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Review

Unsuspected task for an old team: Succinate, fumarate and other Krebs cycle acids in metabolic remodeling

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ABSTRACT

Seventy years from the formalization of the Krebs cycle as the central metabolic turntable sustaining the cell respiratory process, key functions of several of its intermediates, especially succinate and fumarate, have been recently uncovered. The presumably immutable organization of the cycle has been challenged by a number of observations, and the variable subcellular location of a number of its constitutive protein components is now well recognized, although yet unexplained. Nonetheless, the most striking observations have been made in the recent period while investigating human diseases, especially a set of specific cancers, revealing the crucial role of Krebs cycle intermediates as factors affecting genes methylation and thus cell remodeling. We review here the recent advances and persisting incognita about the role of Krebs cycle acids in diverse aspects of cellular life and human pathology.

1. Krebs cycle(s)

As depicted by Hans Adolf Krebs before the Second World War, the mitochondrial catabolism of organic acids is structured around a one-piece cycle, the so-called tricarboxylic acid cycle, also known as the Krebs cycle [1]. However, this proposal should be reexamined to take into account the kinetic split that isolates two segments in the cycle *in vivo* (Fig. 1) [2]. The occurrence of a shortcut resulting from the transamination reaction catalyzed by the aspartate aminotransferase actually allows the two independent segments to function at different rates. This entanglement links amino and organic acid catabolism and confers a key function to the glutamate/aspartate couple in controlling the overall kinetic of Krebs cycle acids (KCA) conversion. An additional level of complexity results from the subcellular distribution of Krebs cycle protein components. While all Krebs cycle enzymes are found in the mitochondrial matrix, a subset of these enzymes are also found, variably according to tissues, in the cytosol with yet unknown functions in most cases [3]. The subcellular compartmentation of the enzymes is combined with a discriminating permeability of the mitochondrial inner membrane towards each KCA [4]. In response to adverse conditions, part of the Krebs cycle enzymes may also functionally associate with additional enzymes. Thus, the α-ketoglutarate dehydrogenase using the NAD^+ generated by the mitochondrial diaphorases may provide succinyl CoA to the succinyl CoA ligase, allowing for an ATP generation in the case of respiratory chain complex I blockade [5]. Hence, it is probably wise to consider that the organization and function of the Krebs cycle is not unique and static but is modulated to fit the fluctuating metabolic demand of each cell type.

To ensure this flexibility, a set of genes encoding the components of the cycle is available in the human genome. Both concerted and individual regulations have been reported to modulate the expression of these genes, making use of the full panoply of regulatory processes, including control by miRNAs with indirect (e.g., miR-378 through PGC-1β) [6] or direct (e.g., miR-183 on IDH2) [7] actions on the members of the Krebs cycle [8].

Flux through the Krebs cycle is determined by both enzyme activities and substrate concentrations. Except under peculiar conditions, including skeletal muscle under intensive exercise, the capacity of the Krebs cycle enzymes exceeds the need as does the respiratory chain, allowing to face variable feeding of substrates and variable cell energetic demand [9,10].

The handling of the KCA within the mitochondria is not independent of the cytosolic fate of these acids. The active malate–aspartate shuttle is

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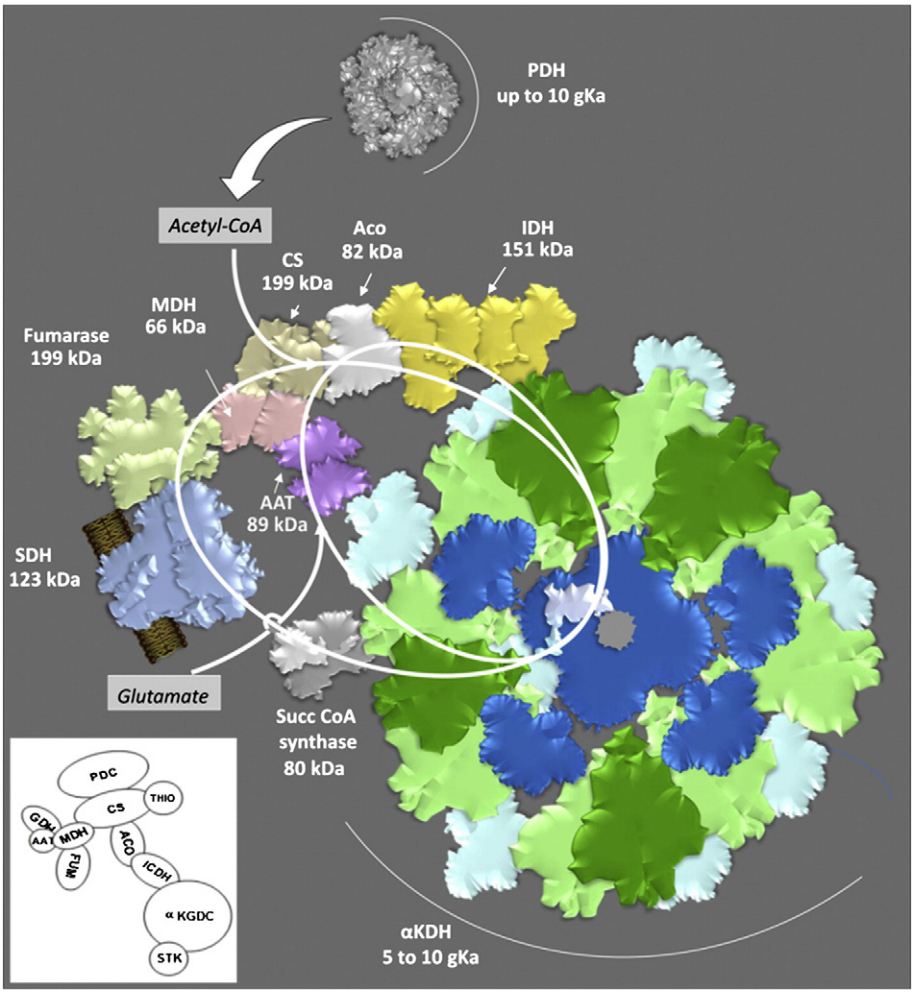

Fig. 1. A schematized view of the Krebs cycle(s) emphasizing the respective size of cycle(s) components and their arrangement as metabolons. Large white circles indicate different metabolic fluxes observed through Krebs cycle segments. The acetylCoA and the glutamate inputs are also indicated in white. The active, polymeric forms of enzymes are depicted with the corresponding molecular weights predicted from the human amino acid sequences (human mitochondrial protein database; http://bioinfo.nist.gov/hmpd/) after presequence cleavage. Protein structures and other data are derived from the RCSB PDB-101 (protein data base; http://www.rcsb.org/pdb/). Inset: The metabolon build from the Krebs cycle components as proposed by P. Srere in 1987 [67]. Abbreviations: AAT, aspartate aminotransferase (EC 2.6.1.1); Aco, aconitase (EC 4.2.1.3); CS, citrate synthase (EC 2.3.3.1); Fum, fumarate hydratase (EC 4.2.1.2); GDH, glutamate dehydrogenase (EC 1.4.1.2); IDH, isocitrate dehydrogenase (EC 1.1.1.41); αKDH (αKGDC), α-ketoglutarate dehydrogenase multienzyme complex (multiple copies of EC 1.2.4.2, EC 2.3.1.61 and EC 1.8.1.4); MDH, malate dehydrogenase (EC 1.1.1.37); PDH (PDC), pyruvate dehydrogenase multienzyme complex (multiple copies of EC 1.2.4.1, EC 2.3.1.12 and EC 1.8.1.4); Succ CoA synthase (STK), succinyl CoA synthase (EC 6.2.1.5); SDH, succinate dehydrogenase (EC 1.3.5.1).

widely admitted to act as a transfer mechanism for reduced equivalents from mitochondrial matrix NADH to cytosolic NAD^+ [11]. However, taking into account the newly described roles of KCA in the cytosol, their proper distribution in the cytosol ensured by this shuttle is presumably of crucial importance as well. There is actually, outside of mitochondria, a plethora of targets for KCA, possibly acting as primary substrates, signal molecules or actors of post-translational modifications (PTMs).

2. Krebs cycle acids and post-translational modifications

PTMs include the acetyl-CoA-dependent acetylation of either the N-terminus or at protein lysine residues [12–14]. Proteins can be also modified by succinylation whereby a succinyl group (–CO-CH2-CH2-CO–), presumably from succinyl-CoA, is added to a lysine residue (Fig. 2, bottom right) [15]. Both acetylation and succinylation of lysine residue modify the charge (from 1 to −1) with more steric hindrance in the case of succinylation. Because of this, succinylation is expected to more readily affect protein properties. In term of cellular targets, lysine modification, including acetylation and succinylation, possibly regulates numerous eukaryotic proteins involved in metabolism, cell cycle, aging, growth, angiogenesis and cancer [14], making PMTs identification an active field in proteomics research [16]. In particular, SOD1 protein is subjected to succinylation, and this appears as a critical factor for growth of lung tumor cells, an effect counterbalanced by SIRT5-dependent de-succinylation of the enzyme [17]. However, low levels of lysine succinylation are observed in eukaryotic cells and many possibly crucial sites remain unidentified. [15]. Mitochondrial matrix proteins can also be modified *via* a widely spread non-enzymatic acylation, dependent on the pH of the mitochondrial matrix and the actual concentration of acyl-CoA inside the mitochondria [18]. The presence of three matrix-located sirtuins (SIRT 3-5) with NAD^+-dependent deacetylase activity further suggests an essential role of mitochondrial protein acetylation in metabolism regulation [19,20].

The PTMs of histones are known to have a general impact on gene expression and DNA repair. Succinylation is one among these PTMs, with thirteen lysine succinylation sites described in HeLa cells so far, thus linking Krebs cycle metabolites to histone biology [21]. Cysteine is another residue that can undergo PTMs by a Michael addition (Fig. 2, top). During this reaction, one fumarate is added to the thiol group of a cysteine to form an S-(2-succinyl) cysteine [22]. An aberrant succination of proteins by fumarate has been observed in hereditary leiomyomatosis and renal cell cancer (HLRCC) syndrome associated with germ line mutations in the *FH* gene [23]. In particular, the succination of the KEAP1 protein results in the stabilization of the

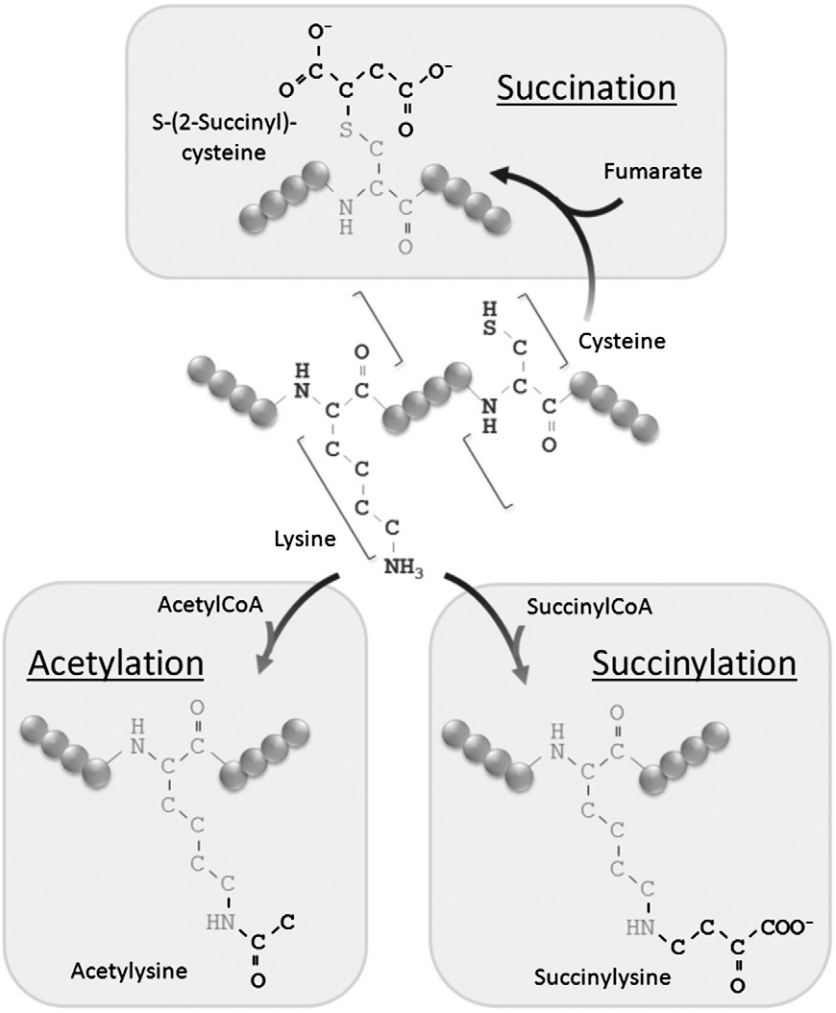

Fig. 2. Examples of protein modification involving Krebs cycle metabolites. Bottom: Acetylation resulting from acetyl CoA addition (left) and succinylation by succinyl CoA (right) on lysine residue; Top: Succination resulting from fumarate addition on cysteine residue.

transcription factor NRf2, causing the activation of a series of genes involved in the stress response [24–26]. Succination has also been suggested to play an important role in obesity and diabetes [27], where the degree of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) succination correlates with the inactivation of the enzyme [28].

3. Krebs cycle acids and their receptors

Succinate and α-ketoglutarate bind to two of the more than 800 G protein-coupled receptors on the plasma membrane of a kidney cell, GPR91 or SUCNR1 (succinate receptor 1) and GPR99 or OXGR1 (oxoglutarate receptor 1), respectively [29,30]. So far, however, physiological roles have been attributed only to SUCNR1, also expressed in retinal pigment epithelium [31], cardiomyocytes [32], blood cells [33], liver [34] and white adipose tissue [35]. In white adipose tissue, while stimulatory G-proteins favor lipolysis in response to low glucose level, when glucose is high, the SUCNR1 signaling reduces lipolysis [35] by decreasing adenylate cyclase-dependent cAMP formation. In cardiomyocytes, the succinate-activated SUCNR1 increases the activity of the protein kinase A and intra-cellular calcium affecting cell contraction, while a prolonged exposure of cardiomyocytes to succinate triggers apoptotic processes [36]. In the liver, SUCNR1, expressed exclusively in quiescent stellate cells, has been suggested to act as an early detector of hepatic stress or damage, enhancing the initial steps of stellate cell activation to restore damaged tissue in the ischemic liver [30,37]. SUCNR1 is also present in blood (hematopoietic precursor cells and many subtypes of blood and immune cells) (Fig. 3A) where it would modulate platelets aggregation and cell proliferation [33]. In retinal ganglion neurons (Fig. 3B), SUCNR1 appears to control the vascularization of the retina through VEGF [38]. In mouse kidney (Fig. 3C), SUCNR1 exerts a signaling effect in the nephron through nitric oxide and prostaglandin E2 release, favoring the excretion of renin from the granular cells of the juxtaglomerular apparatus [39]. This uncovers (in rodents) a role for succinate and SUCNR1 in the control of the glomerular hyper-filtration and the renal renin–angiotensin system. Accordingly, high succinate has been incriminated as a cause of hypertension, obesity and diabetes in rodents [39]. High succinate was however not detected in human suffering similar conditions. In a number of contexts, the exact role of SUCNR1 activation by succinate is still to be firmly established, but it appears that succinate and its receptors are frequently at work under stress conditions (ischemia, hypoxia, metabolic syndrome, diabetes, etc.). It can thus be hypothesized that the receptor acts as a sensor for the "stress-signaling metabolite" succinate, which concentration inversely varies with oxygen concentration, increasing with hypoxia and during exercise, or under various disease conditions [30].

4. Krebs cycle acids, hydroxylases and demethylases

In the cytosol, α-ketoglutarate and succinate can also be channeled to a class of specific non-heme iron oxygenases, the so-called Fe(II)/α-ketoglutarate-dependent dioxygenases. This family of enzymes

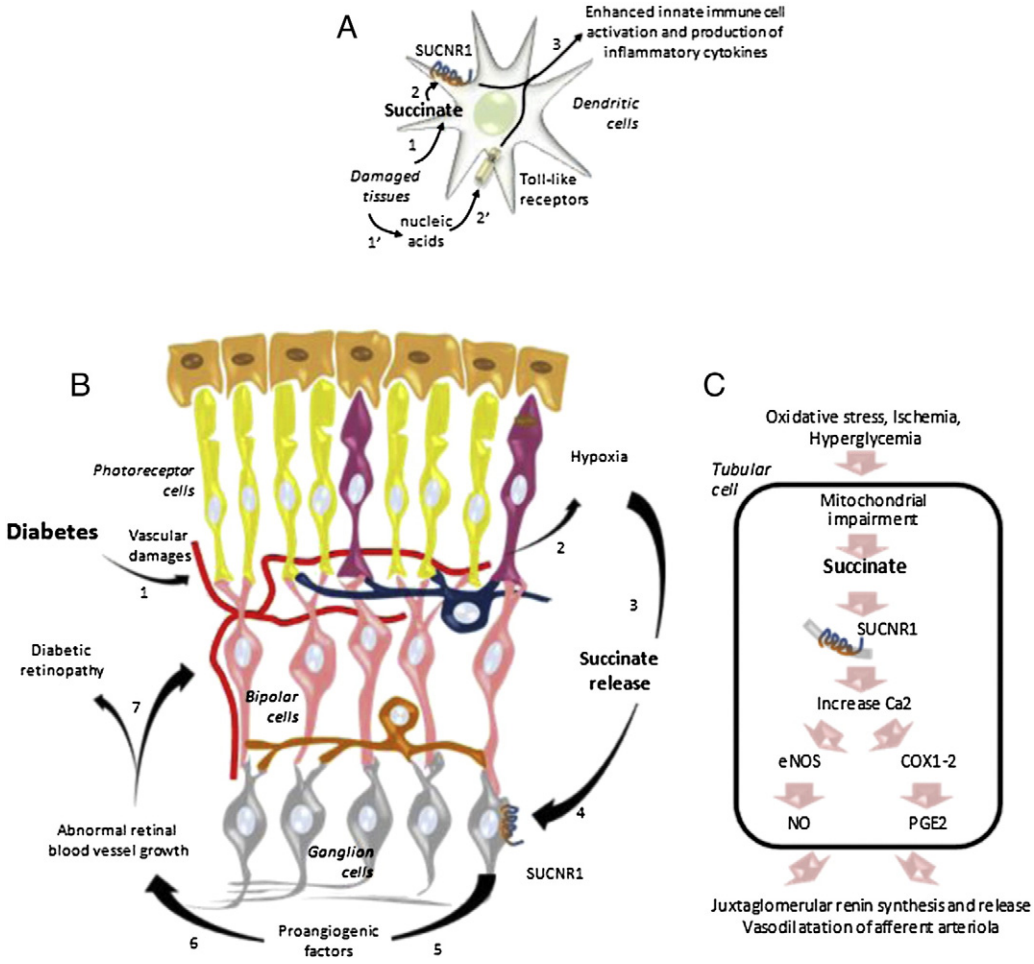

Fig. 3. Examples of succinate receptor SUCNR1 (GPR91) implication in stress response in various tissues. (A) Upon impairment of mitochondrial activity of kidney tubular cells by various type of stresses, released succinate binds to SUCNR1 increasing cell calcium which activates cyclo-oxygenase 1,2 (COX 1,2) and endothelial nitric oxide synthase (eNOS). This triggers the secretion of nitric oxide (NO) and prostaglandin E_2 (PGE_2) that will promote the release of renin from neighboring granular cells of the juxtaglomerular apparatus [68]. (B) Vascular damages consecutive to diabetes condition (1) tend to favor retinal hypoxia (2) which results in succinate release by photoreceptor cells (3). Upon succinate binding to the SUCNR1 of ganglion cells (4), released proangiogenic factors (5) promote anarchic blood vessel growth characteristic of diabetic retinopathy [31]. On the other hand, deficiency of the receptor has also been shown recently to result in outer retinal lesion [69]. (C) Binding of succinate released by damaged tissues (1) on SUCNR1 receptor of innate dendritic cells (2) may induce activation of immune cells and production of inflammatory cytokines (3) [70]. Similar results can be triggered by nucleic acid (1′) released from damaged tissues through Toll-like receptors presents as well on these cells (2′).

uses molecular oxygen to oxidize a primary substrate together with α-ketoglutarate to form succinate and CO_2. Because these enzymes are subject to product inhibition by succinate, their activity is largely dependent on the relative ratio of α-ketoglutarate to succinate. The inhibition of these enzymes by product accumulation has been pinpointed as the instrumental mechanism linking succinate dehydrogenase and fumarate hydratase deficiencies to tumor formation and carcinogenesis in human [40,41]. Two of these dioxygenases appear critically involved in these processes, namely, the one responsible for the hydroxylation of the HIF1α transcription factor and those involved in the control of histone and DNA demethylation.

The transcription factor HIF1 consists of two subunits: HIF1α, an oxygen-sensitive subunit, and HIF1β, a constitutively expressed subunit [42]. HIF1 binds the 5′-RCGTG-3′ consensus sequence found within or in the vicinity of the more than 1,000 HIF1-regulated genes. The stability of the HIF1α subunit is negatively regulated by oxygen, as is HIF2α, a second factor susceptible to interact with HIF1β [43]. At variance with HIF1α that is expressed in all cell types, HIF2α is expressed in a subset of human cell types, including vascular endothelial cells, hepatocytes, type II pneumocytes and macrophages [44]. In a number of cancer cells, both HIF1α and HIF2α proteins are expressed. Notwithstanding an extensive sequence homology, HIF1α and HIF2α have non-overlapping and sometimes even opposing roles [45]. Under normoxic conditions, provided α-ketoglutarate is available, HIF1α is hydroxylated by a prolyl hydroxylase at conserved proline residues (Pro-403 or Pro-564 in human HIF1α). This allows HIF1α recognition and ubiquitination by the von Hippel–Lindau (VHL)/E3 ubiquitin ligase and its rapid degradation by the proteasome (Fig. 4). As the prolyl hydroxylase requires molecular oxygen, the reaction is decreased under hypoxic conditions, resulting in HIF1α stabilization. The accumulation of succinate resulting from a succinate dehydrogenase deficiency causes a similar decrease of the enzyme activity leading to HIF1α stabilization [40,46]. Noticeably, the accumulation of the structurally related fumarate molecule resulting from a fumarate hydratase defect also causes the inhibition of the prolyl hydroxylase reaction [47]. When stabilized, HIF1α up regulates a number of the genes insuring cell resistance to low-oxygen conditions. Thus, even under normoxic conditions, succinate accumulation up regulates a similar set of genes, especially those involved in the vascularization and facilitated oxygen distribution in tissues, as vascular endothelial growth factor (*VEGF*) [48]. Controlled by HIF1 stabilization, this angiogenesis provides favorable conditions for tumor growth and carcinogenesis. Accordingly, germ line loss-of-function mutations in the four genes encoding subunits A-D or assembly factor (SDHAF2) of the SDH cause hereditary paraganglioma/

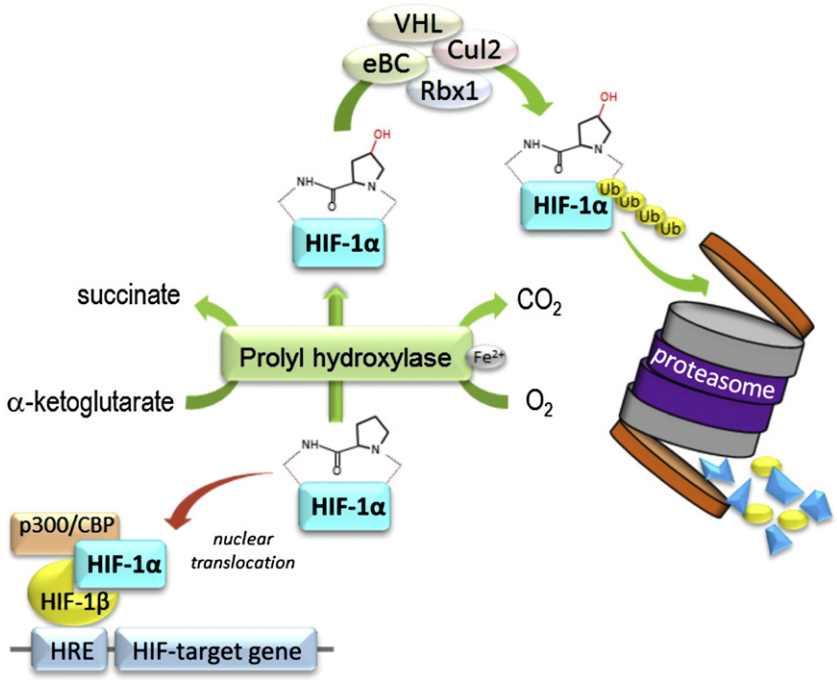

Fig. 4. The control of prolyl hydroxylase activity by organic acids. The continuous degradation of the HIF1α protein by the proteasome (left) is dependent on its ubiquitination by a protein complex (VHL, eBC, Cul2 and Rbx1; top). This ubiquitination is made possible by the initial oxygen-dependent hydroxylation of the HIF1α protein by the prolyl hydroxylase. Under anaerobic conditions, the HIF1α protein translocates to the nucleus where it participates with HIF1β and p300/CBP proteins in the transcriptional activation of a set of genes possessing the hypoxia response element (HRE; bottom). Because α-ketoglutarate and succinate are respectively substrate and product of the prolyl hydroxylase, the activity of this latter enzyme is dependent *in vivo* on the respective concentrations of these two Krebs cycle acids [41]. Abbreviations: HIF, hypoxia-inducible factor; eBC, ElonginB/C; VHL, Von Hippel–Lindau; Cul2, CULLIN 2; Rbx1, RING-box protein 1).

pheochromocytoma syndrome (HPGL/PCC), and these genes are now recognized as tumor suppressor genes [49–51]. The stabilization of HIF1 resulting from KCA accumulation provides a first mechanism linking tumoral processes and the balance between Krebs cycle intermediates, α-ketoglutarate, succinate and fumarate.

More recently described DNA alterations in tumors related to Krebs cycle enzyme defect led to the identification of demethylases as additional targets for KCA and their derivative (R-2-hydroxyglutarate). The observed abnormal methylation of DNA, with an overall hypomethylation of genes and hypermethylation of CpG islands, is a recognized hallmark of cancer, causing transcriptional silencing of a series of tumor suppressor genes [52,53]. In 2011, a hypermethylation of CpG islands [54] has been linked to gain-of-function mutations in *IDH1* and *IDH2* [55]. *IDH* mutations are responsible for diffuse and anaplastic gliomas and secondary glioblastomas, specific types of cartilaginous tumors and leukemias [56]. These oncogenic gain-of-function mutations cause the IDH enzymes to produce (R)-2-hydroxyglutarate from α-ketoglutarate [57]. The 2-hydroxyglutarate acts as a competitive inhibitor of α-ketoglutarate-dependent histone demethylases and of the TET family of 5-methyl-cytosine hydroxylases (Fig. 5). The production of 2-hydroxyglutarate thus results in alterations of the overall DNA methylation in the patient tumoral tissues [54]. Similar targeting of histone and DNA demethylases by accumulated succinate or fumarate has been shown to account for the hypermethylation phenotype observed in paragangliomas/pheochromocytomas resulting from *SDH* and *FH* gene mutations [58]. Thus, succinate, fumarate and (R)-2-hydroxyglutarate are now referred to as "oncometabolites".

5. Krebs cycle acids and differentiations

Finally, by changing the activity of several metabolic pathways, KCA might be important players among factors promoting and regulating differentiation processes. For example, citrate exported from the mitochondria has been shown to favor lipid synthesis, with a number of consequences on cell fate [59]. The cellular citrate concentration is presumably of crucial importance in a number of instances [59]. In keeping with this, while investigating the metabolic enzyme equipment of stem cells, we recently observed that the activity of the couple aconitases/isocitrate dehydrogenases determining cellular citrate concentration profoundly differs between mesenchymal and neural stem cells (Fig. 6). This observation echoes the metabolic alterations known to underlie differentiation of mesenchymal cells into functional osteoblasts, which are dependent on a change from glycolytic to oxidative metabolism [60], and the fine tuning of the activity of the Krebs cycle enzymes [48]. Likewise, the changes in gene expression observed during the maturation of mouse 3T3L1 cells to adipocytes or the differentiation of the parasite *Toxoplasma gondii* have been ascribed to histone succination, acetylation, [61,62] and succinylation [63], respectively. Finally, PTMs, including acetylation and methylation, are now recognized as key factors regulating the pluripotency of human stem cells [64,65].

6. Concluding remarks

Through this short overview of the many functions now recognized to organic acids of the Krebs cycle, it appears that we actually deal with major actors of numerous aspects of cell life. More than the traditional view of their role as reducing power sinks for the respiratory chain and providers of carbon skeletons for anabolism, KCA now appears to be important signaling molecules and instrumental actors for the regulation of gene expression. There is possibly more to learn about the new and unexpected functions of this "old team" of actors. However, their implication in the remodeling of cells under pathological conditions is now firmly established, e.g., cancer [66]. Likewise, considering the nature of the mechanism pinpointed through the studies of cancer cells and tissues, i.e., the effect of KCA on key enzymes controlling signaling cascade(s) and gene expression, one is led to admit that this mechanism

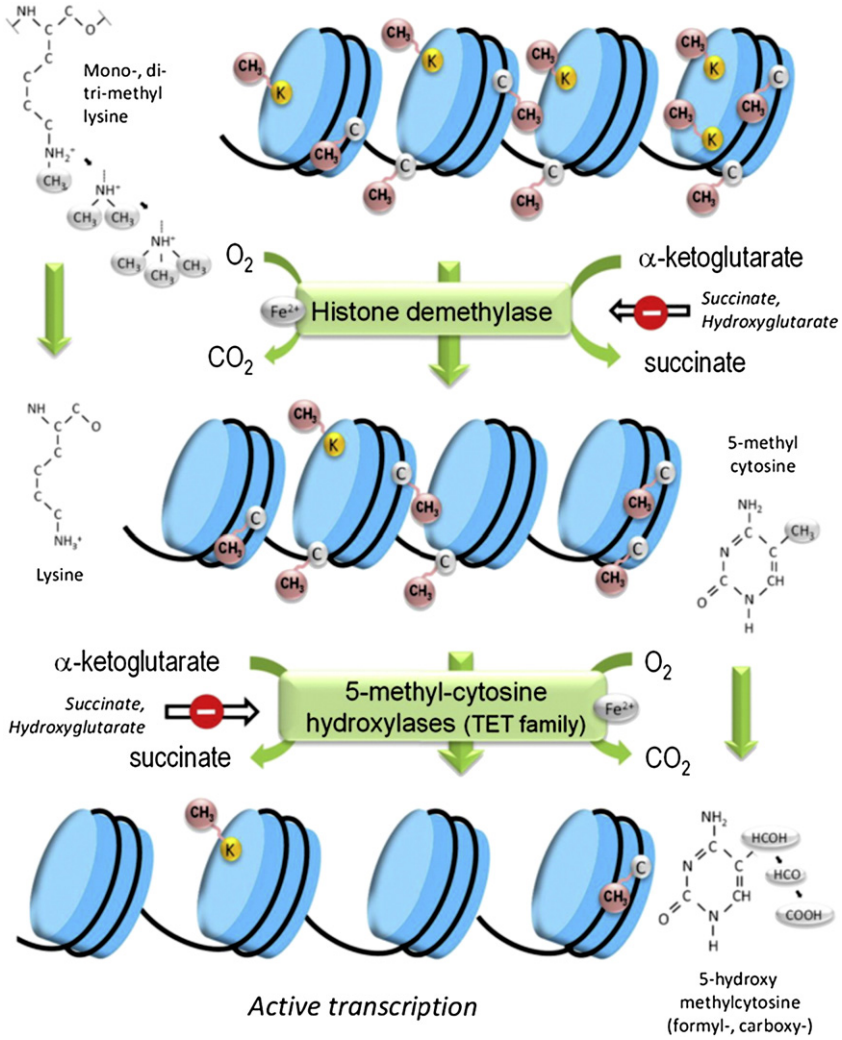

Fig. 5. Histone demethylase and DNA hydroxylase as targets of organic acids. Both di-oxygenase-catalyzed reactions use α-ketoglutarate and succinate as substrate and product, respectively, implicating control of their reaction rates by the respective concentrations of these acids, or by any interfering acids, e.g., hydroxyglutarate produced by mutant IDH enzymes. The histone demethylase reaction (top) modulates the variable methylation (top left) of lysine residues of the histone protein. The hydroxylase of the TET family (bottom) modulates the methylation of the DNA cytosine residue resulting in variable oxidation products (bottom right). Changes in the activity of these two dioxygenases triggered by succinate or hydroxyglutarate accumulation resulting from SDH or IDH mutations, respectively, have been shown to modulate the transcriptional activity of a set of genes and to be instrumental for tumor formation [54,58].

is presumably at work under non-pathological conditions as well. By similar mechanisms, organic acids of the Krebs cycle would not only control pathological remodeling but also direct physiological modeling. This finally closes the loop involving genes, non-coding RNAs, proteins and now metabolites in the interplay ensuring proper organismal development and adaptation.

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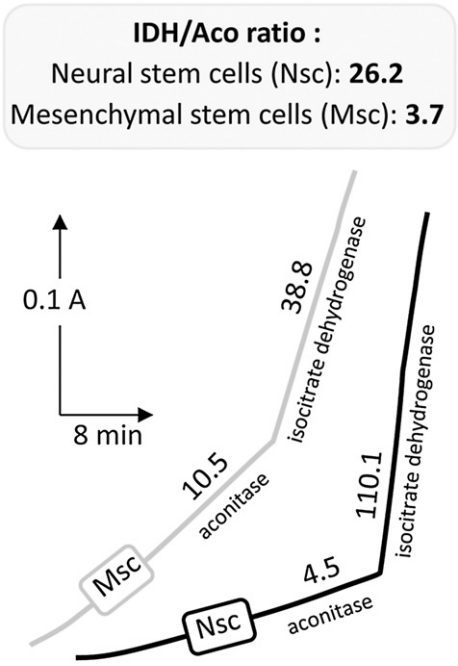

Fig. 6. Stem cell differentiation is accompanied by a profound modification of Krebs cycle enzyme activity. Upon the derivation from human-induced pluripotent stem cells (iPSCs), the enzyme equipment controlling Krebs cycle acids, i.e., aconitase and isocitrate dehydrogenase (IDH), strongly diverges between mesenchymal and neural stems cells, resulting in a 7-fold difference in activity ratios. Numbers along the traces are nmol/min/mg prot. Enzyme activity measured as previously described [71].

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