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Review

Metabolic challengers selecting tumor-persistent cells

Caterina Mancini [1,3], Giulia Lori [1,3], Erica Pranzini [2,*] and Maria Letizia Taddei [1,*]

Resistance to anticancer therapy still represents one of the main obstacles to cancer treatment. Numerous components of the tumor microenvironment (TME) contribute significantly to the acquisition of drug resistance. Microenvironmental pressures arising during cancer evolution foster tumor heterogeneity (TH) and facilitate the emergence of drug-resistant clones. In particular, metabolic pressures arising in the TME may favor epigenetic adaptations supporting the acquisition of persistence features in tumor cells. Tumor-persistent cells (TPCs) are characterized by high phenotypic and metabolic plasticity, representing a noticeable advantage in chemo- and radio-resistance. Understanding the crosslink between the evolution of metabolic pressures in the TME, epigenetics, and TPC evolution is significant for developing novel therapeutic strategies specifically targeting TPC vulnerabilities to overcome drug resistance.

Highlights

Microenvironmental pressures foster intratumor heterogeneity.

Metabolic adaptations induced by the tumor microenvironment modify the epigenetic landscape of cancer cells.

Epigenetically primed cells represent the reservoir for the selection of tumor-persistent cells which may sustain the emergence of therapy-resistant clones and drive cancer recurrence.

TME-induced epigenetic heterogeneity: the breeding ground for the emergence of TPCs

Therapy resistance still represents the main obstacle in developing effective weapons in the fight against cancer. **TH** (see Glossary) is a major driver of resistance to anticancer therapy [1,2]. Cancer cells continuously deal with challenges arising in the evolving **TME** by dynamically modifying their phenotype and acquiring selective **adaptations** in response to stress. Even though several favorable interactions between cancer cells and the different components of the TME have been described, environmental alterations emerging during tumor progression represent critical challenges to be overcome. In fact, evolving microenvironmental hardships in the TME represent a double-edged sword over the process of tumorigenesis: while on one side they impair cancer-cell proliferation and survival, on the other side they represent positive selective pressures supporting the success of pre-existing persistent clones or forcing the acquisition of surviving features in resident cells [3,4] (Figure 1). Growing evidence emphasizes the crucial contribution of **epigenetic** changes in this adaptive process [5]. Indeed, epigenetic alterations significantly foster TH and sustain the generation of resistant clones in primary and metastatic sites. **TPCs** are a subpopulation of rare cells which contribute to tumor recurrence by surviving initial anticancer treatment, acquiring a slower proliferative rate, and serving as founders for disease relapse [6,7]. TPC populations include cells adopting different slow-proliferating strategies, such as cancer stem cells (CSCs), senescent cells, and therapeutic-resistant cells. Differential natures of cells belonging to the TPC populations must be considered while investigating their role in therapy resistance [8].

Drug resistance has for a long time been associated with genetic mutations emerging from the **selection** of pre-existing mutant clones or the genetic evolution of drug-tolerant cells [9]. However, it is now clear that cancer-drug tolerance is strongly supported by non-genetic variations, and TPCs arise from the evolution of tumor cells undergoing epigenetic [10–13] and mRNA translation reprogramming [14]. Indeed, epigenetics fosters the acquisition of a persistent phenotype

[1]Department of Experimental and Clinical Medicine, University of Florence, Viale Morgagni 50, 50134 Florence, Italy
[2]Department of Experimental and Clinical Biomedical Sciences, University of Florence, Viale Morgagni 50, 50134 Florence, Italy
[3]These authors contributed equally to this work.

*Correspondence:
erica.pranzini@unifi.it (E. Pranzini) and
marialetizia.taddei@unifi.it (M.L. Taddei).

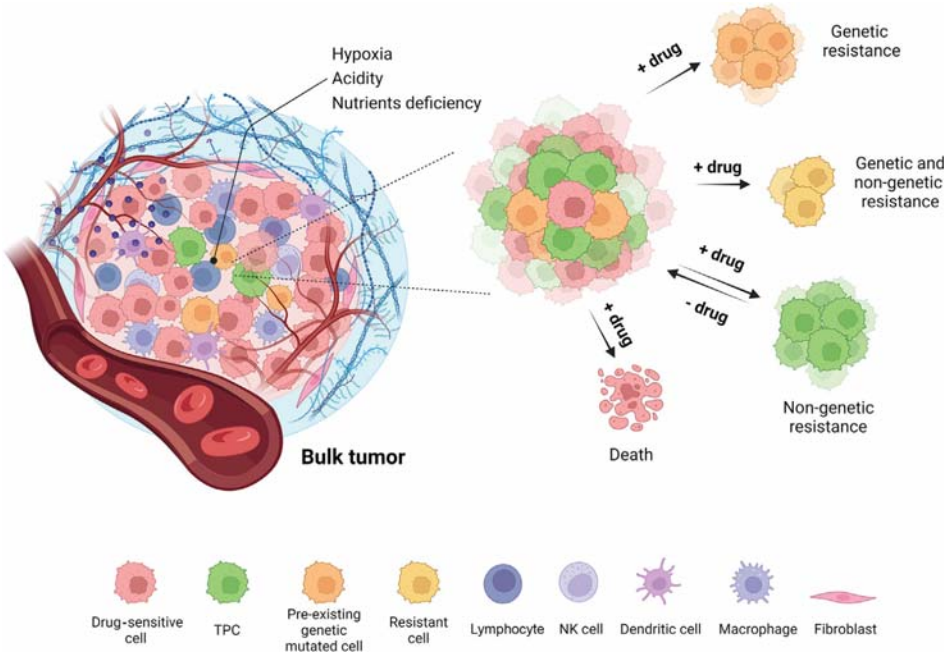

Figure 1. The tumor microenvironment (TME) plays a crucial role in tumor heterogeneity (TH) and tumor-persistent cell (TPC) selection by acting as a driver for the emergence of drug resistance. Cancer cells experience extreme conditions within TME, such as hypoxia, acidity, and nutrient deficiency, promoting cancer-cell aggressiveness. These microenvironmental pressures arising during tumorigenesis induce metabolic reprogramming and epigenetic modifications in cancer cells, leading to increased TH and the selection of metabolic reprogrammed TPCs (green). Metabolic adaptation to a harsh TME could allow cancer cells to foster drug resistance. The tumor bulk is constituted by drug-sensitive (pink) TPCs and pre-existing genetically mutated cells (orange), leading to irreversible genetic resistance, independent of TME pressure. Drug treatment selects drug-tolerant cells, which can be reversed into sensitive cells again after drug removal or can develop into an irreversible drug-resistant state through both genetic and non-genetic mechanisms (yellow). Abbreviation: NK cell, natural killer cell. Figure created using BioRender.

[12,13] by guaranteeing excellent plasticity and advantageous forbearance capacity to harsh microenvironmental conditions [5].

In this review we underline the close connection between metabolism, epigenetics, and therapy resistance. Despite the growing interest in the role of TPCs in driving drug resistance, a deep description of the relationship between TME and TPCs is lacking. Thus, we analyze the role of the microenvironmental pressures in dynamically defining the epigenetic profile of TPCs, fostering the insurgence of drug-resistant clones. This will be of great support in identifying novel approaches against therapy resistance.

Environmental pressures fostering the development of TPCs

During cancer progression, tumor cells interact with all the components of the TME, generating a supportive milieu which is characterized by significant metabolic/nutritional challenges. Different actors dynamically modulate the metabolic composition of the TME during cancer evolution: (i) cancer cells alter the metabolic landscape of the TME as a result of their high metabolic activity, (ii) blood vessels remove waste products and provide oxygen/nutrients, and (iii) stromal and immune cells consume/produce metabolites and secrete signaling molecules and extracellular matrix (ECM) components. Collectively, all of these contribute to the generation of an acidic, hypoxic, and nutrient-deprived tumor niche (Box 1) [15], which exerts an evolutionary pressure on

Glossary

Adaptation: changes made by a cell in response to adverse or varying environmental pressures.
Autophagy: a regulated, physiological mechanism of cellular component degradation and recycling. It acts as an adaptive response under nutrient starvation and participates in intracellular clearance of unneeded macromolecules/organelles.
Cancer dormancy: a condition during cancer progression where tumor cells stop dividing in the primary or in the metastatic sites but survive in a quiescent state while waiting for appropriate environmental conditions to start regrowth.
Diapause: in animals, a period of delayed development or growth accompanied by reduced metabolism and inactivity.
Epigenetic drug: a drug that targets the proteins involved in writing, reading, or erasing the epigenetic marks.
Epigenetics: the study of heritable changes in gene expression that, unlike mutations, do not occur through alterations in the sequence of DNA but are due to chemical modifications of DNA bases and changes to the chromosomal superstructure in which DNA is packaged.
Haploinsufficiency: a genetic condition in which a single copy of the wild-type allele at a locus in heterozygous combination with a variant allele is insufficient to produce the wild-type phenotype.
Immune surveillance: processes by which cells of the immune system (such as natural killer cells, cytotoxic T cells, or macrophages) recognize and destroy premalignant or malignant cells in the body.
Metabolic fitness: the optimal configuration of metabolism to respond to the energetic/biosynthetic demands of the cell.
Metabolic flexibility: the ability to use different nutrients.
Metabolic plasticity: the ability of an organism to process the same nutrient differently by metabolic adaptations aimed at maintaining metabolic fitness across different states and environments.
Methuosis: non-apoptotic cell death characterized by accumulation of cytoplasmic vacuoles derived from macropinosomes and late endosomes.

Box 1. Metabolic stress arising within the TME

Cancer cells display **metabolic flexibility** and **metabolic plasticity** making them highly resilient and adaptable entities [104]. Cancer-cell metabolic reprogramming results from the interplay between stable and transient changes in response to extrinsic fluctuations of metabolism-related factors in the TME [105]. During tumor growth, nutrient availability gradually becomes insufficient to sustain metabolic requirements while waste products may accumulate, overall becoming important selective pressures triggering malignant transformation and forcing cancer cells to reorganize their metabolism. The ability of cancer cells to sense stressful conditions and adjust their metabolism is central to obtaining an optimal **metabolic fitness** [106].

Hypoxia

Tumor hypoxia is the outcome of numerous events that add up during tumorigenesis: enhanced cell metabolism, dysregulated proliferation and organization of vascular endothelial cells, increased distance between tumor cells and blood vessels, and vascular deformation [95]. 'Acute hypoxia' results from transient perturbation in perfusion and supports cell survival and autophagy. 'Chronic hypoxia' arises due to limited oxygen diffusion during tumor mass expansion and determines long-term cellular changes. Under chronic hypoxia, cancer cells switch from OXPHOS to anaerobic glycolysis, with consequent constant lactate production and acidosis. Acute and chronic hypoxia are primarily mediated by HIF1α and HIF2α and are associated with tumor progression and increased aggressiveness [107]. However, the oxygenation state that better reflects the *in vivo* state is 'cycling hypoxia', characterized by oxygen fluctuations with periods of intermittent hypoxia; it provokes a 'reoxygenation injury' with radical formation and oxidative stress thereby contributing cancer aggressiveness [108].

Acidosis

The extracellular pH of tumors is generally acidic due to increased production of lactate and a stoichiometric amount of H^+ ions as a consequence of the 'glycolytic shift' of cancer cells, inefficient wash-out of H^+ from the extracellular milieu because of the disorganized tumor vasculature, and hydration of CO_2 produced in the more oxidative tumor areas. Adaptation to acidosis requires (post-)transcriptional changes in cancer cells and leads to increased metastatic potential and therapy resistance [109].

Nutrient deprivation

Cancer cells suffer from nutrient deprivation because of limited vasculature/lymphatics supply and their high metabolic demand caused by deregulated cell growth. Moreover, cancer cells experience strong competition/sharing for nutrients with other cell types in the TME, further influencing their metabolic profile. Remarkably, cancer cells in different tumor types and tissue localization and over various proliferation states are not universally limited by the availability of the same nutrient [110].

cancer cells and goes from being a simple obstacle to cell proliferation to a selective advantage for tumor cells, finally fostering the emergence of TPCs. Several studies have highlighted the importance of cancer metabolism in supporting drug resistance, evidencing specific strategies promoting adaptive traits to counteract the action of anticancer drugs [16–19].

Due to the reprogramming of cellular metabolism toward glycolysis, tumor cells are generally characterized by an acidic extracellular pH and an alkaline intracellular pH. Acidosis of the TME is a critical determinant in the acquisition of aggressive cancer characteristics and radio-/chemo-resistance. The alkaline intracellular pH of tumor cells favors cancer-cell survival and prevents mitotic arrest induced by DNA damage [20]. Increased TME acidity frequently results in a lowly proliferating phenotype in tumor cells by increasing the percentage of cells in phase G0 and reducing the activation of the Raf/ERK pathway [21]. Furthermore, an acidic environment may also alter tumor **immune surveillance**. Cancer-cell-mediated TME acidification leads to tumor-associated macrophages' functional polarization toward a noninflammatory phenotype [22], which fosters a pro-dormancy state within the primary tumor [23]. An acidic TME may, therefore, become a productive environment for selecting slowly proliferating cells able to escape the toxic effect of therapeutics, representing a dangerous reservoir of drug-resistant cells.

Altered cell metabolism, dysregulated proliferation, and abnormal organization of blood vessels result in the generation of low-oxygen regions within the tumor mass. Low oxygen levels may

Neoadjuvant: refers to any cure that is given before radical treatment intervention.
Oncometabolites: metabolites whose abundance increases in cancer cells through loss-of-function or gain-of-function mutations in specific enzymes involved in their production. Dysregulation of many oncometabolites is linked to epigenetic changes in cancer-sustaining tumor progression.
Organoid: a self-assembled 3D structure, typically derived from stem cells, that recapitulates key functional, structural, and biological aspects of the corresponding tissue *in vivo*.
Proteasome inhibitors (PIs): a new class of anticancer targeted therapy (i.e., bortezomib) that targets the proteasome, which is involved in protein degradation.
Pyroptosis: an inflammation-driven, caspase-1-dependent programmed cell death, triggered by various pathological stimuli such as stroke, heart attack, cancer, and microbial infection.
Selection: the outgrowth of individual cells in a genetically heterogeneous cancer-cell population following drug exposure.
Tumor heterogeneity (TH): subpopulations of tumor cells with distinct genotypes and phenotypes that may harbor divergent biological behaviors within a primary tumor and its metastases, or between tumors of the same histopathological subtype (intra- and inter-tumor, respectively).
Tumor microenvironment (TME): the cellular (endothelial, immune, and stromal cells) and non-cellular (signaling molecules, extracellular matrix, hypoxia) components surrounding a tumor which are in close and constant interaction with cancer cells.
Tumor-persistent cells (TPCs): a subpopulation of cancer cells which evade cell death from chemotherapy and targeted therapy by entering a reversible slow proliferation state. They may resume proliferation and drug sensitivity after drug discontinuation, or they eventually may evolve in drug-resistant cells under continuous treatment.
Vascular normalizing therapy: the application of low doses of anti-angiogenic drugs to restore more structurally and functionally normal vasculature by favoring the balance between pro- and anti-angiogenic signaling.

promote TPC emergence. Indeed, hypoxic cells acquire radio-/chemo-resistant phenotypes by different mechanisms, including decreased drug activity in the absence of oxygen, increased DNA errors and chromosomal instability, and inhibition of the G1/S cell cycle checkpoint [24]. In glioblastoma, hypoxia induces protein phosphatase 2A (PP2A) to mediate **cancer dormancy** through G1/S arrest [25]. In prostate cancer, the entry into a dormant state is promoted by the hypoxia-inducible factor (HIF)-mediated induction of C-X-C motif chemokine receptor 4 (CXCR4) and N-MYC downstream-regulated gene-1 (NDRG1) [26,27]. These adaptive responses promote the selection of slowly proliferating subpopulations of cells prone to survive under drug treatment. In epidermal mutant growth factor receptor (EGFR) lung cancer, hypoxia induces the expression of the negative regulator of ERBB signaling, MIG6/ERRFI1/RALT/Gene33, thereby preventing heterodimer formation of ERBB family RTKs, and suppressing their downstream signaling. MIG6 induction mediates cancer dormancy and results in EGFR–tyrosine kinase inhibitor (TKI) resistance [28]. In primary breast cancer, hypoxic regions foster the generation of clusters of quiescent cells with reduced immune infiltration. The activation of HIF1α, specifically in quiescent tumor cells but not in immune cells, orchestrates the generation of a microniche characterized by suppressive fibroblasts, impaired ability of dendritic cells to recruit and activate T cells, reduced T cell infiltration, and enhanced T cell exhaustion [29]. The hypoxic microenvironment in primary lesions may give rise to a subpopulation of tumor cells that display a long-term dormancy-like program which is maintained even when normoxic conditions are re-established [30]. Following re-oxygenation, hypoxia-imprinted cells do not maintain high expression of hypoxia markers but are prone to enter an NR2F1-dependent dormant state and evade chemotherapy toxicity [30].

Xenograft: a graft of tissue or an organ derived from a (donor) species different from the recipient species.

Moreover, the uneven vasculature and the competition among different cellular populations within the TME also result in regional alterations in nutrient availability [31]. Transformed cells adapt to such adverse conditions by increasing the expression of nutrient/ion transporters and modifying metabolic enzyme activity [32]. However, the existence of differential nutritional niches imposes a specific metabolic zonation within the tumor mass and encourages the emergence of cancer-cell populations characterized by proliferative adaptations, ultimately favoring the emergence of TPCs. Cells adjacent to blood vessels are highly aggressive and more resistant to chemo-/radiotherapies through a mammalian target of rapamycin (mTOR)/nutrient-sensing-dependent mechanism [33]. In colorectal cancer (CRC) cells, serum deprivation induces the transition toward a dormant state characterized by the absence of proliferation and cell death, metabolic suppression, and resistance to chemotherapy. Mechanistically, serum starvation induces Nanog overexpression through the fatty acid oxidation (FAO)/ATP citrate lyase (ACLY)-dependent pathway: increased ACLY expression promotes the synthesis of acetyl-coenzyme A (CoA), which accelerates H3K27 acetylation of the Nanog promoter. Nanog up-regulation finally increases the transcription of the dormancy-related factors P21 and P27 [34]. Under nutrient-deprived conditions, **autophagy** is activated in tumor cells as a stress-induced mechanism of resistance [35]. In ovarian cancer cells, the GTPase DIRAS3 can be transcriptionally upregulated under nutrient-poor conditions. Mechanistically, amino acid withdrawal decreases the binding of E2F1/4 to the autophagy-inducer DIRAS3 promoter, thus up-regulating DIRAS3 transcription and activating the autophagic response, which sustains dormant cell viability following chemotherapy [36]. In CRC, standard chemotherapy treatment (CPT-11) leads cancer cells to enter a drug-tolerant persistent state by activating the evolutionarily conserved survival strategy of **diapause**, characterized by inactivation of the mTOR pathway and increased autophagy [37]. Interestingly, these adaptations make slow-cycling cells particularly sensible to autophagy inhibition [38]. Treating dormant breast cancer cells with the inhibitor of Unc-51-like autophagy-activating kinases 1 (ULK1) sensitizes them to CPT-11 toxicity [39]. However, reinforcing the autophagic response in slowly proliferating cells also appears to be a valid approach to impair cell survival. Indeed, treatment with Saikosaponin A, a major triterpenoid saponin isolated from

Bupleurum D.C., leads to cell death by breaking the 'autophagy equilibrium' in quiescent prostate cancer cells, boosting the already activated autophagy processes [40].

Together, the evolving harsh conditions in the TME – such as acidity, hypoxia, and nutrient deprivation – are major drivers of TPC establishment. Cancer cells' plasticity fosters the adaptations necessary to survive these adverse microenvironmental conditions, participating in the selection of cells able to survive.

The epigenetic landscape of TPCs

The slow-cycling TPC populations constitute a reservoir from which drug-resistant cells may emerge, and epigenetic modifications are central non-genetic mechanisms in the adaptability and survival of drug-tolerant cells [41]. Indeed, drug-tolerant persister cells can reversibly transit through a non-permanent 'drug-tolerant' state or stably enter an irreversible drug-resistant state [42,43] (Figure 1). Owing to this strong dynamism, drug-tolerant cells can re-acquire sensitivity to the original treatment after a drug withdrawal period ('drug holiday'). This is why many refractory tumors are responsive to the original treatments following a period of drug removal [42]. Besides, a strong disruption of the epigenetic regulatory network may equally favor the selection of TPCs by promoting a defective transcriptional response to environmental stress. Epigenetically disrupted cells acquire a 'phenotypic inertia' status, increasing cancer cells' tolerance to harsh conditions and conferring a competitive advantage during tumor progression. Indeed, the loss of main epigenetic regulators induces a 'long-term adaptation' status to cancer cells as they fail to halt proliferation under stress conditions and raise the stress-suffering threshold [44]. Disrupted epigenetic landscape and epigenetically driven ability to transit between cellular states are, therefore, two antithetic non-genetic strategies promoting targeted therapy resistance: while the epigenetic plasticity favors stress adaptation through transcriptional rewiring, epigenetic disruption promotes an unresponsive phenotype [4].

Extensive transcriptional changes for histones and epigenetic modifiers in different tumor models (**organoids**, **xenografts**, and cancer patient samples) support a diapause-like molecular adaptation associated with suppressed Myc activity and overall biosynthesis to persist during treatment [45]. In chronic lymphocytic leukemia (CLL), the relevance of epigenetic alterations in chemoresistance is documented by single-cell RNA sequencing (scRNAseq), revealing that drug resistance is based on genetic and epigenetic changes, thus suggesting that the strategy of discontinuous treatment must be preferred to time-limited administration to avoid the insurgence of resistance [46]. In patients with BRAF-mutated myeloma, BRAF/MEK inhibition indicates that the development of refractory disease is associated with rapid epigenetic changes and metabolic rewiring of TPCs [47]. The generation of drug-tolerant persister cells in EGFR-mutant non-small cell lung cancer (NSCLC) in response to EGFR-TKI (erlotinib) treatment revealed significant histone tail modifications. Specifically, upon drug exposure, these cells exhibit decreased histone acetylation, reduced levels of H3K4me3, and increased global repressive histone H3 modifications which maintain heterochromatin formation mainly located over LINE-1 regions. These alterations prevent drug/stress-induced gene expression and allow cell survival under drug exposure [10]. These epigenetic changes are reversible upon a 'drug holiday' period, allowing a restored drug response [48]. Concordantly, stress-induced multidrug-tolerant cells originating from different cancer cell lines show diffuse epigenetic alterations. In particular, a common loss of the H3K4me3 and H3K27me3 and gain of H3K9me3 marks, associated with an upregulation of histone methyltransferases (SETDB1 and SETDB2), are observed in response to drug exposure or nutrient starvation [48].

Accordingly, epigenetically related histone lysine demethylase (KDM) family members (KDM2, KDM3, KDM5, KDM6, KDM7) play a significant role in generating drug-tolerant cells. In particular, KDM2, KDM3, KDM6, and KDM7 expression is largely upregulated in these cells [49]. In

melanoma, KDM5B emerged as a critical epigenetic modulator fostering TH by governing the transition of a subpopulation of cells toward a drug-tolerant/CD34^{-} state upon exposure to BRAF inhibitors alone or in combination with MEK inhibitors [50]. In patients with NSCLC, overexpression of KDM5B positively correlates with advanced tumor stages and poor overall survival. Coherently, *in vitro* models show increased expression of KDM5B in cisplatin/doxorubicin-resistant cells displaying enhanced cancer stem-cell features [51]. A recent study suggests that histone methyltransferases G9a and EZH are involved in drug-tolerant cell survival [52].

Beside chromatin remodeling, DNA epigenetic modifications frequently also result, affecting TPCs. Indeed, DNA hypermethylation has been reported in the proximal genomic elements concerning the transcription start site of downregulated genes characterizing TPCs [53]. By performing a transcriptomic and proteomic comparative analysis enabling clustering of patients undergoing **neoadjuvant** aromatase inhibitor therapy with letrozole as 'dormant' and 'resistant', 5-methylcytosine and 5-hydroxymethylcytosine were found to be significantly reduced in resistant tumors after extended letrozole treatment [54]. Interestingly, an alteration in the expression of three DNA methyltransferases, DNMT3A, DNMT3B, and DNMT1, in resistant cells compared with untreated cells has also been observed [53].

Finally, some studies also report altered histone deacetylase (HDAC) activity and histone acetylation patterns in TPCs. In particular, a glioblastoma TPC *in vitro* model displays enhanced histone H3K27 acetylation [12], whereas lung cancer TPCs present a global decrease in H3K acetylation [10].

In cancer types carrying altered chromosomal stability, such as BRAF mutation and PTEN loss, epigenetic reprogramming supports drug tolerance going beyond the contribution of genetic alterations [55]. The importance of epigenetic modulation in those models is evident in V600E BRAF-mutated melanoma cell lines, where it induces the insurgence of rare viable and quiescent cells able to regrow following drug removal owing to alterations in the expression of epigenetic regulator genes. Coherently, in this model, epigenetic inhibition reduces proliferation and triggers apoptosis in the surviving population [56]. In BRAFV600-mutant melanoma, the **haploinsufficiency** of SIRT6 HDAC fosters persistence in the presence of MAPK inhibitors through H3K56 acetylation of the IGFBP2 locus and the consequent activation of the IGF-1 receptor [57]. Drug-resistant cells not only emerge from treatment-mediated selection of subpopulations that are present before the start of therapy, but also arise from adaptive processes occurring during therapy exposure, owing to epigenetic modifications. Multiple myeloma cells exposed to **proteasome inhibitors (PIs)** enter a slow-cycling and reversible drug-tolerant state associated with epigenetic plasticity. A combination treatment with HDAC inhibitors and high-dosage intermittent therapy is effective in preventing the emergence of PI-tolerant cells [58].

Taken together, this evidence indicates that epigenetic alterations are essential drivers in TPC selection, establishment, and survival, underlying their potential role as therapy targets in managing anticancer therapy resistance.

Metabolic features of TMEs fostering epigenetic remodeling

Metabolic adaptations appearing in response to the pressure of the TME may promote epigenetic dynamics in cancer cells, finally resulting in the emergence of TPCs under drug exposure. Indeed, many epigenetic modifiers respond to oxygen/nutrient availability and use metabolites as cofactors/substrates (Box 2).

The metabolic adaptations characterizing a hypoxic environment sustain altered DNA methylation and histone post-translational modifications in several cancers [49]. In NSCLC cell lines, histone

Box 2. Metabolites with epigenetic functions

Cellular metabolism fosters epigenetic reprogramming by modulating intracellular levels of specific metabolites.

S-adenosylmethionine (SAM) is the major methyl donor in DNA and histone methylation [111]. Metabolites of the TCA cycle affect the activity of DNA and histone demethylases.

α-Ketoglutarate (α-KG) is a cofactor for Jumonji (JmjC)-domain-containing histone demethylases and TET family proteins (TET1, TET2, and TET3), which mediate DNA demethylation [112].

Succinate and fumarate are inhibitors of TET and JmjC-containing members of the demethylase family.

D-2-hydroxyglutarate (D-2-HG), produced by mutated isocitrate dehydrogenase 1 and 2 (IDH), is a competitive inhibitor of TETs and Jmj-C family demethylases. Besides, the L enantiomer of 2-HG (L-2-HG), accumulates in kidney tumors and, similarly to D2HG, inhibits TETs [113]. These metabolites are categorized as **oncometabolites** as their intracellular accumulation arises due to loss-of-function or gain-of-function mutations in the specific enzymes involved in their production [114].

Succinyl-CoA promotes succinylation of histone H3 on lysine 79 and it is involved in tumor progression [115].

Histone acetylation depends on the equilibrium between the action of histone acetyltransferases (HATs) and histone deacetylases (HDACs) and strongly depends on availability of acetyl-CoA, a key metabolite for energy production via OXPHOS and for anabolic pathways [116].

Histone deacetylation is catalyzed by zinc-dependent (classes I, II, and IV) and NAD^+-dependent (class III) deacetylases. Increased NAD^+/NADH ratio stimulates HDAC III histone deacetylation, the tight wrapping of DNA, and consequently gene silencing. Butyrate, a C4 short-chain fatty acid, and the ketone body β-hydroxybutyrate (β-OHB) suppress respectively HDACs I, II, and IV and HDACs I and IIa [117].

Lactate is frequently excessively produced by cancer and tumor-associated cells [118] and is a substrate for histone 'lactylation' [119,120].

Uridine diphosphate N-acetylglucosamine (UDP-GlcNAc) is an activated substrate that provides GlcNAc for O-GlcNAcylation, produced in the hexosamine biosynthetic pathway. Protein O-GlcNAcylation is carried by the opposite actions of O-GlcNAc transferase (OGT) and O-GlcNAcase (OGA) [121]. O-GlcNAc acts as a nutritional sensor controlling the destiny of nuclear and cytosolic target proteins according to glucose and nutrient availability [116]. OGT is associated with TETs to facilitate O-GlcNAcylation of histone H2B activating gene transcription; as a part of the polycomb group proteins, OGT is required to form H3K27me3 for silencing of tumor suppressor genes [122].

The AMP-activated protein kinase (AMPK), a sensor of cellular energetic status, is activated by decreased ATM/AMP ratio. AMPK phosphorylates histone H2B on serine 36, promoting gene expression and sustaining tumor survival [116].

demethylase LSD1 drives hypoxia-induced gefitinib resistance [59]. In esophageal squamous-cell carcinoma, hypoxia promotes radio-resistance by increasing KDM3A/KDM6B expression, enhancing cell survival, decreasing DNA damage accumulation, and impairing apoptosis [60].

A key role in epigenetically driven drug tolerance is given by intratumoral variation of nutrient availability [61]. Cells surviving and adapting to nutrient deprivation undergo non-genetic adaptations, allowing them to reversibly switch among different metabolic states [62].

Glutamine restriction, by lowering intracellular α-ketoglutarate (α-KG) levels, promotes stemness via the Wnt signaling pathway and blocks cellular differentiation in CRC organoids. Conversely, α-KG supplementation stimulates DNA hypomethylation and histone H3K4me3 loss, supporting the reactivation of the transcription program of differentiation-associated genes and the downregulation of Wnt target genes [63]. Similarly, melanoma cancer cells often experience low glutamine availability, promoting cell de-differentiation [64]. In patient-derived (V600E)-BRAF melanoma cells, low glutamine induces histone hypermethylation, promoting cancer-cell de-differentiation and resistance to BRAF inhibitor treatment [65].

Changes in methionine availability affect chromatin state, modulating S-adenosylmethionine/S-adenosylhomocysteine (SAM/SAH) concentrations [66,67]. In taxane-resistant triple-negative breast cancer (TNBC) cells, modified epigenome induced by altered methionine metabolism enables evasion from the viral mimicry response, allowing the growth of TNBCs and supporting drug resistance [68]. In prostate cancer, the emergence of the neuroendocrine prostate cancer variant resistant to androgen receptor-targeted therapies is promoted by the serine-mediated accumulation of SAM, which in turn epigenetically impacts the expression of genes controlling cell type specification [69]. Serine deficiency is a metabolic stress often observed in breast cancer [70]. Interestingly, serine deprivation impairs glucose flux through glycolysis and tricarboxylic acid (TCA) cycle, limits acetyl-CoA generation, and decreases global H3K27ac and locus-specific H3K27ac abundance at the promoter region of estrogen-receptor pathway genes. Mechanistically, low serine levels foster breast cancer progression to an endocrine-resistant state [71].

Environmental acetyl-CoA levels are dynamic and directly dependent on nutrient availability [72]. Tumor cells adapting to survive under hypoxia and nutrient deprivation reprogram lipid metabolism and direct exogenous acetate to maintain acetylation of histones [73]. The overexpression of acetyl-CoA synthetase short-chain family member 2 (ACSS2) supports cisplatin resistance in esophageal squamous carcinoma cells when exposed to nutrient stress [74]. Together with acetyl-CoA, histone acetylation levels also depend on the availability of the metabolite nicotinamide adenine dinucleotide (NAD^+) [75].

Lactate, a cancer-related metabolite, acts directly as a substrate for lysine modification, promoting histone lactylation [76]. In prostate and lung adenocarcinomas, lactate accumulation leads to histone lactylation and transcriptional upregulation of neuroendocrine-associated genes, resulting in cell plasticity, neuroendocrine differentiation, and resistance to targeted therapy [77].

Apart from canonical mechanisms, other metabolic adaptive strategies are emerging which counteract exposure to chemotherapeutics agents by inducing epigenetic alterations. **Pyroptosis** is a mechanism of inflammatory cell death induced in stressful conditions such as endoplasmic reticulum (ER) stress. High methionine-to-taurine metabolic flux represents a strategy for cells to persist by evading TKI-induced pyroptotic osmotic crisis. An enhanced flux of methionine to taurine promotes epigenetic changes, increasing DNA hypermethylation of genes regulating metal ion balance and intrinsic immune response, allowing TPCs to gain proliferative advantages. TPCs undergoing such a metabolic adaptation show increased sensitivity to the hypomethylating agent decitabine [78]. Collectively, different metabolic strategies sustain the emergence of epigenetically modified TPCs, ultimately contributing to drug resistance. Epigenetics may therefore represent an Achille's heel in eradicating TME-adapted TPCs and counteracting tumor relapse.

Targeting TPC epigenetic and metabolic adaptation to overcome drug resistance

Regardless of their high heterogeneity and flexibility, common features of TPCs can be identified to selectively target this small subpopulation of cells surviving under acute stress. In particular, a crucial targetable trait of TPCs is their altered epigenetics [79]. Emerging **epigenetic drugs** (epi-drugs) are designed to target enzymes that regulate aberrant epigenetic modifications implicated in all the well-known hallmarks of cancer [80]. Combining epi-drugs with standard therapies can boost the efficacy of principal anticancer treatments [81]. Importantly, epi-drugs also represent an excellent opportunity to target TPCs and overcome drug resistance. Nevertheless, because of the dynamic nature of TPCs, identifying effective treatment and timing for targeting their epigenetic alterations is challenging. A concomitant continuous administration can be used to obtain a synergic effect when the two drugs have slight efficacy as monotherapies. However, providing specific epi-drugs following the treatment with standard therapies would be more effective in eliminating TPCs that contribute to therapy

resistance through epigenetic modifications [81]. Despite promising results, the overall response to the combination of anticancer therapies with epi-drugs is not completely satisfactory, mainly due to the systemic toxicity and poor efficacy of this approach compared with monotherapy [80].

Otherwise, these studies have opened up the scenario for promising novel approaches selectively affecting metabolic adaptations promoting TPC epigenetic flexibility (Figure 2). Cells entering the TPC state generally activate mitochondrial metabolism and develop a strong antioxidant response to counteract the accumulation of reactive oxygen species (ROS) [82]. Targeting mitochondrial oxidative phosphorylation (OXPHOS) may therefore be instrumental in eradicating TPCs. Indeed, inhibiting mitochondrial activity has emerged as an option to successfully eradicate drug-resistant stem cells in hematological malignancies [83,84]. Similarly, in melanoma TPCs,

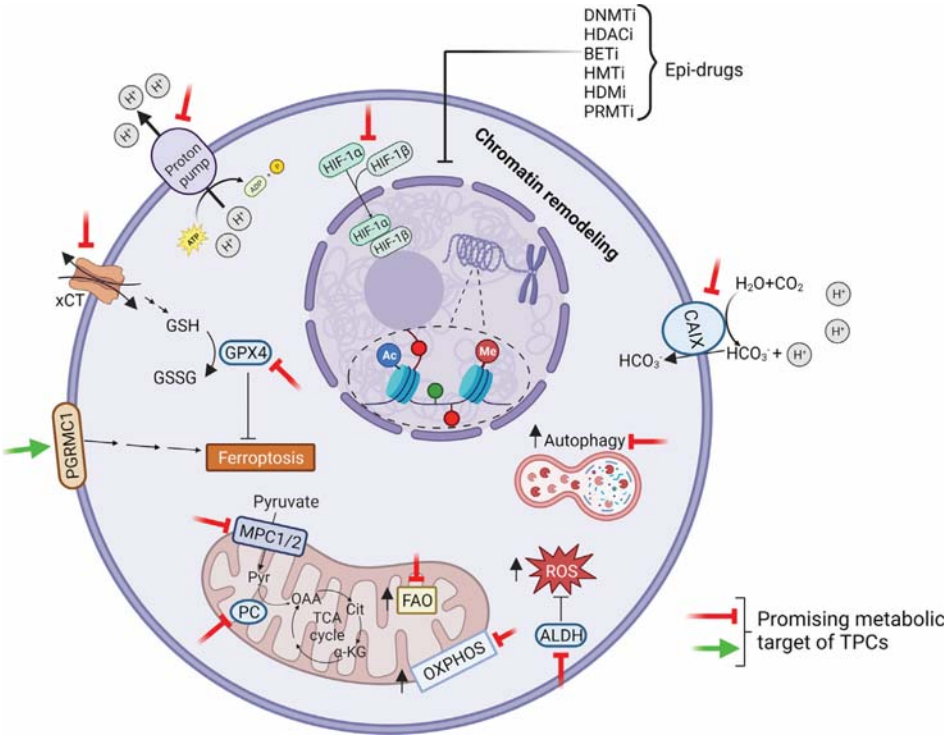

Figure 2. Targeting metabolic features of tumor-persistent cells (TPCs) as a promising approach to overcoming drug resistance. Novel approaches that selectively target the metabolic adaptations characterizing TPCs in combination with conventional (epi-)drugs are emerging to eradicate TPCs. Targeting TPCs' increased mitochondrial metabolism – mainly oxidative phosphorylation (OXPHOS) and upregulated fatty acid oxidation (FAO) – are possible strategies. TPCs also exploit aldehyde dehydrogenase (ALDH) activity to prevent reactive oxygen species (ROS)-derived toxic effects, avoiding apoptosis. The combination of ALDH inhibition with standard therapy could enhance treatment efficacy. TPCs trigger autophagy under stress, offering another potential targetable mechanism. Some TPCs show higher amounts of the mitochondrial carrier pyruvate 1/2 (MPC 1/2) and higher pyruvate carboxylase (PC) activity, suggesting pyruvate anaplerosis as a novel target. A potential strategy to hit TPCs could be the induction of the ferroptosis pathway by inhibiting GPX4 or activating progesterone receptor membrane component 1 (PGRMC1). Finally, modifying TME nutrient/ion composition is an additional approach to overcome drug resistance by eliminating factors favoring TPC selection. To this end, promising strategies include targeting the intratumor pH and the hypoxia-activated pathways. Abbreviations: BETi, bromodomain and extra-terminal domain inhibitor; CAIX, carbonic anhydrase IX; DNMTi, DNA methyltransferase inhibitor; GSH, glutathione-reduced state; GSSG, glutathione-oxidized state; HDACi, histone deacetylase inhibitor; HDMi, histone demethylase inhibitor; HIF, hypoxia-inducible factor; HMTi, histone methyltransferase inhibitor; PRMTi, protein arginine methyltransferase inhibitor. Figure created using BioRender.

targeting the electron transport chain significantly compromises cell survival [85]. Pancreatic dormant cancer cells surviving oncogene ablation display an overexpression of genes involved in mitochondrial functionality; OXPHOS inhibition specifically targets surviving cells and counteracts tumor recurrence [86]. Disrupting redox homeostasis is also a valid approach to delete TPCs. Her2-downregulated persistent breast cancer cells rewire cellular metabolism and reestablish redox homeostasis, upregulating the antioxidant transcription factor NRF2, which in turn mediates *de novo* nucleotide synthesis and facilitates recurrence. The reactivation of quiescent cells can be prevented by glutaminase inhibition, suggesting a novel approach to target NRF2 highly dormant and recurrent cancer [87]. The acquisition of a drug-tolerant persister phenotype in different multidrug-resistant cancer models is characterized by the NPC1L1-mediated uptake of vitamin E to survive chemotherapy-induced oxidative stress. In these models, NRF2 is involved in the transcriptional regulation of NPC1L1 via decreased DNA promoter methylation. Treatment with an NPC1L1 inhibitor strongly enhances combinatorial therapy's effect by inducing **methuosis** and preventing recurrence *in vivo* [88]. The inhibition of aldehyde dehydrogenase (ALDH), which normally protects drug-tolerant persister cells from ROS-derived toxic effects, leads to DNA damage and apoptosis. In particular, combining the ALDH inhibitor disulfiram with TKIs delays treatment relapse *in vitro* and *in vivo* [89]. Interestingly, pyruvate anaplerosis has recently been identified as a targetable vulnerability in persistent leukemic stem cells (LSCs). These cells have increased mitochondrial pyruvate carrier 1/2 (MPC1/2) levels and pyruvate carboxylase activity compared with their normal counterparts. Genetic ablation of pyruvate anaplerosis sensitizes chronic myeloid leukemia (CML) cells to imatinib [90]. Conversely, in liver cancer, sorafenib-tolerant TPCs suffer mitochondrial dysfunction and rely on glycolysis by increasing the expression of hexokinase 2. The combined treatment with the glycolytic inhibitor 2-deoxy-D-glucose and sorafenib promotes TPC apoptosis *in vitro* and reduces tumor growth in mouse models [91].

A shift toward FAO has been frequently observed in TPCs. In this regard, in BRAF(V600E) mutant melanoma, TPCs display a shift from glycolysis to OXPHOS supported by peroxisomal FAO, transcriptionally regulated by peroxisome proliferator-activated receptor α (PPARα). A combinatory treatment of BRAF/MEK inhibitors with the FAO inhibitor thioridazine significantly decreases the emergence of TPCs [92]. This strong dependency on the lipid peroxidase pathway in TPCs causes increased vulnerability to ferroptotic cell death. Interestingly, paclitaxel-TPCs are highly sensitive to xCT inhibitors through ferroptosis induction by the expression of progesterone receptor membrane component 1 (PGRMC1)-dependent lipophagy. Thus, the activation of PGRMC1 represents a powerful strategy to eradicate TPCs via ferroptosis [93]. Similarly, drug-tolerant persister cells are also specifically vulnerable to the inhibition of lipid hydroperoxidase GPX4, and co-treatment of standard anticancer therapies with a GPX4 inhibitor effectively reduces the residual persistent cell pool. A staggered treatment strategy that combines anticancer therapy and pre- or post-treatment with GPX4 inhibitors, rather than co-treatment, is effective in depleting the pool of persistent cells by inducing ferroptotic cell death *in vitro* and preventing tumor relapse *in vivo* [94].

As previously reported, TPCs may activate autophagy under stress, providing another possible approach to counteract tumor relapse. Drug-tolerant persister cells in CRC are strongly dependent on autophagy for survival, and targeting the autophagic program together with chemotherapy results in a significant suppression of tumor growth and negligible recovery [37].

Metabolic challenges arising in the TME are crucial drivers in selecting TPCs. Therefore, therapeutic strategies to modify TME composition would represent novel approaches to overcome drug resistance by eliminating factors favoring TPCs' achievement. A series of strategies has been proposed for overcoming hypoxia in solid tumors, including targeting hypoxia-activated pathways

(i.e., interference with HIF signaling), metabolic intervention, and recently designed approaches such as siRNA-mediated gene therapies and recombinant anaerobic bacteria [95]. It is well known that hypoxia both generates and is caused by aberrant tumor vascularization. In line with this, **vascular normalizing therapies** are capable of enhancing the effectiveness of chemo/radiotherapy and immunotherapy not only by favoring a deeper penetration of chemotherapeutics in the tissue and facilitating the migration of functional immune cells [96], but also by preventing cancer cells from acquiring more aggressive phenotypes in the hypoxic microenvironment [97]. Similarly, modulating intratumor pH is another promising approach for overcoming TPC success in refractory tumors. To this aim, pH-regulating proteins – such as proton pump H^+-ATPase [98], sodium–hydrogen antiporter 1 (NHE1) [99], and carbonic anhydrase IX (CAIX) [100] – are emerging as attractive therapeutic targets for treating hypoxic tumors. In addition, oral or parenteral administration of buffer systems (sodium bicarbonate) to neutralize tumor acidity can be applied in combination with standard therapies to increase their effectiveness [101].

Increasing evidence suggests that therapeutic approaches specifically targeting metabolic adaptations induced by microenvironmental pressures represent a valid strategy to hinder TPC survival and tumor recurrence (Table 1).

Concluding remarks and future perspectives

The plasticity of TPCs is driven mainly by epigenetic modifications. Despite sustained efforts to better identify the origin, biology, and specific markers of TPCs, factors triggering their occurrence still need to be clarified, making it impossible to develop effective therapies targeting TPCs (see Outstanding questions). Challenging microenvironmental conditions such as hypoxia, nutrient starvation, and acidic pH impose on cancer cells selective pressures, requiring their continuous metabolic rewriting. This review describes how such metabolic stresses fuel epigenetic adaptations supporting TPC emergence. This novel perspective underscores that modulating the metabolic landscape of the TME is a promising innovative strategy to hinder the epigenetic flexibility of TPCs. Future studies are required to understand how to selectively target TPCs within the heterogeneous population of tumor cells, as well as to define the best timing of such treatments. Recent advances in clinical imaging methods, such as magnetic resonance imaging and positron emission tomography, to specifically detect intratumor O_2 levels and acidity in patients will aid in addressing the complexity of TH and in choosing the most efficacious TME-targeted therapeutic approach [102,103]. In conclusion, data reported herein underline that a

Table 1. Therapeutic approaches to target metabolic features of TPCs

Target	Molecular mechanism	Refs
Mitochondrial OXPHOS	Inhibition of electron transport chain (in melanoma) Inhibition of ATP synthase	[85,86]
Redox homeostasis	Glutaminase inhibition to target NRF2 antioxidant effect Inhibition of NPC1L1 by inducing apoptosis ALDH inhibition	[87–89]
Pyruvate anaplerosis	Inhibition of MCP1/2	[90]
Glycolysis	Apoptosis promotion by glycolysis inhibition (2-DG)	[91]
Peroxisomal FAO	Peroxisomal FAO inhibition (thioridazine)	[92,94]
Ferroptotic cell death	Ferroptosis induction by the expression of PGRMC1 Inhibition of GPX4	[93]
Autophagy	Pharmacological inhibition of ULK1 Treatment with Saikosaponin A to break the 'autophagy equilibrium'	[39,40]
Intratumor pH	Inhibition of H^+-ATPase, NHE1, and CAIX	[98–100]

Outstanding questions

The definition of TPCs includes subpopulations of cells with similar phenotypic characteristics, such as dormant cancer cells and CSCs, but characterized by different genetic defects, allowing them to resist chemo/radiotherapy. Which is the contribution of genetic and epigenetic modifications in defining the nature of persistent cells? What criteria can be applied to define them? Is it possible to define a common profile of these cells in different types of tumors?

Available *in vitro* models are only partially reliable for studying TPCs as they constitute a complex and under-represented system. Besides the recently developed lineage tracing method – clustered regularly interspaced short palindromic repeats transcriptional activation (CRISPRa) tracing of clones in heterogeneous cell populations (CaTCH) – useful to isolate very rare cell populations, will it be possible to develop other adequate clinically relevant models for TPC study?

Can we reprogram the epigenome of cancer cells modifying the metabolic composition of the TME to halt cancer progression?

The use of epigenetic drugs together with standard therapy has been described for many years. Still, although they can improve the response to monotherapy, no epi-drug has moved into therapeutic guidelines for cancers. Could the development of therapy targeting microenvironmental pressure be helpful to hinder TPC maintenance and counteract drug resistance?

Which might be the best temporary window to target metabolic adaptations in TPCs during tumor progression and drug treatment?

combined strategy targeting epigenetic adaptations in cancer cells and the evolving TME landscape represents a necessary approach in the fight against resistance to anticancer therapy.

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Declaration of interests

The authors declare no competing interests.

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