**. In contrast, glycolytic capacity, as well as glycolytic reserve were significantly reduced in the acetoacetate group."	(Denoon et al., 2020) (13)

ATP, adenosine triphosphate; BSA, bovine serum albumin; DMEM, Dulbecco's Modified Eagle Medium; ECAR, extracellular acidification rate; FBS, fetal bovine serum; FCS, fetal calf serum; HIF1α, hypoxia-inducible factor 1α; LDH, lactate dehydrogenase; OCR, oxygen consumption rate; OXPHOS, oxidative phosphorylation; RPTC, renal proximal tubular cells; TCA, tricarboxylic acid cycle.

Salabei et al., 2015). Remarkably, it was shown that the Crabtree effect is not restricted to cells kept in high glucose medium (10–25 mM), but also persist at more physiologic glucose concentrations (5.0–5.6 mM) (Griner and Schnellmann, 1994; Heywood et al., 2010; Krützfeldt et al., 1990; Nowak and Schnellmann, 1996). Only when glucose levels were further lowered to concentrations of 0.1–5.0 mM, an increased shift towards OXPHOS was observed in cells (Heywood et al., 2010, 2014; Krützfeldt et al., 1990). In this context, *Mot et al.* and *Arend et al.* described that reversal of the Crabtree effect only occurred after total glucose depletion: fibroblasts and astrocytes cultured in 2.0–5.0 mM glucose first completely consumed the available glucose to lactate, after which cells were forced to utilize the accumulated lactate as energy substrate via mitochondrial OXPHOS in order to fulfill their energy requirements (Arend et al., 2019; Mot et al., 2016).

3.2.2. *Replacing glucose by other primary substrates*

A second series of studies evaluated the use of glucose-free cell culture medium (Table 1**)** (Dott et al., 2014; Gohil et al., 2010; Griner and Schnellmann, 1994; Hu et al., 2018; Kishi et al., 2015; Kuchka et al., 1981; Nagy et al., 1859; Nowak and Schnellmann, 1996; Salabei et al., 2015; Zeidler et al., 2017). In these studies, the cell culture medium was often enriched with other, exogenous energy substrates such as carbohydrates (galactose), amino-acids (glutamine), fatty acids (palmitate), pyruvate or ketone bodies (acetoacetate).

Most of the glucose-replacement studies evaluated the effect of substituting glucose by galactose in the culture medium (Griner and Schnellmann, 1994; Dott et al., 2014; Gohil et al., 2010; Kishi et al., 2015; Kuchka et al., 1981). The rationale behind this strategy is that slow oxidation of galactose to pyruvate yields not sufficient ATP per unit time, and thus impels cells to rely on OXPHOS - mostly by an increased glutaminolysis - to generate ATP for cellular homeostasis (Dott et al., 2014; Mot et al., 2016; Rossignol et al., 2004). It was concluded that cells grown in galactose-medium (5.0–10.0 mM), had a lower glycolytic rate, and an increased reliance on OXPHOS (Griner and Schnellmann, 1994; Dott et al., 2014; Gohil et al., 2010; Kishi et al., 2015; Kuchka et al., 1981). Furthermore, cells cultured in galactose-enriched medium are more sensitive to the mitochondrial inhibitors rotenone and antimycin A, confirming the increased reliance on mitochondrial respiration to meet energy requirements (Dott et al., 2014; Griner and Schnellmann, 1994).

A more direct enforcement of OXPHOS dominated ATP synthesis includes the exclusive provision of substrates in glucose-free medium that are metabolized in the tricarboxylic acid cycle (glutamine, pyruvate, acetoacetate and/or fatty acids).

Kuchka et al. showed that human fibroblasts increased glutamine oxidation in hexose-free medium, and hence enhanced OXPHOS (Kuchka et al., 1981). A similar phenomenon was observed in microglial cells (Nagy et al., 1859).

Similarly, replacement of glucose by pyruvate as the sole substrate increased mitochondrial respiration in microglial cells (note that the conversion from pyruvate to lactate does not generate ATP, and pyruvate is therefore forced to enter the mitochondria via acetyl coenzyme A for ATP production) (Nagy et al., 1859).

A similar phenomenon has been described for human pluripotent stem cell-derived cardiomyocytes, in which simulation of their in vivo preference for β-oxidation by culturing cells in fatty-acids-only medium (deprived of glucose), glycolytic rates reduced and OXPHOS rates increased (Hu et al., 2018).

Likewise, supplementation of growth medium (5 mM glucose) with the ketone body acetoacetate - the preferred energy substrate of proximal tubules cells - increased mitochondrial respiration and decreased glycolytic capacity in LLC-PK_1 cells (Denoon et al., 2020).

3.3. *Glycolytic inhibitors*

Conceptually, an obvious approach to prevent excessive aerobic

Table 2
Summary of studies aimed at circumventing the Crabtree effect with glycolytic inhibitors.

	Cells	Initial medium	Intervention(-medium)	Incubated or continued	Duration of intervention	Author's key findings	Author - Year
Glycolytic inhibitors	Rat proximal tubular epithelial cells	DMEM/F-12 (17.5 mM glucose), $NaHCO_3$, 5% FBS.	HBSS, 5% FBS, 0, 5 or 20 mM glucose and: **0.03–1 mM iodoacetic acid** or DMEM/F12, 5% FBS**, 20 mM 2-deoxyglucose**	Incubated	1 day	○ "Oxidative respiration could be restored by uncoupling mitochondria with **CCCP** or by inhibition of glycolysis with **iodoacetic acid** and **2-deoxyglucose** which indicates that respiration is inhibited by glycolysis (also known as the Crabtree effect)."	(Chi et al., 1995) (9) *(Also in Table 3)*
	Deep and superficial chondrocyte cells	Isolated chondrocytes	5 mM glucose, **50 mM 2-deoxyglucose**	Incubated	Not specified	○ "Both populations expressed the Crabtree phenomena, with oxygen consumption increasing ± 2.5-fold in response to glycolytic inhibition by **glucose deprivation** or **2-deoxyglucose**."	(Heywood et al., 2010) (26) *(Also in Table 1)*
	Cerebellar granule cells	Basal Medium Eagle (5.6 mM glucose), 5 mM KCl, 2 mM glutamine, 10% FCS.	**0.1 mM dichloroacetate**	Incubated	1 day	○ "Cells subjected to an apoptotic stimulus, activate a Warburg-effect like mechanisms, i.e. suppression of OXPHOS combined with activation of aerobic glycolysis as the main pathway for ATP synthesis." ○ "D**ichloroacetate** was shown to reverse the glycolytic phenotype – which characterizes the early phase in our model of apoptosis - lowering lactate level and raising mitochondrial ROS production, which is associated with the enhanced consumption of oxygen in the OXPHOS reaction."	(Bobba et al., 2015) (8)
	Cardiac progenitor cells	DMEM/F12 (17.5 mM glucose), 2.5 mM glutamine, 0.5 mM pyruvate, 10% embryonic stem cell FBS and supplements.	DMEM (without substrates) and 0–5 − 25 mM glucose. DMEM, 1 mM L-glutamine, 0–5 − 25 mM glucose, **5 μM koningic acid**.	Incubated Incubated	4 days Not specified	exposure of cells to 25 mM Glc resulted in a 40% increase in glycolytic flux compared with cells in 5 mM Glc ($p < 0.01$), and this was inhibited by the GAPDH inhibitor, KA (Fig. 2B) ○ "Exposure of cells to 25 mM glucose resulted in a 40% increase in glycolytic flux compared with cells in 5 mM glucose." ○ "The increase in glycolysis due to glucose treatment was almost completely inhibited by **koningic acid**."	(Salabei et al., 2015) (52) *(Also in Table 1)*
	Primary and BV-2 microglial cells	BV-2: DMEM (25 mM glucose), 4 mM glutamine, 10% FCS. Primary cells: MEM (5.6 mM glucose), 4 mM glutamine, 10% FCS.	ACSF assay medium, 10 mM glucose, **10 mM sodium oxamate**	Incubated	± 1.5 h	○ "Inhibition of lactate dehydrogenase by **oxamate** stimulated the entry of glycolytic pyruvate into mitochondria, increased cellular respiration and decreased the rate of acidification in both primary and BV-2 microglial cells."	Nagy et al. 2018 (40) *(Also in Table 1)*

ATP, adenosine triphosphate; DMEM, Dulbecco's Modified Eagle medium; FBS, fetal bovine serum; FCS, fetal calf serum; OXPHOS, oxidative phosphorylation; ROS, reactive oxygen species.

Table 3
Summary of studies aimed at circumventing the Crabtree effect with mitochondrial interventions.

	Cells	Initial medium	Intervention (-medium)	Incubated or continued	Duration of intervention	Author's key findings	Author – Year
Mitochondrial interventions	Rat lung cells	Primary isolated cells in Krebs-Ringer bicarbonate buffer.	**0.25 mM dinitrophenol**	Incubated	Not specified	○ "The addition of 5 mM glucose decreased respiration rates approximately 20%, suggesting the presence of a Crabtree effect." ○ "The classical Crabtree effect, glucose-induced depression of oxygen utilization was prevented by 0.25 mM **dinitrophenol**."	(Ayuso et al., 1973) (4)
	Rat heart muscle cells	Isolated heart cells incubated in Krebs-Henseleit saline (5 mM glucose) and 6 mU/ml insulin.	**0.05 mM 2,4-dinitrophenol**	Incubated	Not specified	○ "Exogenous glucose (plus insulin) decreased oxygen consumption by the cells, providing evidence for the Crabtree effect." ○ "Oxygen consumption and glucose utilization by the cells were increased greatly by the uncoupler of oxidative phosphorylation, **2,4-dinitrophenol**."	(Farmer et al., 1977) (17)
	Rat lung cells	Primary isolated cells* in Krebs-Ringer bicarbonate buffer.	**0.25 mM dinitrophenol**, Krebs-Ringer bicarbonate buffer, defatted bovine serum albumin.	Incubated	± 5 min	○ "Uncoupling of respiration from oxidative phosphorylation by **dinitrophenol**, in agreement with previous studies on neoplastic tissues, released the inhibitory effect of glucose on lung cells respiration."	(Ayuso-Parrilla et al., 1978) (5) Methods: (Pérez-Diaz et al., 1977) (46)
	Rat proximal tubular epithelial cells	DMEM/F-12 (17.5 mM glucose), $NaHCO_3$, 5% FBS.	HBSS, 5% FBS, glucose (conc. not specified), **4 μM CCCP**	Incubated	1 day	○ "Oxidative respiration could be restored by uncoupling mitochondria with **CCCP** or by inhibition of glycolysis with **iodoacetic acid** and **2-deoxyglucose** which indicates that respiration is inhibited by glycolysis (also known as the Crabtree effect)."	(Chi et al., 1995) (9) *(Also in Table 2)*

glycolysis in cultured cells, is the inhibition of glycolytic enzymes (Fig. 2**,** Table 2). For example, inhibiting the glycolytic enzyme hexokinase by 2-deoxyglucose (20.0–50.0 mM), effectively reduces glycolytic rates, and increases OXPHOS (Chi et al., 1995; Heywood et al., 2010). Alternatively, dichloroacetate (0.1 mM) inhibits pyruvate dehydrogenase kinase and reversed the glycolytic phenotype (Bobba et al., 2015). Other glycolytic inhibitors reported to reverse the Crabtree effect include iodoacetic acid (0.03–1.0 mM) and koningic acid (5.0 μM) through inhibition of the enzyme glyceraldehyde-3-phosphate dehydrogenase (Chi et al., 1995; Salabei et al., 2015). Also inhibition of lactate dehydrogenase (LDH) by 10.0 mM sodium oxamate reduced lactate accumulation, and – as described by the authors – 'slightly' (significance was not provided) enhanced cellular respiration due to an increased entry of pyruvate into mitochondria (Nagy et al., 1859).

3.4. Mitochondrial interventions

Four identified studies assessed the potential of mitochondrial uncouplers (Table 3) and demonstrated that the proton motive force needed to generate ATP by ATP synthase is dissipated, causing mitochondrial respiration to be maximal (Ayuso et al., 1973; Ayuso-Parrilla et al., 1978; Chi et al., 1995; Farmer et al., 1977). Three studies conducted in the seventies, showed that the glucose-induced depression of oxygen respiration in rat lung cells and heart muscle cells, was prevented by the uncoupler dinitrophenol (0.025–0.25 mM) (Ayuso et al., 1973; Ayuso-Parrilla et al., 1978; Farmer et al., 1977). In 1995, *Chi et al.* concluded that uncoupling the mitochondria with 4.0 μM carbonyl cyanide-p-chlorophenylhydrazone restored oxidative respiration in rat proximal tubular epithelial cells (Chi et al., 1995). A critical question, however, is whether the increased uncoupled respiration indicates circumvention of the Crabtree effect because the uncoupled maximal oxygen consumption rates do not contribute to mitochondrial ATP generation.

3.5. Miscellaneous strategies

Several indirect strategies (Table 4**)**, such as *in vitro* supplementation with irisin (which increases the expression of PGC-1α) (Vaughan et al., 2014) or hydrogen sulfide (which attenuates hyperglycemia-induced formation of reactive oxygen species) (Suzuki et al., 2011), have been reported to effectively switch the cellular metabolism from glycolysis to OXPHOS.

Also the addition of L-ascorbic acid 2-phosphate in rabbit renal proximal tubular cells (RPTC), inhibited glycolysis and promoted OXPHOS by stimulating pyruvate utilization in mitochondria (Nowak and Schnellmann, 1996). An alternative, molecular approach is based on the premise that autocrine production of TGF-β1 is responsible for the stimulation of glycolysis in long-term cultures of RPTC (Nowak and Schnellmann, 1996). Indeed, RPTC treated with anti-TGF-β antibodies exhibited decreased glycolytic dominance (Nowak and Schnellmann, 1996).

In contrast to more general approaches to improve oxidative metabolism (i.e. adaptations in medium substrates, or supplementation of specific compounds), an alternate, more holistic strategy was proposed by *Felder et al.* (Felder et al., 2002). The authors argued that factors as cell proliferation rate (Nowak and Schnellmann, 1995; Tang et al., 1989; Tang and Tannen, 1990), culture medium substrate composition (Aleo and Schnellmann, 1992; Balaban and Mandel, 1988), and hypoxic conditions of the culture method itself (Aleo and Schnellmann, 1992; Nowak et al., 1996) might trigger the increased glycolytic phenotype in the renal epithelial LLC-PK_1 cell line. On these grounds, the authors developed a cell culture device (EpiFlow) in which continuous nutrient

Table 4
Summary of studies aimed at circumventing the Crabtree effect with miscellaneous interventions.

	Cells	Initial medium	Intervention (-medium)	Incubated or continued	Duration of intervention	Author's key findings	Author – Year
Miscellaneous interventions	Rabbit renal proximal tubular cells	DMEM/F-12 without phenol red and pyruvate, **5 mM glucose**, 0.44 mM L-alanine, 5 mM lactate, 15 mM $NaHCO_3$	**25–1,000 µM L-ascorbic acid 2-phosphate (AscP)**	Incubated	6–12 days	○ "**AscP** reduced glycolysis, increased net lactate consumption by 38%, and stimulated net glucose production by 47%." ○ "Basal O2 consumption increased by 39% in RPTC grown in the presence of **AscP** and was equivalent to that in freshly isolated proximal tubules." ○ "Supplementation of media with **AscP** further improves RPTC culture conditions by promotion of cellular growth and stimulation of in vivo-like respiration, lactate utilization and net glucose synthesis."	(Nowak et al., 1996) (44) *(Also in Table 1)*
	Rabbit renal proximal tubular cells	DMEM/F-12 without phenol red and pyruvate, 5 mM glucose, 0.44 mM L-alanine, 2 mM glycine, 5 mM lactate, 15 mM $NaHCO_3$, (**5 µg/ml anti-TGF-β antibodies**).	- **5 µg/ml anti-TGF-β antibodies**	Continued Incubated	12 days 6 days	○ "**TGF-β1** stimulates glycolysis, decreases respiration and, at higher concentrations, induces RPTC apoptosis and phenotypic changes." ○ "Glycolysis was not stimulated in RPTC grown in the presence of **anti-TGF-β antibodies**." ○ "RPTC treated with **anti-TGF-β antibodies** exhibited decreased glycolysis, and lactate metabolism shifted from net production to net consumption."	(Nowak et al., 1996) (42)
	LLC-PK_1 cells	DMEM (5 mM glucose), 2 mM L-glutamine, 2mMpyruvate, 30 µM phenol red.	**EpiFlow** cell culture perfusion device: medium perfusion rate of 2 ml/hour and air delivery rate of 5–10 ml/min.	Incubated	5 days	○ "**EpiFlow** maintained cells exhibited an improved oxidative metabolism as evidenced by 1) a decreased activity of glycolytic enzymes, 2) an increase in the activity of mitochondrial phosphate-dependent-glutaminase, 3) an increase in cellular ATP content, and 4) an improved morphology."	(Felder et al., 2002) (18)
	bEnd3 microvascular endothelial cells	DMEM (5.5 mM glucose), 2 mM glutamine, 1% nonessential amino acids, 10% FBS.	DMEM (5 mM or 40 mM glucose), **0–300 µM H_2S**	Incubated	7 days	○ "In vitro hyperglycemia resulted in a switch from OXPHOS to glycolysis, an effect that was partially corrected by **H_2S supplementation**." ○ Treatment of the cells with **H_2S** resulted in an improvement of mitochondrial respiration, whereas the hyperglycemia-induced increase in the glycolytic activity of the cells was normalized."	(Suzuki et al., 2011) (56)
	Murine C2C 12 myocytes	DMEM (25 mM glucose), 10% heat inactivated FBS.	**0.78 – 50 nM irisin**	Incubated	1 day	○ "Basal glycolytic metabolism was reduced following treatment with **irisin** at 2.5, 5.0 or 10.0 nM for 24 h." ○ "**Irisin treatment** for 24 h at either 2.5, 5.0 or 10.0 nM significantly elevated basal oxidative metabolism by 31.4 ± 3.5, 14.8 ± 0.5 and 8.5 ± 2.3%, respectively." ○ "The oxidative reliance (represented by the ratio of oxidative metabolism to	(Vaughan et al., 2014) (60)

(*continued on next page*)

Table 4 (*continued*)

Cells	Initial medium	Intervention (-medium)	Incubated or continued	Duration of intervention	Author's key findings	Author – Year
					glycolytic metabolism) was significantly increased in all 24 h-treated doses." ○ "**Irisin** significantly elevated metabolic gene expression including PGC-1α, NRF1, TFAM, GLUT4 and UCP3 leading to increased mitochondrial biogenesis."	
Human Pluripotent Stem Cell-Derived Cardiomyocytes (hPSC-CMs)	Glucose medium (GLM) or Glucose fatty acids medium (GFAM). *See* table 1.	**Small molecule inhibition of HIF1α** with CTM, or **small molecule inhibition of LDHA** with GSKA (in GLM or GFAM cultured CMs) **siRNA inhibition of HIF1α** in GFAM cultured CMs	Incubated Incubated	7 days 96 h	○ "**Small molecule or siRNA inhibition of HIF1α** or small **molecule inhibition of LDHA** results in a metabolic switch from aerobic glycolysis to oxidative phosphorylation, a more mature metabolic phenotype."	(Hu et al., 2018) (29) *(Also in* Table 1*)*

AscP, L-ascorbic acid 2-phosphate; ATP, adenosine triphosphate; DMEM, Dulbecco's Modified Eagle Medium; FBS, fetal bovine serum; GLM, glucose medium ; GFAM, glucose fatty acids medium; HIF1α, hypoxia-inducible factor 1α; hPSC-CMs, human pluripotent stem cell-derived cardiomyocytes; LDH, lactate dehydrogenase; OXPHOS, oxidative phosphorylation; RPTC, renal proximal tubular cells; TCA, tricarboxylic acid cycle; TGF β, transforming growth factor beta.

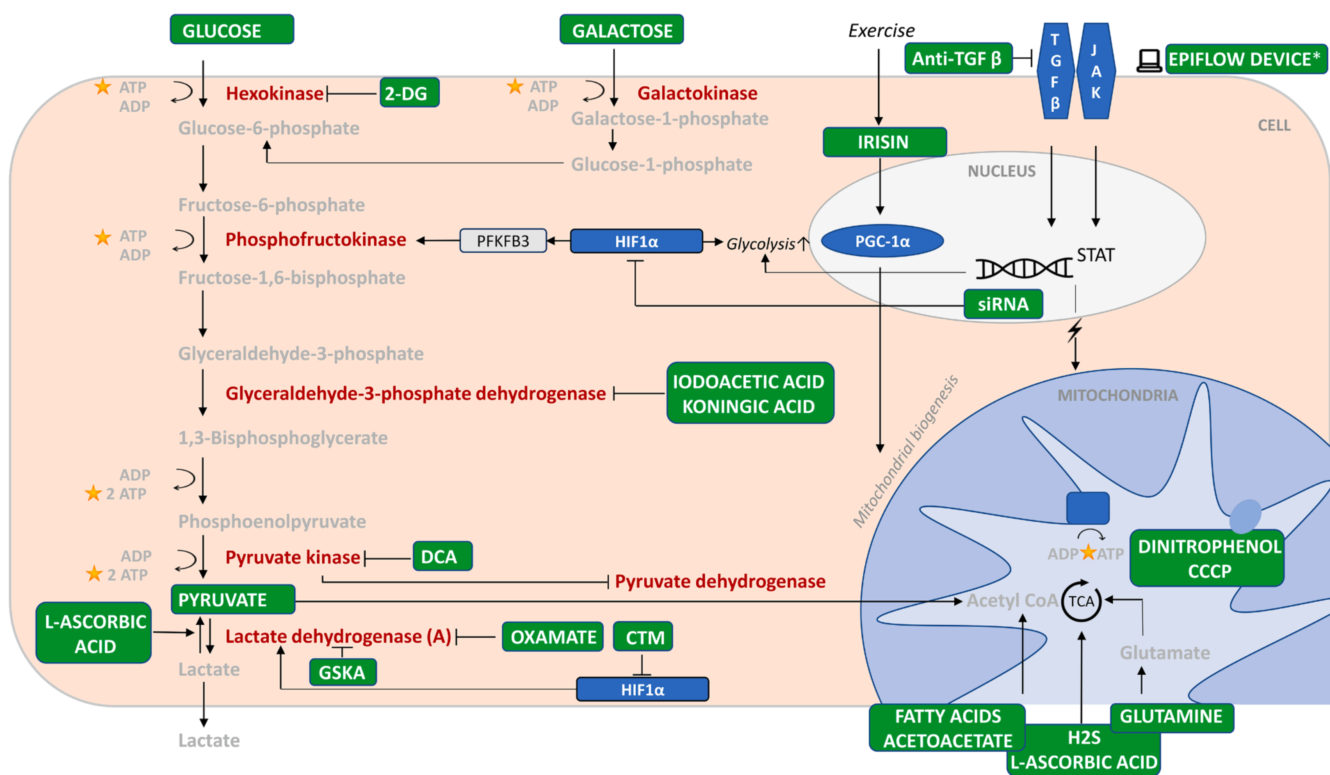

Fig. 2. Graphical summary of reported strategies aimed at circumventing the Crabtree effect. * A cell culture device in which continuous nutrient supply is combined with continuous oxygenation (18). 2-DG, 2-deoxyglucose; ADP, adenosine diphosphate; AMPK, adenosine monophosphate-activated protein kinase; anti-TGF β, anti-transforming growth factor beta; ATP, adenosine triphosphate; CCCP, carbonyl cyanide-p-chlorophenylhydrazone; DCA, dichloroacetate; HIF1α, hypoxia-inducible factor 1α; mTOR, mammalian target of rapamycin; TCA, tricarboxylic acid cycle.

supply is combined with continuous oxygenation. It was reported that this device resulted in a more physiologic metabolism (Felder et al., 2002).

A final, molecular approach to promote a switch from glycolysis-dominated to OXPHOS-dominated catabolism was described by *Hu et al.* (Hu et al., 2018). The authors observed that cardiomyocytes in the presence of glucose (concentration not specified) upregulate hypoxia-inducible factor 1α (HIF1α) and its downstream target LDH-A. Small interference RNA inhibition of HIF1α, as well as small molecule inhibition of HIF1α or LDH-A effectively restored physiologic dependence on OXPHOS (Fig. 2) (Hu et al., 2018).

4. Discussion

For decades, cell culture is still a widely used technique, and will also serve as an important technique in the future to study the wide-ranging processes of metabolism. However, the Crabtree phenomenon that arises in cultured cells fundamentally interferes in these studies (i.e. studies focusing on susceptibility of mitochondrial toxicants (Marroquin et al., 2007; Orlicka-Płocka et al., 2020), mitochondria-related processes (Mot et al., 2016), metabolic flexibility (Goodpaster and Sparks, 2017; Smith et al., 2018) and mitochondrial diseases (Robinson, 1988). If not taken into account, or even not recognized, this may lead to erroneous interpretations regarding mitochondrial susceptibility (reduced sensitivity to mitochondrial toxicants), the detection of non-existent metabolic shifts, and inappropriate clinical implications. Thus, in order to restore OXPHOS dominance for ATP synthesis, this study provides a comprehensive overview of reported strategies that circumvent the Crabtree effect.

The results of this review show that circumvention of the Crabtree effect - i.e. reverting the cellular metabolism from glycolysis to OXPHOS for ATP generation - is possible. Identified strategies are roughly based on suppression of glycolysis or alternatively on metabolic reprogramming. Suppression of glycolysis is either achieved by direct (e.g. use of glycolytic inhibitors) or indirect (e.g. use of galactose medium) inhibition of the glycolytic pathway. As a consequence, cells are forced to rely on other substrates than glucose that rely on OXPHOS for ATP generation. Alternatively, metabolic reprogramming aims to reprogram cells without excluding the glycolytic pathway. Reported strategies include small molecule or siRNA inhibition of HIF1α, and treatment with anti-TGF-β antibodies. While the large majority of identified reports are based on suppression of glycolysis and substrate modulation, this strategy interferes with the physiologic metabolic flexibility of mammalian cells.

So far, the mechanism of the Crabtree effect by which glucose inhibits OXPHOS remains unexplained and is probably multifactorial (de Alteriis et al., 2018; Marroquin et al., 2007; Vander Heiden et al., 2009). A widely accepted hypothesis is that glycolytic enzymes (i.e. phosphoglycerate kinase and pyruvate kinase) in the cytosol and ATP synthase compete for the phosphorylation of the available ADP pool (Diaz-Ruiz et al., 1807; Hammad et al., 1857; Glycolysis, 1972). According to this competition theory, the high glycolytic flux would result in low cytosolic ADP levels. As a result, the exchange of cytosolic ADP for mitochondrial ATP across the inner mitochondrial membrane diminishes and regulatory feedback loops (i.e. pyruvate dehydrogenase inhibition by mitochondrial ATP) will be activated. Despite its attractiveness, it is important to point out that in vivo the Michaelis constant (Km) for adenine nucleotide translocator (ANT) - which facilitates the exchange of ADP and ATP across the mitochondrial inner membrane - is approximately 100 times lower than that of glycolytic enzymes (Diaz-Ruiz et al., 1807; Hammad et al., 1857). This implies that even under rather extreme conditions, in which the activity of glycolytic enzymes increases, the cytosolic ADP would preferably be transported into the mitochondria (Diaz-Ruiz et al., 1807; Hammad et al., 1857).

A further aspect to take into account is that the high glycolytic rates observed in cultured cells reflect the high proliferation rate of cell lines. Glycolytic switching is part of the physiological responses that accompany cell division processes in order to cope with the enhanced energy requirements and to deliver glycolytic intermediates as building blocks for the proliferating cells (Lunt and Vander Heiden, 2011). However, if glycolysis increases to meet the metabolic requirements for cellular growth and division, one may ask why pyruvate is converted to lactate, and not transported into the mitochondria for more ATP production. This may be explained by the fact that the high glycolytic flux of proliferating cells exceeds the maximum pyruvate dehydrogenase activity (Curi et al., 1988; Lunt and Vander Heiden, 2011). Another explanation might be that the conversion of pyruvate to lactate regenerates NAD^+, which is necessary for maintenance of the redox balance and continued glycolytic flux (Lunt and Vander Heiden, 2011). Although NADH is also converted into NAD^+ by mitochondrial complex I, this process is kinetically much slower than the conversion from pyruvate to lactate by LDH (Lunt and Vander Heiden, 2011).

Activation of transcription factor HIF1α - induced by hypoxic conditions in the cell culture medium - is another proposed aspect that may contribute to the high glycolytic rate in cultured cells. Yet, while shaking culture dishes – to provide an aerobic environment – partially reduces the induction of glycolysis (Griner et al., 1993; Griner and Schnellmann, 1994), oxygen tensions in standard culture conditions (140 mmHg) are found to be significantly higher than those in tissues (e.g. kidney 15–70 mmHg, brain 21–47 mmHg and uterus 15–19 mmHg) (Ast and Mootha, 2019). Even in lung alveoli, which have the highest partial pressure of oxygen in the body, oxygen tensions are lower than in standard culture conditions (±110 mmHg versus 140 mmHg) (Ast and Mootha, 2019). However, as static culture may come with large diffusion gradients - depending on cellular oxygen consumption rates, cell density, medium thickness and barometric pressure - significantly lower oxygen tensions may be present at the cellular level (Keeley and Mann, 2019; Pettersen et al., 2005; Place et al., 2017).

Overall, the Crabtree effect can be considered a complex, and probably multifactorial phenomenon involving both external and internal factors. This is also reflected by the wide-ranging reported strategies aimed at circumventing the Crabtree effect. A first series of strategies focuses on the adjustment of external conditions.

The most widely reported 'external' strategy to circumvent the Crabtree effect, is replacement of glucose for galactose in the cell culture medium. Phosphorylation of galactose is depending on galactokinase, which is less active and can have an 8-fold slower metabolic rate than hexokinase, the first enzyme in the phosphorylation of glucose (Dott et al., 2014; Wagner et al., 1991). Consequently, as the glycolytic rate with the use of galactose decreases, cells have to rely on alternative routes (e.g. fatty acids or glutamine- driven oxidative metabolism) to maintain adequate ATP levels for cellular survival. Importantly however, although galactose is the most widely reported, and a relatively simple strategy to circumvent the Crabtree effect, it should be stressed that galactokinase kinetics differ among cell types, and some cells are even unable to metabolize galactose at all (Elkalaf et al., 2013; Mailloux and Harper, 2010; Mot et al., 2016). As such, the use of galactose to achieve mitochondrial dependency can be inconsistent among different cell types. Irrespective of this concern, one should realize that the use of galactose is in fact an indirect means of reducing the cellular glucose flux.

A more direct means of modulating the cellular glucose flux was evaluated by culturing cells in physiologic or low glucose conditions. The Crabtree effect remains clearly present in cells cultured at physiological levels of glucose. And although the passage of glucose through the cell-membrane is often the most rate-limiting step (Murray et al., 2000), it might not be surprising that the Crabtree effect persists at physiological glucose concentrations as the affinity of glucose for GLUTs (with exception of GLUT2, Km ± 20 mM) is relatively high (GLUT1, Km ± 2 mM; GLUT3, Km 1–2 mM; GLUT4, Km ± 5 mM) (Holman, 2020). Few studies identified a metabolic switch from glycolysis towards OXPHOS in hypoglycemic culture conditions. However, this presumably reflects a state after which all the available glucose is consumed, and cells subsequently consume other substrates that recruit OXPHOS for ATP generation. This notion is supported by *Mot et al.* and *Arend et al.* who state that cells in hypoglycemic conditions prefer glycolysis above OXPHOS until glucose is no longer available (Arend et al., 2019; Mot et al., 2016). Although the *Km* for glucose for hexokinase is only 0.05 mM (Murray et al., 2000), the affinity of glucose for the glucose transporters can be assumed to be the limiting factor. In this light, it could be argued that achieving a Crabtree circumventing state requires almost total depletion of glucose from the cell culture medium.

Total depletion of glucose from the cell culture medium requires the inclusion of alternative metabolic fuel sources, and reported alternatives

include the use of pyruvate, lactate, glutamine or fatty acids as single metabolic fuels. While this can be considered a feasible way of circumventing the Crabtree effect, it should be stressed that this strategy leads to complete elimination of the glycolytic pathway, and thus interferes with the metabolic flexibility of mammalian cells. This may explain the observed cessation in cell proliferation and differentiation when cells are cultured in the absence of glucose (Denoon et al., 2020; Mot et al., 2016).

An alternative strategy of regulating the glycolytic flux is with use of glycolytic inhibitors. In the context of the Warburg phenomenon many glycolytic inhibitors, targeting various levels of the glycolytic pathway, have been evaluated. So far, the variety of glycolytic inhibitors evaluated in the context of Crabtree is limited, and well-known inhibitors such as 3-bromopyruvate and 3-(3-pyridinyl)-1-(4-pyridinyl)-2-propen-1-one (3PO) are not yet studied. Although these inhibitors would also be effective in limiting the glycolytic flux, one should realize that it results in metabolic inflexibility.

In summary, circumventing the Crabtree effect is possible by adjustment of external culture conditions. Alternatively, few studies focused on internal genetic reprogramming as a strategy to circumvent the Crabtree effect (i.e. inhibition of HIF1α, anti-TGF-β-antibodies, H2S and irisin). Although these strategies are promising, as it internally changes the setpoints but at the same time preserves the cellular metabolic flexibility, interventions are possible cell specific, reports are heterogenous and external validation is missing.

5. Conclusions

In conclusion, the Crabtree effect is a major hurdle in cell based, metabolic studies. Circumvention of the Crabtree phenomenon is possible and can be achieved by several strategies. The choice of strategy, however, should be dictated on the scientific question. For research focusing on mitochondria, it is feasible to meet mitochondrial dependence by substrate modulation in which cells are forced to rely on OXPHOS as a result of direct or indirect inhibition of glycolysis. However, the need for persistent inhibition of glycolysis interferes with restoration of normal metabolic flexibility, and would therefore severely impact studies focusing on metabolic flexibility. In this respect, it would be more preferable to use galactose-medium or metabolic reprogramming strategies.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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