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Proline metabolism shapes the tumor microenvironment: from collagen deposition to immune evasion

Emily J Kay[1], Sara Zanivan[1,2] and Alessandro Rufini[3,4]

Proline is a nonessential amino acid, and its metabolism has been implicated in numerous malignancies. Together with a direct role in regulating cancer cells' proliferation and survival, proline metabolism plays active roles in shaping the tumor microenvironment (TME). Cancer-associated fibroblasts (CAFs) display high rates of proline biosynthesis to support the production of collagen for the extracellular matrix (ECM). Indeed, impaired proline metabolism in CAFs results in reduced collagen deposition and compromises the growth and metastatic spread of cancer. Moreover, the rate of proline metabolism regulates intracellular reactive oxygen species (ROS) levels, which influence the production and release of cytokines from cancer cells, contributing toward an immune-permissive TME. Hence, targeting proline metabolism is a promising anticancer strategy that could improve patients' outcome and response to immunotherapy.

Addresses
[1] CRUK Beatson Institute, Switchback Road, Glasgow G61 1BD, UK
[2] School of Cancer Sciences, University of Glasgow, Switchback Road, Glasgow G61 1QH, UK
[3] Dipartimento di Bioscienze, University of Milan, Via Celoria 26, 20133 Milan, Italy
[4] Leicester Cancer Research Centre, University of Leicester, Leicester LE1 7RH, UK

Corresponding authors: Zanivan, Sara (s.zanivan@beatson.gla.ac.uk), Rufini, Alessandro (Alessandro.Rufini@unimi.it)

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Introduction

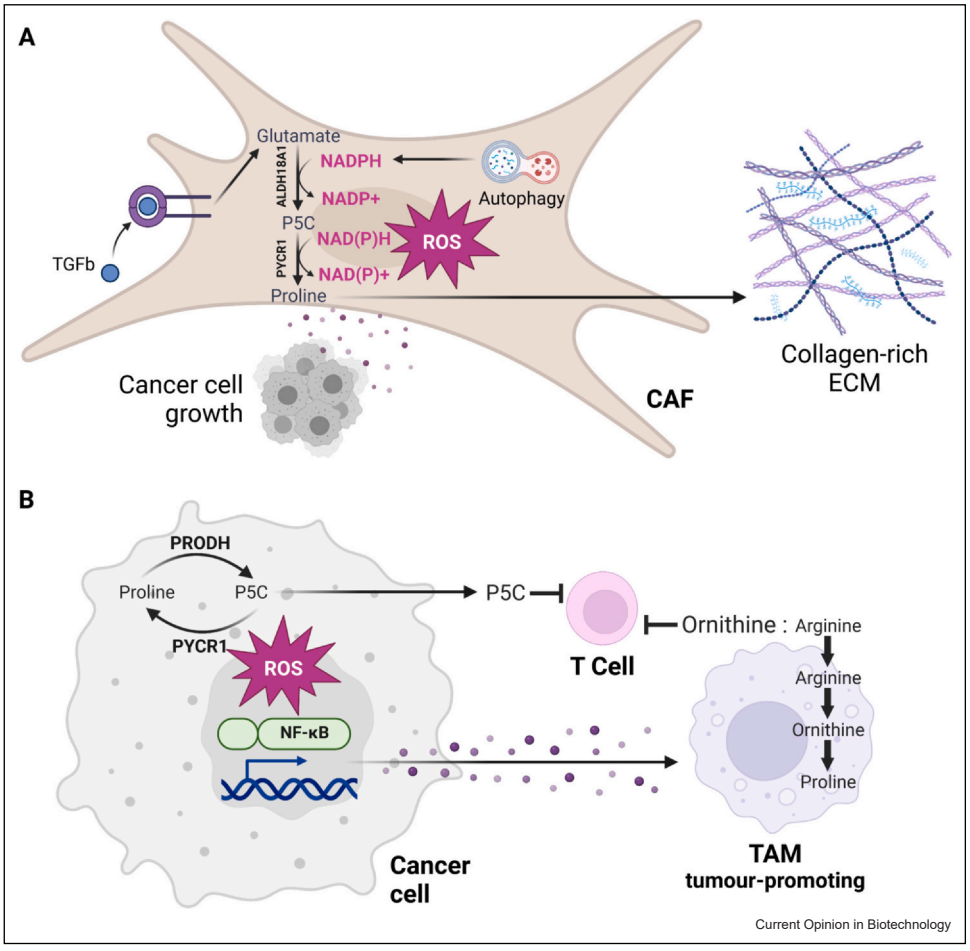
Proline is a nonessential amino acid with a unique structure. Owing to the nitrogen group binding the alpha carbon to form a five-membered ring, proline lacks the primary amine group present in other amino acids and instead has a secondary amine. This unique conformation grants L-proline essential properties that influence the 3D structure of proteins. Proline is synthesized from glutamine and ornithine through the common precursor pyrroline-5-carboxylate (P5C). Ornithine is converted to P5C by the mitochondrial enzyme ornithine aminotransferase (OAT), whereas glutamine-derived glutamate is converted to P5C by the mitochondrial 1-pyrroline-5-carboxylate synthase (P5CS, encoded by *ALDH18A1* gene). Then, P5C is reduced to proline by one of the nicotinamide adenine dinucleotide (phosphate) (NAD(P)H)-dependent P5C reductases (P5CRs, encoded by the pyrroline-5-carboxylate reductase, *PYCR* genes): mitochondrial PYCR1 and PYCR2, and cytosolic PYCR3. The first step in the catabolism of proline is the flavin adenine dinucleotide (FADH)-dependent oxidation of proline to P5C catalyzed by the proline dehydrogenase enzyme (PRODH). P5C is then converted to ornithine by OAT or oxidized to glutamate by the NAD-dependent mitochondrial enzyme P5C dehydrogenase (P5CDH, encoded by *ALDH4A1* gene) [1].

Proline metabolism has been widely associated with cancer. Many studies highlighted a direct, cell-autonomous role of proline biosynthesis or catabolism in regulating the growth and survival of cancer cells [1]. However, *in vivo*, cancer cells exist within a complex tumor microenvironment (TME) consisting of a collagen-rich extracellular matrix (ECM) and different cell types, such as cancer-associated fibroblasts (CAFs), endothelial, and immune cells. Proline metabolism regulates the crosstalk between cancer cells and the TME, influencing tumor progression and response to therapy. Intuitively, proline metabolism contributes toward the synthesis of proline-rich collagen, but it also shapes the immune TME and the efficacy of immune therapy in cancer patients (Figure 1). Here, we summarize the diverse functions of proline metabolism relevant to the TME and consider the implications for targeting proline metabolism in cancer therapy.

Proline metabolism in stromal-derived extracellular matrix

CAFs produce ECM proteins. ECM acts as a physical barrier to prevent drug delivery and infiltration of immune cells, it directly influences immune cell phenotype, and fuels cancer cell growth and metastatic dissemination [2]. Collagen proteins are abundant in CAF-derived ECM and are rich in proline owing to the repeating Gly–Pro–Hyp

Figure 1



Schematic summary of the main roles of proline metabolism in the TME. **(a)** Both autophagy and TGF-β signaling increase proline biosynthesis in fibroblasts to stimulate collagen production for ECM deposition. The production of proline is tightly linked to cellular redox potential and ROS production through regulation of NAD(P)+/NAD(P)H ratio. **(b)** Proline metabolism regulates the crosstalk between tumor cells and immune cells. By regulating intracellular ROS levels in cancer cells, proline metabolism can activate the transcription factor and master regulator of inflammation NF-kB, thus leading to production and secretion of cytokines that promote a pro-tumorigenic polarization of macrophages. Moreover, metabolites in the proline metabolic pathway, namely P5C and ornithine, have been suggested to behave as immunosuppressive molecules, inhibiting the activation or proliferation of T lymphocytes.

motif. Early studies connecting collagen to proline were performed in the context of wound healing, a process relying on collagen production and where skin fibroblasts are activated similarly to CAFs. Fibroblasts preferentially synthesize their own proline from glutamine rather than using extracellular proline [2,3]. However, proline supplementation stimulates collagen synthesis in human skin fibroblasts, accelerating wound healing in rats [4], and an oral supplement containing proline and arginine accelerates wound healing in humans [5]. Interestingly, fibroblasts at different stages of wound healing correspond with different CAF subpopulations [6]. Fibroblasts from the early wound resemble the myCAF subpopulation in tumors, which are myofibroblastic and enriched for collagen and ECM production [7]. In breast cancer, PYCR1 is upregulated in CAFs compared with normal fibroblasts, particularly in the myCAF subpopulation [8]. PYCR1 levels are also upregulated in wound-associated fibroblasts during the proliferative phase when collagen production peaks [6], and in fibrotic diseases where collagen production is high [9], emphasizing how the upregulation of enzymes in the proline synthesis pathway is a universal response to increase collagen production in fibroblasts. In addition, proline synthesis is directly linked to collagen production in mammary CAFs. Depletion of proline by genetic and pharmacological inhibition of PYCR1 induces ribosome stalling on collagen mRNA, thus reducing collagen production. Collagen levels are restored by supplementation of supraphysiological levels of proline, confirming that CAFs rely on proline to maintain translation of collagen proteins. Co-transplantation of cancer

cells and PYCR1-depleted CAFs into mice reduces the amount of collagen in tumors and decreases tumor growth and metastatic spread [8]. NIH-3T3 fibroblasts acutely stimulated by trasforming growth factor (TGF)-β upregulate collagen production and proline synthesis from glutamine (Figure 1a). In this instance, however, individual deletion of PYCR enzymes has no effect on proline or collagen levels, whereas deletion of the *ALDH18A1* gene, encoding for the upstream enzyme P5CS, reduces collagen production, which is rescued by the addition of exogenous proline [10]. Furthermore, co-transplantation of *Aldh18a1* knockout CAFs with pancreatic adenocarcinoma (PDAC) cancer cells diminishes intratumoral collagen [11]. Similarly, in PDAC, when CAFs are deactivated by inhibiting autophagy or mitophagy under low glucose, there is a corresponding decrease in proline and collagen synthesis. Collagen levels are restored by proline supplementation, suggesting that autophagy controls collagen production indirectly by promoting proline biosynthesis (Figure 1a) [11]. Interestingly, targeting proline synthesis in CAFs likely has wide-ranging effects on the CAF phenotype other than collagen production and affects translation of other proteins that maintain a pro-tumorigenic CAF phenotype, as conditioned media from *Aldh18a1* knockout CAFs reduces cancer cell proliferation independently of collagen. Notwithstanding, in PDAC, collagen within the ECM promotes growth of cancer cells by acting as a reservoir of proline. Indeed, especially in low-nutrient conditions with limited glutamine availability, PDACs catabolize collagen-derived proline through PRODH1 to fuel the tricarboxylic acid cycle (TCA) cycle [12].

A mediator of proline biosynthesis rate in CAFs is Kindlin-2, a protein first identified as an integrin binder at focal adhesions. In both lung adenocarcinoma and idiopathic lung fibrosis, a disease in which fibroblasts are activated similarly to CAFs and produce excess ECM, PYCR1 forms a complex with Kindlin-2, which can translocate into the mitochondria [9,13]. Ablation of Kindlin-2, either from lung adenocarcinomas or from lung fibroblasts, reduces proline synthesis and collagen production, suggesting that this complex formation enhances PYCR1 activity, although the mechanism for this is still unclear. Additionally, in lung adenocarcinoma, the interaction of Kindlin-2 with PYCR1 is stimulated by increased ECM stiffness, suggestive of a positive-feedback loop whereby increased ECM stiffness reinforces proline synthesis and ECM production.

The upregulation of proline synthesis to sustain collagen production is a common mechanism in fibroblasts. Although the specific point in the pathway best-suited to intervention may vary depending on the model and disease, there are now several potential targets to reduce proline synthesis and pro-tumorigenic collagen in the TME, either by directly targeting proline synthetic enzymes or interacting proteins.

Proline as a redox regulator in the tumor microenvironment

Another function of the proline metabolic pathway is to maintain redox balance in cells. Proline synthesis results in a net production of NAD(P)+, whereas its degradation produces NAD(P)H. Proline cycling can therefore be used to balance the redox state of the cell and protect against ROS (Figure 1). This property of proline metabolism is very important in the TME, which experiences high levels of ROS and hypoxia. A burst in intracellular ROS levels follows TGF-β activation in NIH-3T3 fibroblasts, but is quenched as proline synthesis is subsequently activated. Importantly, fibroblasts with *ALDH18A1* deletion and impaired proline synthesis are unable to resolve TGF-β-induced ROS [10]. In this instance, TGF-β promotes collagen biosynthesis for ECM deposition and increases mitochondrial oxidation to meet the consequent bioenergetic demand. In parallel, TGF-β signaling activates proline biosynthesis from glutamine with two purposes: newly synthesized proline is incorporated into nascent collagen fibers and increased flux through the proline pathway averts excess mitochondrial redox potential and ROS production through regeneration of oxidized NAD^+ [10]. As discussed above, autophagy- or mitophagy-deficient CAFs from PDAC display reduced collagen production because of decreased proline synthesis [11]. This results from an altered redox balance, as autophagy/mitophagy inhibition reduces mitochondrial NADPH generation via impaired activity of NADK2, suggesting that proline synthesis is downregulated to conserve the NADPH/NADP+ balance [11,14,15].

Proline metabolism and the immune tumor microenvironment

Proline metabolism shapes the immune TME by influencing the crosstalk between cancer cells and immune cells (Figure 1b). PYCR1 is a prognostic factor in clear-cell renal cell carcinoma (ccRCC) [16–18], where high expression of PYCR1 correlates with a higher risk of resistance to immunotherapy and increased infiltration of immunosuppressive myeloid-derived suppressor cells, Tregs, and tumor-infiltrating macrophages (TAMs) [17]. PYCR1 also promotes the polarization of TAMs toward a pro-tumorigenic subtype in oral squamous cell carcinoma. Kuo and colleagues [19] showed that the expression of the mitochondrial chaperone Lon in cancer cells increases ROS production, which in turn activates a cascading signaling involving the p38 mitogen activated protein kinase (MAPK) and the pro-inflammatory transcription factor Nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB). This signaling cascade increases production and secretion of cytokines from cancer cells, including IL-4, IL-6, and IL-13, which are known to steer TAMs toward a tumor-promoting phenotype [20–22]. Although the role of PYCR1 in the

production of pro-inflammatory cytokines was not tested directly, PYCR1 was necessary, through direct interaction with the Lon chaperone, for the increase in ROS levels that triggered the pro-inflammatory phenotype of cancer cells [19]. Of note, TAMs can have a direct role in shaping the tumor ECM [23], as increased matrix stiffness synergizes with TGF-β signaling in inducing a collagen–ECM signature in TAMs. Consequently, stiffness-induced TAMs engage in collagen biosynthesis, contributing toward ECM remodeling and increased stiffness and promoting the physical exclusion of cytotoxic T lymphocytes from the TME. Hypoxia has also been associated with an immune-depleted TME, through multiple mechanisms [24]. Of note, hypoxia also promotes proline biosynthesis through increased expression of *ALDH18A1* and *PYCR1* and alters collagen deposition and matrix stiffness [25–31]. It is therefore possible that changes to the TME might contribute to the immune depletion in hypoxic tumors. Proline catabolism influences cytokine production in cancer cells as well. In lung cancer, PRODH expression is increased and associated with poor prognosis [32]. One mechanism by which PRODH enhances lung cancer progression is by stimulating the production of pro-inflammatory cytokines Cxcl1, Lcn2, and IL17C in a ROS-dependent fashion. Expression of the indicated cytokines also predicts poor survival in patients with lung cancer. Mechanistically, PRODH induces the formation of ROS, which in turn are responsible for the phosphorylation of the IκB kinase (IKK)α kinase, which is part of the IKK complex that activates NF-kB. Phosphorylated IKKα within the nucleus directly regulates the transcription of pro-inflammatory cytokines. Although a direct activation of NF-kB has been observed in PRODH-overexpressing lung cancer cells, its role in the stimulation of cytokine production has not been characterized [32]. Notwithstanding, the observations in lung cancer resemble the PYCR1/ROS/NF-kB axis responsible for the release of pro-inflammatory cytokine in squamous carcinoma. More tellingly, the ability of PRODH to stimulate cytokine transcription is abolished in the absence of PYCR enzymes. Indeed, although PRODH is primarily responsible for ROS production, PYCR enzymes are necessary to replenish proline from P5C to sustain PRODH activity [32]. This suggests the existence of a futile proline–P5C cycle in cancer cells, which fuels ROS production and the activation of pro-inflammatory NF-kB to influence the immune TME. An alternative ROS-dependent mechanism by which proline metabolism promotes immune evasion was discovered in prostate cancer, where high PRODH expression in xenografted cancer cells increases tumor growth and reduces T-cell infiltration in the TME [33]. In this instance, high levels of PRODH expression in cancer cells lead to the secretion of its metabolite product P5C into the TME. P5C then acts as an immunosuppressive metabolite that inhibits the proliferation, but not the activation, of T lymphocytes, by stimulating ROS production through inhibition of complex III of the mitochondrial electron transport chain. The immunosuppressive role of P5C might be compounded by another metabolite from the proline metabolism pathway, namely ornithine. Indeed, proline biosynthesis from both glutamine and arginine in TAMs is stimulated by the combined action of ECM stiffness and TGF-β signaling. Of note, this metabolic rewiring alters the metabolite ratios within the TME, leading to decreased arginine and increased ornithine content in tumors with stiff ECM. Arginine is an essential metabolite that contributes to the activation of cytotoxic T lymphocytes [34,35]. So, its decrease can impair antitumor immunity in cancer with a stiff ECM. Moreover, ornithine exerts a direct immunosuppressive activity by reducing the efficacy of immune checkpoint blockade therapy and by inhibiting the cytotoxic activity of T lymphocytes [23].

Conclusions

Proline metabolism sustains tumorigenesis promoting the growth of cancer cells and the establishment of a permissive TME. Therefore, targeting proline metabolism is an attractive therapeutic opportunity. Since proline is one of the most abundant amino acids in collagen [10,36], inhibiting proline synthesis may reduce collagen translation and ECM production. Reducing or 'normalizing' the ECM is a promising therapeutic avenue, however, sometimes, ablating collagen can free tumors of the constraint of the ECM barrier and accelerate tumor progression [37–39]. Therefore, a balance needs to be struck between reducing and eradicating the ECM to optimize the antitumor effects and improve response to therapy. Moreover, despite the contribution of proline metabolism to immune evasion, activated T lymphocytes express P5CS, PRODH, and OAT to increase flux of glutamine toward ornithine and polyamine biosynthesis [35,40], whereas overexpression of PRODH2 augments the anticancer activity of chimeric antigen receptor-T cells [41]. Future research efforts using advanced technologies, such as spatial transcriptomics and image mass spectrometry, and focused on clarifying the different immune-regulatory roles of proline metabolism, will be fundamental to our ability to exploit this pathway to bolster immunotherapy.

CRediT authorship contribution statement

EK, SZ, and AR participated in the conception of the review text and figures. EK and AR wrote the paper. SZ prepared the figures. All authors read and commented on the article and approved of the final version.

Data Availability

No data were generated for the research described in this article.

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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- • of special interest
- •• of outstanding interest

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