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Review Article

Cancer stem-like cells in uveal melanoma: novel insights and therapeutic implications

Alessandra Loda [a], Francesco Semeraro [b], Silvia Parolini [a,c,d], Roberto Ronca [a,d,1], Sara Rezzola [a,*,1]

[a] Department of Molecular and Translational Medicine, University of Brescia, Brescia, Italy
[b] Eye Clinic, Department of Medical and Surgical Specialties, Radiological Sciences and Public Health, University of Brescia, Brescia, Italy
[c] National Center for Gene Therapy and Drugs based on RNA Technology – CN3, Padova, Italy
[d] Consorzio Interuniversitario per le Biotecnologie (CIB), Italy

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ABSTRACT

Uveal melanoma (UM) is the most common primary ocular tumor in the adult population. Even though these primary tumors are successfully treated in 90% of cases, almost 50% of patients ultimately develop metastasis, mainly in the liver, *via* hematological dissemination, with a median survival spanning from 6 to 12 months after diagnosis. In this context, chemotherapy regimens and molecular targeted therapies have demonstrated poor response rates and failed to improve survival. Among the multiple reasons for therapy failure, the presence of cancer stem-like cells (CSCs) represents the main cause of resistance to anticancer therapies. In the last few years, the existence of CSCs in UM has been demonstrated both in preclinical and clinical studies, and new molecular pathways and mechanisms have been described for this subpopulation of UM cells.

Here, we will discuss the state of the art of CSC biology and their potential exploitation as therapeutic target in UM.

1. Introduction

Ophthalmic tumors are a family of rare cancers that develop within the eyeball. These cancers originate from different cell types localized in different areas of the eye and include retinoblastoma, intraocular lymphoma, eyelid carcinoma, lacrimal gland tumor, conjunctival melanoma and uveal melanoma (UM) [1–3]. Among them, UM is the most common intraocular tumor in the adult population [1]. UM arises from melanocytes located in the uveal tract, a pigmented vascularized region that includes the iris, the ciliary body, and the choroid [4,5]. The iris is a contractile diaphragm with a central opening, the pupil, regulating the amount of light passing through and reaching the retina [4,5]. The ciliary body is located behind the iris and it includes the ciliary epithelium, the ciliary stroma, and the ciliary muscle [5]. The ciliary body is involved in mediating many ocular functions; for instance, the ciliary epithelium secretes the aqueous humor, while the ciliary muscle is necessary to adjust focus of vision [4,5]. Finally, the choroid consists mainly of blood vessels and melanocytes, and it exerts the essential function of providing nutrients and oxygen to retinal neurons. The choroid is firmly attached on its inner surface to the retinal pigment epithelium (RPE), while its outer surface adheres to the sclera [4,5].

Most frequently, UM develops in the choroid (almost 90% of total cases), followed by the ciliary body (6%) and the iris (4%) [6] (Fig. 1). Even though primary tumors are successfully treated in 90% of cases, almost 50% of patients ultimately develops metastasis *via* hematological dissemination, mainly to the liver (95%), followed by lungs (24%), bones (16%), and skin (11%), with a median survival after diagnosis spanning from 6 to 12 months [7]. This may be due to early seeding of tumor cells from the primary ocular site to distant organs, where cancer cells originate dormant subclinical micrometastasis [8]. In addition, resistant tumor cells can survive conventional treatments and contribute to the onset of an asymptomatic minimal residual disease that is undetectable by conventional screenings [9].

Cancer stem-like cells (CSCs) are nowadays a well-recognized subpopulation in tumor parenchyma characterized by low replication rate and undifferentiated phenotype and molecular fingerprint. Due to the variable molecular markers and their low percentage inside the tumor, CSCs have been difficult to identify and characterize. Nevertheless, CSCs

* Corresponding author at: University of Brescia, viale Europa 11, 25123 Brescia, Italy.
E-mail address: sara.rezzola@unibs.it (S. Rezzola).
[1] These authors share the last/senior authorship.

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remain a key issue in cancer therapy due to their intrinsic capacity to resist to chemo- and radiotherapies, and to fully recapitulate and restore tumor growth.

Here below the main features and advances on the understandings and targeting of CSCs in UM will be discussed.

2. Biology of uveal melanoma

UM accounts for 5% of all melanomas, with an incidence of approximately 4.6 million cases per year, variable according to age, ethnicity, and latitude [10]. Risk factors for UM include light-colored eyes, fair complexion, ocular melanocytosis, and excessive exposure to natural/artificial ultraviolet and blue light. Tumors are frequently asymptomatic, and diagnosis typically occurs during routine ophthalmic screening. Still, discoloration of the iris or pupillary distortion may be detected by patients when tumors affect the anterior portion of the eye, thus allowing earlier diagnosis. In comparison, posterior tumors can remain latent until a disruption of the visual field manifests. Moreover, larger posterior tumors can be associated to complications such as the formation of an exudative retinal detachment [6,11].

UM and cutaneous melanomas are characterized by extremely different genetic signatures. Indeed, UM lacks the classical *BRAF* and *NRAS* alterations of cutaneous melanoma, whereas gain-of-function and oncogenic mutations mainly occur in *GNAQ* and *GNA11*. These alterations are mutually exclusive and lead to the constitutive activation of Gq-proteins associated to transmembrane receptors and of their respective downstream signaling pathways [2].

Early genetic alterations in UM include monosomy of chromosome 3 (M3), gain of chromosome 8, and loss of chromosome 1p. In particular, M3 occurs in approximately 50–60% of primary tumors and is associated to a worse prognosis [12]. Similarly, gain of 8p takes place in 40–60% of primary lesions, while co-occurrence of both M3 and gain of 8p results in a higher 5-year mortality rate (equal to 66%) [13]. Additionally, loss of chromosome 1p is associated with M3 in approximately 20–30% of cases and can be considered a negative prognostic factor [14]. Other alterations include loss of 8p and gain of 6p, which are associated with a worst and a better prognosis, respectively [13,15].

Inactivating mutations of the tumor-suppressor gene *BAP1* are present in over 80% of metastatic UM and are linked to lower disease-free survival rates. Patient survival is drastically affected by the co-presence of *BAP1* mutations and M3 [16]. Indeed, loss-of-function mutations of *BAP1*, which is located on 3p21, usually follow M3 occurrence. The decreased expression of BAP1 *mRNA* and protein correlate with a global DNA methylation state that is distinct from UM patients with both copies of chromosome 3 (UM-D3) and it has been associated with an increased risk of developing later-onset metastases [17]. In addition, approximately 15% of UM patients display mutations in the splicing factor 3B subunit 1 (*SF3B1*) gene, which encodes for a member of the spliceosome. Indeed, alterations in the spliceosome component can cause intron retention and aberrant alternative splicing of several genes [18]. Recently, *SF3B1* mutations have been linked to the development of metastatic disease and a worse prognosis within UM-D3 patients [17].

Epigenetic regulation, especially methylation, plays an important role in UM by affecting tumor suppressor genes, including cyclin-dependent kinase inhibitor 2 A (*p16INK4a)*, RAS associated domain family 1 isoform A (*RASSF1A),* as well as *BAP1*. Of note, even though major *BAP1* alterations consist of loss-of-function mutations, *BAP1* methylation has also been reported and it might represent a prognostic indicator for the development of metastatic lesions [16,19].

Recently, the molecular profiling of UM based on a specific set of 15 genes allowed the classification of patients into three prognostic groups: low-risk (class 1 A), intermediate (1B), and high-risk (class 2). Class 1 tumors genetic profile resembles that of normal melanocytes, whereas melanocytic genes are downregulated in class-2 tumors, in favor of genes of primitive neural/ectodermal stem cell lineages. This suggests that class-2 tumors lose their melanocytic identify and revert to a more aggressive, stem-like phenotype [20]. This classification can be further refined by assessing a set of antigens preferentially expressed in melanoma (PRAME), which are linked to an increased risk of metastasis and poorer survival [21].

2.1. Therapeutic strategies for primary and metastatic UM

Several therapeutic strategies are employed in the clinical practice to eradicate primary eye tumors, preserving the globe and vision, and preventing the occurrence of distant metastasis.

Brachytherapy is a technique that allows the direct administration of radiotherapy to the tumor site through the application of a radioactive plaque on the sclera, promoting tumor regression within 2 months of therapy. The most frequently used radioisotopes are ruthenium-106 and

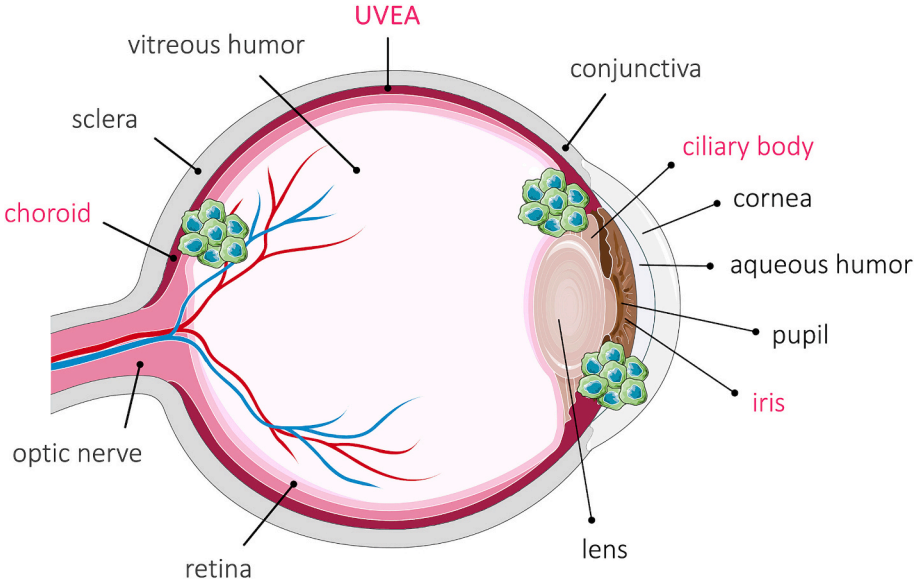

Fig. 1. Schematic representation of ocular anatomy and UM localization.
UM originates from melanocytes located in the uvea (pink), a pigmented vascular layer which provides trophic support to the retina, and consists of the choroid, the iris, and the ciliary body. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

iodine-125, according to tumor size [22]. Transpupillary thermotherapy (TTT) targets the tumor with an infrared laser passing through the pupil and causing hyperthermia up to 4 mm in depth. Currently, TTT is mainly administered to reduce tumor size before radiotherapy and is indicated for small tumors arising distant from the macula and the optic nerve [23]. Photodynamic therapy (PDT) is a less common procedure in which a photosensitive dye is injected intravenously to induce photochemical toxicity, causing vascular closure and tumor necrosis. Finally, local tumor resection can be a valid treatment for tumors unsuitable for radiotherapy due to their localization or dimensions, thus allowing for globe preservation and vision retention. Before the advent of brachytherapy, enucleation, *i.e.* the surgical removal of the eye, was the first line treatment for UM. Currently, it is limited to large tumors that cannot be treated otherwise, whereas exenteration, which includes the removal of nerves, muscles, and fatty tissue, is applicable in the presence of an extensive extraocular involvement [24]. In any case, whole body examination should be performed to exclude the presence of metastasis before treatment of the primary tumor since local treatment may be deferred in favor of systemic therapy if metastatic foci are detected.

Frequently, UM disseminates during the early stages of the disease, but metastatic growth is delayed over time, as demonstrated by the fact that clinical metastases are rare at time of diagnosis of primary tumors and usually appear decades later [25]. For the treatment of metastatic UM, therapies can be grouped into the following categories: liver-directed therapies, chemotherapy approaches, molecular-targeted therapies, and immunotherapy [26].

Given that the liver is the most common site of metastatic dissemination, liver-directed therapies such as surgical resection, chemoembolization, radioembolization, and percutaneous hepatic infusion of chemotherapeutic drugs are often employed [11]. Partial hepatectomy should also be considered in the presence of limited and accessible hepatic lesions [11,27]. Nevertheless, systemic management of metastatic disease remains extremely complex. Indeed, chemotherapy regiments using dacarbazine, temozolomide, cisplatin, and fotemustine have demonstrated poor response rates and failed to improve patient survival, both as single agents and in combination therapies [27].

As an attempt to overcome therapy resistance, molecular targeted therapies, aimed at blocking specific signaling pathways that regulate the biological behavior of tumor cells, have been tested in UM. Studies have focused on hampering downstream mediators of constitutively activated GαQ and Gα11, including Mitogen-Activated Protein Kinase (MAPK) and Phosphatidylinositol 3-Kinase (PI3K)/AKT/ Mechanistic Target of Rapamycin (mTOR) kinase. Despite the promising results of these inhibitors in experimental *in vitro* models, they exerted limited efficacy in clinical trials [28].

Advances in immunotherapy, such as immune checkpoint inhibitors targeting the cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4) and the programmed cell death 1 (PD-1)/PD-L1 axis, have significantly improved the treatment of cutaneous melanoma. However, immunotherapy approaches are unsuccessful in UM due to the low mutational burden of these tumor cells [29]. Of note, recent single cell RNA-sequencing studies on primary and metastatic UM samples have opened to the possibility of active immune surveillance in low-risk tumors. Indeed, mutations of *SF3B1* and of the eukaryotic translation initiation factor 1 A X-linked (*EIF1AX*) could result in the generation of tumor neoantigens, favoring immune response [30]. By contrast, it has been hypothesized that in high-risk (class 2) UM tumors the wide genomic aberrations and increased aneuploidy could be responsible for the creation of an immunosuppressive tumor microenvironment (TME) which promotes immune escape and sustains metastasis formation [30]. In this regard, in a recent analysis performed in 12 human cancer types, tumor aneuploidy has been correlated with markers of immune evasion and resistance to immunotherapy [31] and this appears to be mediated through the activation of the cytosolic DNA-sensing pathway (cGAS–STING) and the activation of the nuclear factor kappa B (NF-κB) signaling [32].

Nevertheless, a new drug has recently been approved for the pharmacological treatment of HLA-A*02:01-positive unresectable or metastatic UM. Tebentafusp belongs to the immune mobilizing monoclonal T-cell receptors against cancer (ImmTAC) class of bispecific T-cell engagers, where an anti-CD3 single-chain antibody fragment is bound to a monoclonal high affinity T-cell receptor directed against a specific cancer-related antigen. Specifically, tebentafusp recruits and directs CD3+ T cells against UM cells presenting a melanoma-associated antigen glycoprotein 100 (gp100)-derived peptide, normally involved in the maturation of melanosomes and highly expressed by tumor cells [33]. The safety profile of tebentafusp is encouraging, with manageable adverse effects, mainly skin reactions, occurring during the first administrations. Therefore, this paves the way for further exploration on the efficacy of novel immunotherapy approaches to improve the clinical outcome of high-risk UM patients [34].

In general, while primary UM results clinically manageable, the improvement of metastatic UM treatment remains a major clinical challenge. Among many possible causes for this therapy failure, a major feature resides in the presence of slow-cycling or dormant UM cells within the liver microenvironment, which are able to evade the cytotoxic effect of the vast majority of anti-tumor drugs. In this frame, the selective pressure induced by cytotoxic approaches triggers the enrichment in a cancer stem-like component, since CSCs are favored by their reduced proliferative rate and by the activation of drug resistance molecular mechanisms [35,36]. Therefore, it is reasonable to assume that CSCs are actively involved in hindering the response to systemic and local therapies in metastatic UM and in UM recurrence.

3. Cancer stem-like cells

CSCs represent a small subset of tumor cells characterized by the ability to self-renew and to differentiate into multiple lineages within the tumor mass through symmetric and asymmetric cell division [37]. Additionally, CSCs are responsible for tumor initiation and growth, and they are involved in metastatic dissemination, resistance to therapy, and tumor relapse (Fig. 2) [38].

The exact process of CSC formation in tumors is still unclear; however, two main hypotheses are currently being discussed: on one side, the idea that CSCs could derive from normal stem cells undergoing mutations or epigenetic changes; on the other, the notion that differentiated cancer cells could activate oncogenic reprogramming, leading to the acquisition of stem-like properties [39]. Nevertheless, a certain degree of plasticity occurs between CSCs and differentiated cancer cells, suggesting that both are capable of phenotypical transition in response to environmental stimuli [40]. Moreover, CSCs are strictly dependent on the TME, which is a complex network of signals and cell types (*i.e.* endothelial and perivascular cells, fibroblasts, and immune cells) that sustain stem cells, while contributing to their differentiation into stromal lineages [41].

The first evidence of cancer stem cells dates back to 1997, when it was demonstrated that a subpopulation of $CD34^{+}/CD38^{-}$ leukemia cells could initiate the disease when inoculated in severe combined immunodeficient (SCID) mice [42]. Currently, the presence of CSCs has been described in several tumor types, including lung [43], liver [44], breast [45], stomach [46], pancreatic [47], bladder [48], and colon cancer [49], as well as cutaneous and, more recently, uveal melanoma [50,51]. Cell surface markers, such as CD24, CD34, CD44, CD90, CD123, CD133, CD166, and the epithelial cell adhesion molecule (epCAM), are essential tools to guide the identification of CSCs in both solid and hematological malignancies [52,53]. Additionally, CSCs may be recognized through the evaluation of distinctive stem-like properties, such as the enhanced expression of enzymes belonging to the aldehyde dehydrogenases (ALDH) superfamily and the ability to grow *in vitro* as three-dimensional spheres [54,55]. However, markers for CSCs can be extensively variable among tumor types and no universal marker has been identified yet. Moreover, the majority of these markers are shared by tissue-resident

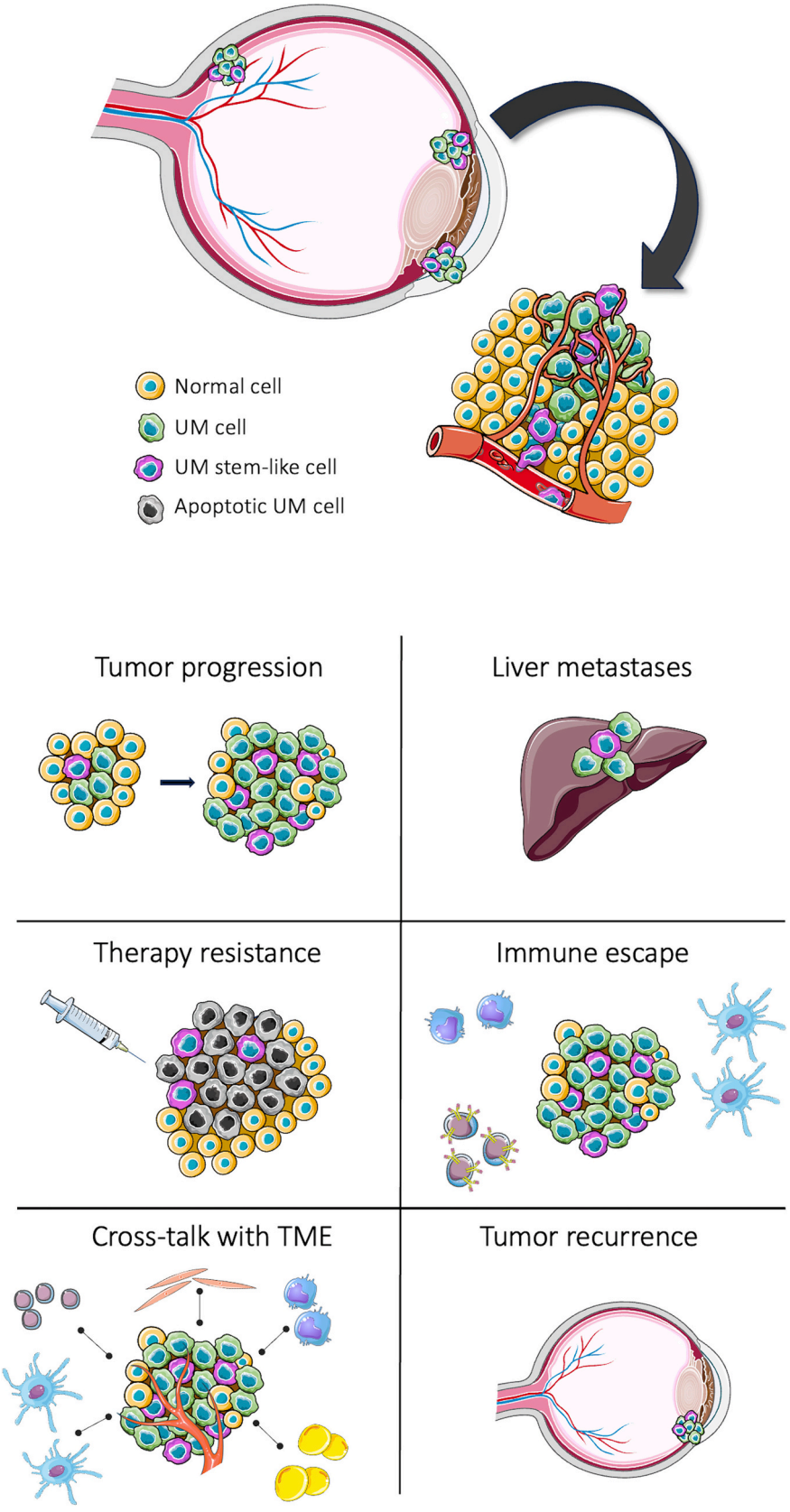

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Fig. 2. Cancer stem-like cells (CSCs).
UM-CSCs (purple) represent a small subset of total UM cells (green). CSCs are able to self-renew and to differentiate into various cell lineages, promoting tumor growth. Additionally, CSCs are actively sustained by an intricate cross-talk with different cell types located in the tumor microenvironment (TME), such as endothelial cells, fibroblasts, and immune cells. CSCs may acquire a transient epithelial-to-mesenchymal phenotype, which favors dissemination and metastasis. Moreover, CSCs can actively evade immune surveillance through the production of immune checkpoint molecules as well as growth factors, cytokines, and metabolites. Due to their low proliferation rate, the upregulation of anti-apoptotic proteins, and the activation of DNA repair machinery, CSCs can escape therapy-induced apoptosis; therefore, while conventional therapies eliminate only bulk cells (grey), remaining CSCs lead to recurrence. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

and embryonic stem cells [52].

CSCs are characterized by many distinctive traits, including the activation of stemness-associated signaling pathways. Indeed, CSCs upregulate transcription factors and molecules that control self-renewal and pluripotency, such as octamer-binding transcription factor 4 (OCT4), homebox protein NANOG, Sry-related HMG box 2 (SOX2), c-MYC, NF-κB, signal transducer and activator of transcription 3 (STAT3), Kruppel-like factor 4 (KLF4), Hedgehog (Hh), and Notch [56–60]. Additionally, CSCs may acquire a transient epithelial-to-mesenchymal phenotype, which allows them to easily migrate, invade the surrounding tissue, and drive metastasis formation [61], as suggested by the expression of several key regulators of epithelial-to-mesenchymal transition, such as twist-related protein 1 (TWIST1), zinc-finger protein SNAI1 (Snail), zinc-finger *E*-box binding homeobox 1 (ZEB1), and ZEB2 [62,63].

A major challenge in cancer therapy revolves around the onset of chemoresistance and the risk of recurrence. CSCs are crucially involved in these processes since they are able to resist to conventional therapies through several mechanisms. First, their low proliferation rate, the upregulation of anti-apoptotic proteins, and the timely activation of DNA repair machinery protects them from therapy-induced cell death [64,65]. Moreover, CSCs overexpress transporters and enzymes, such as the ATP-binding cassette (ABC) transporters and the ALDH enzyme superfamily, that inactivate and eliminate drugs. Indeed, ABC transporters actively mediate the efflux of various drugs from the cell, while ALDH enzymes are involved in detoxification processes, by lowering levels of intracellular reactive oxygen species (ROS) and reactive aldehydes [66,67].

To overcome these mechanisms of resistance, several therapeutic strategies are currently being investigated in cancer treatment, as briefly reported hereafter:

- Targeting the signaling pathways involved in CSC maintenance, proliferation, and differentiation. Inhibitors directed against Notch, Wnt/β-catenin, and Hh signaling pathways have been developed, showing positive results in clinical trials across different tumor types [68,69]. Additionally, other potential targets are being investigated, including TGFβ, NF-κB, and JAK-STAT [70].
- Designing selective monoclonal antibodies to target CSC membrane antigens. For instance, common surface markers such as CD44, CD47, and CD133 are being assessed as promising targets [71]. However, this approach is hindered by the redundance of these surface antigens on CSCs and normal stem cells [72].
- Hitting the TME to hamper the stem-cell niche. Despite the complexity of the TME, direct targeting of stromal cells, such as endothelial cells, tumor-associated fibroblasts, and tumor-associated macrophages, may provide an alternative approach for disrupting the intricate cross-talk of growth factors, cytokines, and chemokines that fosters CSC formation and survival [73].

Over the years, great improvement in cancer treatment has been achieved. However, eliminating CSCs still represents a critical step to reach long lasting and complete tumor eradication. Therefore, a better understanding of the complex mechanisms regulating CSCs is essential for developing novel approaches and lowering the risk of recurrence.

3.1. Cancer stem-like cells in UM

In the last few years, the existence of CSCs in UM has been described thanks to experimental studies aimed at assessing the expression of common markers of stem-like cells (Fig. 3). In this context, a $CD133^{+}$/$Nestin^{+}$ subpopulation has been identified in Mel270, OMM2.3 and OMM2.5 human UM cells. Notably, the fact that these lines are derived respectively from primary tumor and liver metastasis of the same patient suggests the involvement of $CD133^{+}$/$Nestin^{+}$ cells in metastatic dissemination [74]. Additionally, immunohistochemical analysis of paraffin-embedded primary tumors revealed the expression of CD133, Pax6, Musashi, Nestin, SOX2, and ABCB5 [74]. The upregulation of Nestin and CD166 has been demonstrated in short-term cultures of primary UM cells compared to normal choroidal melanocytes. Finally, an enrichment of the CSC subpopulation has been suggested by the high levels of CD166, Nestin, and CD271 found in UM cells resistant to anchorage-dependent cell death [75]. Interestingly, the upregulation of the stem-cell marker CD271 in UM has been previously linked to vasculogenic mimicry patterns and to an increased metastatic potential [76]. Despite these promising results, a stemness-related marker signature has not been identified in UM, thus hampering the possibility to sort, isolate and characterize this subpopulation.

To overcome these limitations, various strategies relying on the evaluation of distinctive properties of stem-like cells, *e.g.* sphere-formation capability and ALDH activity, have been developed. In the sphere-formation assay, CSCs are propagated by three-dimensional *in vitro* spheres growing in non-adherent and serum-free conditions, to form the so called melanospheres (Fig. 3). In these conditions the sphere-forming capacity is directly related to the number of CSCs present in culture [77].

On the other hand, the assessment of enzymatic activity levels of ALDH by flow cytometry is a reliable strategy to discriminate between CSCs, identified as an $ALDH^{+}$ (or $ALDH^{bright}$) population, and non-CSCs, which represent the $ALDH^{-}$ (or $ALDH^{low}$) fraction [78]. In this frame, Jin and colleagues have validated the assessment of enzymatic levels of ALDH as a marker for CSCs in UM, by confirming the presence of an $ALDH^{+}$ population of UM cells and proving the enhanced tumorigenic capacity *in vivo* of $ALDH^{+}$ cells compared to the $ALDH^{-}$ counterpart [79]. Moreover, it has been demonstrated that Mel270 and OMM2.5 UM cells, respectively derived from the primary tumor and liver metastasis of the same patient, are able to form melanospheres within 2 weeks of non-adherent culture [51]. Accordingly, the enhanced sphere-formation ability of cells derived from primary tumors of UM patients with poor-prognosis confirmed a correlation between stemness and more aggressive tumor types in the clinical setting [75].

4. Targeting cancer stem-like cells as a therapeutic strategy in uveal melanoma

Targeting CSCs is a necessary process to improve the efficacy of current anti-tumor treatments, especially for UM. Therefore, different therapeutic approaches may be implemented, and they can be theoretically categorized according to their mechanism(s) of action into: *i)* targeting CSC surface markers; *ii)* inhibiting CSC-associated/deregulated molecular pathways; *iii)* targeting molecules or cells that favors the CSC niche(s) within the tumor microenvironment.

Unfortunately, given the absence of reliable surface markers for UM-

A

UM CSC markers	
NESTIN	• Mel270, OMM2.3, OMM2.5 UM cell lines [77] • Primary UM tumors [76] • Short-term UM cultures [77]
CD133	• Mel270, OMM2.3, OMM2.5 UM cell lines [77] • Primary UM tumors [76]
CD166	• Short-term UM cultures [77]
PAX6	• Primary UM tumors [76]
MUSASHI	• Primary UM tumors [76]
SOX2	• Primary UM tumors [76]
ABCB5	• Primary UM tumors [76]

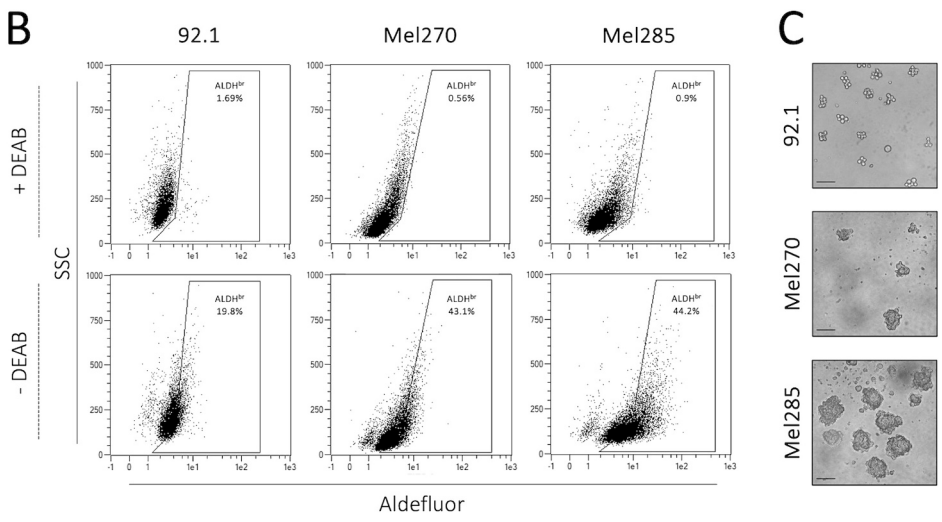

Fig. 3. Features of CSC subset in UM.
The presence of CSCs in UM has been validated by demonstrating the expression of common markers of stemness on UM cells lines, primary UM tumors and/or short-term UM cultures (A). Moreover, UM-CSCs are characterized by increased enzymatic activity of aldehyde dehydrogenase (ALDH), which can be assessed by flow cytometry (B), and by the capability to form melanospheres in non-adherent, serum-free conditions (scale bar = 100 μm) (C). B, C) Adapted from [106] and licensed under the Creative Commons Attribution 4.0 International License.

CSCs, to date a direct targeting of CSCs through surface-specific antigens is still poorly pursued. Nevertheless, new experimental approaches focused on targeting either signaling pathways involved in stemness maintenance or the tumor microenvironment are emerging in positive preclinical and clinical studies (Fig. 4).

4.1. *Targeting UM-CSC signaling pathways*

Recently, promising approaches involving the direct or indirect inhibition of signaling pathways that contribute to the maintenance of stemness have been described.

Two inhibitors of NF-κB and Wnt/β-catenin, the triterpenoid

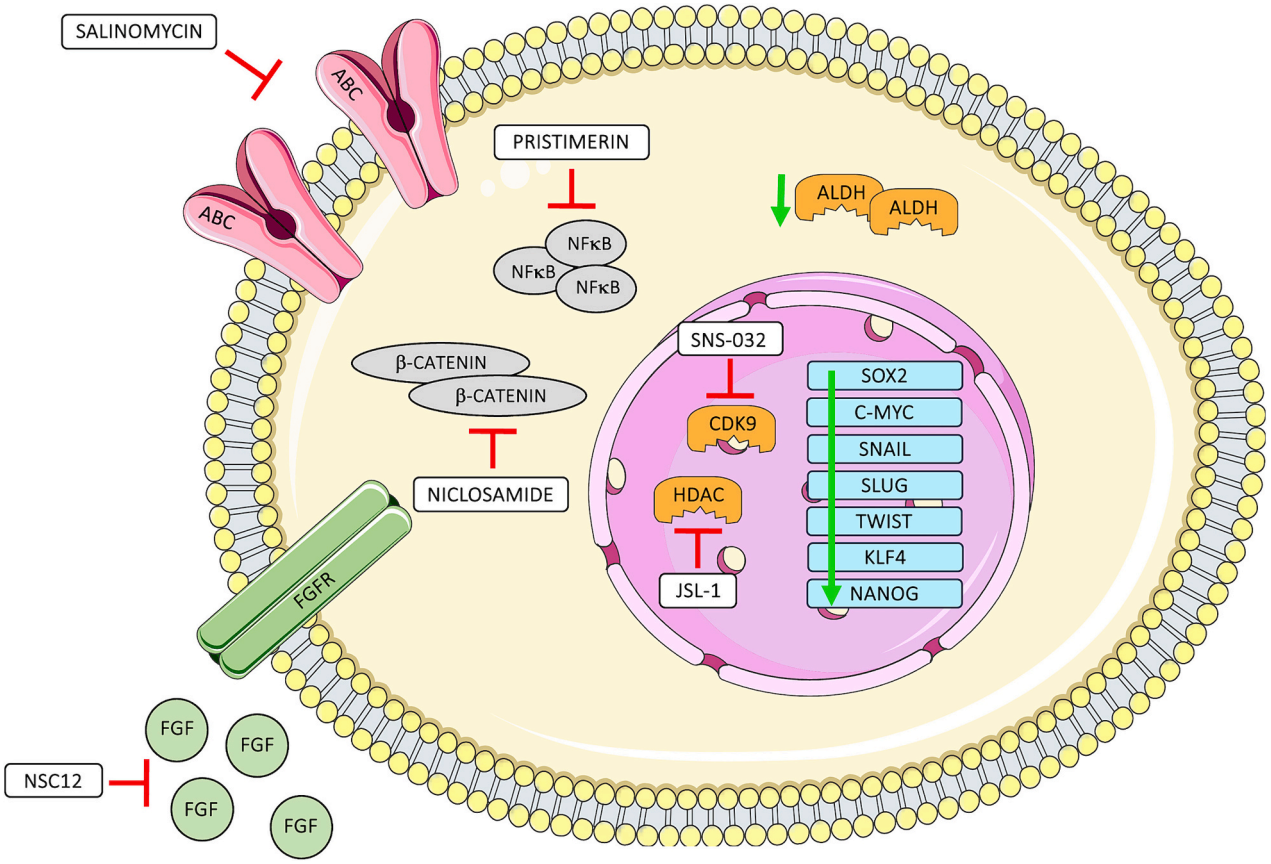

Fig. 4. Experimental targeting of UM-CSCs.
In the Figure are represented the experimental approaches carried out to inhibit CSC-associated pathways in UM.

pristimerin and the salicylanilide niclosamide, have been shown to impair the clonogenic potential and invasiveness of UM cells and affect cell viability promoting the production of ROS and triggering apoptosis. Additionally, they significantly reduce the $ALDH^+$ and sphere-forming cell subpopulation, while downregulating the stemness-associated transcription factors SOX2, Slug, and c-MYC [80,81]. Of note, the FDA-approved drug niclosamide safely used in human for over 50 years, displayed a strong anti-tumor effect in UM xenografts *in vivo*, with minimal cytotoxicity to normal tissues [81].

Salinomycin, a monocarboxylic polyether with antibiotic activity, has been recognized as a selective CSC inhibitor in breast cancer, colon cancer, renal carcinoma, and leukemia. Mechanistically, salinomycin exerts its anti-CSC effect by interfering with ABC transporters and inhibiting stemness-associated transcription factors SOX2, Snail, c-MYC, Hedgehog, and Wnt/β-catenin [82,83]. Relevant to UM, it has been demonstrated that salinomycin impairs cell viability, clonogenic potential, invasiveness, and migration of UM cell lines [84]. *In vivo*, treatment with salinomycin significantly reduces tumor growth of UM xenografts in immunocompromised mice as well as the formation of liver metastasis following intrasplenic injection of UM cells. Additionally, salinomycin hampers the $ALDH^+$ UM stem-like component, lowering the expression of the stemness-related factors *SOX2* and *TWIST1* [84]. Given that both *TWIST1* and *SOX2* correlate with increased risk of metastasis and enhanced mortality in UM patients, these results represent a significant starting point for further investigation for the potential clinical application of salinomycin in UM patients.

Another promising approach involves the targeting of cyclin-dependent kinase 9 (CDK9), which is overexpressed by several UM cell lines, through the selective inhibitor SNS-032. Indeed, CDK9 inhibition by SNS-032 hampers the activity of the transcription activator YAP, which is required for Gαq/11-driven tumorigenesis. Accordingly, SNS-032 significantly reduces cell viability of UM cells, but not in a retinal pigment epithelial cell line. Moreover, treatment with SNS-032 inhibits colony formation and activates apoptosis, exerting a synergic effect in combination with the chemotherapeutic drug vinblastine [85]. In line with the targeting of the CSC population, these effects are paralleled by a decrease of the $ALDH^+$ and sphere-forming CSC subpopulation, and by reduced cell migration and invasiveness, as indicated by the downregulation of metalloproteinases and the impairment of actin polarization/invadopodia formation. Finally, SNS-032 hinders tumor growth *in vivo* and suppresses liver metastasis formation by targeting the stem-like component, as suggested by the reduced expression of Slug and KLF4, two mediators that strongly correlate with increased mortality and lower metastasis-free survival in UM patients [85].

Similarly, enhancer of zeste homolog 2 (EZH2) is a known regulator of stemness in multiple tumor types and is associated to a higher risk of metastasis and to a shorter survival in the clinical settings. Immunohistochemical staining on UM samples and choroidal tissue from healthy donors showed the overexpression of EZH2 in 88% of tumor cases. Moreover, a direct correlation between the overexpression of EZH2 and enhanced aggressiveness of primary UM tumors as well as reduction of the overall survival was observed. In this context, EZH2-transfection in UM cells promotes a more aggressive phenotype by enhancing cell proliferation, clonogenic potential, and invasiveness of UM cells, which where instead affected by *EZH2* knock-down. Additionally, depletion or inhibition of EZH2 impairs the stem-like component, as well as *in vivo* tumor growth and formation of hepatic metastasis, suggesting the relevance of EZH2 as a potential therapeutic target [79].

Histone deacetylase (HDAC) inhibitors have emerged as promising therapeutic agents in many tumor settings, due to their strong selectivity and low toxicity to normal tissues. Currently, four drugs have been approved by the FDA and the European Medicine Agency for the treatment of T-cell lymphoma and multiple myeloma, and their efficacy has been assessed also in experimental models of UM, showing promising results [86]. Indeed, the novel HDAC inhibitor JSL-1 efficiently targets UM-CSCs *in vitro* and *in vivo*, successfully reducing cell proliferation, migration, and invasiveness of UM cells. Moreover, JSL-1 significantly impairs sphere-formation and serial replating capacity, as well as the percentage of $ALDH^+$ cells. Finally, treatment with JSL-1 triggers an apoptotic response in UM cells and significantly impairs tumor growth in a human UM xenograft model, thus confirming a potent anti-tumor activity *in vivo* [87]. Of note, the combined administration of JSL-1 and the chemotherapeutic agent vinblastine exerts a synergistic effect, demonstrating the potential improvement of combining HDAC inhibitors with conventional therapies [87].

Finally, promising results have been obtained by targeting UM cell metabolism. Indeed, the combination of a sodium/calcium (Na^+/Ca^{2+}) exchanger SLC8A1 inhibitor with the mitochondrial antioxidant MitoQ able to target Ca^{2+} homeostasis and oxidative stress, respectively, strongly inhibited the growth of specific subsets of metastatic UM *in vitro* and in *in vivo* xenografts [88]. Given the pleiotropic role played by ROS in modulating CSC biology [89], it would be interesting to assess the effect of this drug combination on UM-CSC metabolic vulnerabilities.

4.2. Targeting UM-CSC supportive microenvironment

An additional strategy to impair the CSC subpopulation inside the tumor mass consists in targeting the TME elements involved in the complex formation and maintenance of the so called “stem niche”. This is an extremely broad approach since many secreted factors, proteases, ECM components and metabolic mediators may contribute to this aspect. To date, few reports have correlated the “manipulation” of TME elements with UM stemness inhibition.

Among the variety of extracellular proteases contributing to the dynamism of TME, ADAMTS1 has been correlated to the acquisition of an endothelial-like phenotype in tumor cells, as an alternative mechanism of neovascularization, though the reversion to a stem-like state [90]. Given its high expression in UM patients during the early stages of the disease, the role of ADAMTS1 in UM stemness regulation has been investigated by CRISPR-Cas9 technology. Notably, the knockout of ADAMTS1 significantly reduced melanosphere-formation capacity, endothelial-like properties of UM cells, and downregulated genes involved in vascular remodeling. Moreover, *ADAMTS1* deficient cells displayed a reduced tumorigenic potential *in vivo*, and the explanted tumors were characterized by a significant downregulation of stemness-associated genes such as *NANOG*, *OCT4*, *PROM1*, and *SOX2*. Also, these tumors had alterations in vascular density, thus supporting the hypothesis that ADMATS1 may sustain the development of UM through the induction of stemness [91].

Within the tumor microenvironment, the fibroblast growth factor (FGF) family plays a pivotal role in assisting tumor growth and further promoting cancer cell proliferation and survival [92,93]. In the TME different cell types, including cancer associated fibroblasts and macrophages, mast cells, endothelial cells, and cancer cells themselves, actively produce FGFs to sustain autocrine and paracrine pro-tumor loops [94–97]. Accordingly, blockade of the FGF/FGF receptor (FGFR)-mediated signaling has been demonstrated to hamper tumor growth of FGF-dependent murine and human cancers, including UM [3,98–100]. In addition, FGF family reportedly participates in self-renewal of stem cells both in physiological conditions as well as in several type of tumors [101–105]. Relevant to UM, it has been demonstrated that sequestration of FGFs by a pan-FGF trap is able to hit and unmask a subpopulation of cells characterized by stem-like properties, including the expression of stemness-related transcription factors, enhanced ALDH activity, and tumor-sphere formation capacity. *In vitro* and *in vivo* “targeting” of this subpopulation resulted in the loss of the stem-like component and in the reduction of UM tumor growth [106]. These findings, together with the evidence that FGF/FGFR expression and stemness are strictly linked in UM patients and are associated to a worst disease-free survival [3,106], provide the rationale of targeting FGF/FGFR to strike UM-CSCs and for repositioning of FDA-approved FGFR inhibitors.

Hypoxia is a feature of solid tumors triggered by the fast growth and metabolic rate of tumor cells and by the deficient tumor-supporting vasculature. It has been shown that hypoxia activates the signaling pathways required for CSC survival and that adaptation of cancer cells to hypoxia requires the activation of hypoxia-responsive genes *via* the hypoxia-inducible factor 1α (HIF-1α). In UM, HIF-1α has been found highly expressed and associated with metastatic spread. Interestingly, the genetic silencing as well as the pharmacologic blockade of HIF-1α resulted in reduced UM cell growth and invasiveness and impacted the Notch pathway components [107]. Given the fact that CSC niche is typically a hypoxic context and that the Notch pathway contributes to the onset of stemness, it would be worth to further investigate the effect of hypoxia-targeting/impairing agents in the context of UM.

Finally, tumor-associated macrophages (TAMs) have been recognized to sustain CSC survival and progression. Moreover, they protect CSC niche from other immune cell recognition [108]. In the context of UM, TAMs actively mediate pro-angiogenic/pro-tumor functions, thus contributing to tumor metastasis [109]. Accordingly, *in vivo* depletion of macrophages resulted in a strong inhibition of intraocular melanoma growth in a syngeneic mouse model [110]. In this context, how TAMs sustain UM-CSCs represents an interesting, still unexplored field that may open new therapeutic possibilities to target the staminal compartment in UM.

5. Conclusions

In the last decades, cancer treatment has seen an incredible acceleration in the development of therapeutic approaches against new targets. This leap forward has been possible thanks to the last generation molecular approaches that allow for the characterization and manipulation of single cells. Nevertheless, since tumor adaptation and relapse remain an issue for most tumor types, understanding the mechanisms leading to therapy failure represents a challenging goal.

In this context, it is now widely demonstrated that CSCs play a pivotal role in tumor progression, dissemination, and relapse; therefore, the scientific community points to the development of CSC-targeted therapies as promising approaches for the treatment of solid cancers [38,40,52]. Nevertheless, despite the huge number of preclinical studies in this field, some general limitations hampered the effective translation of this therapeutic approach to the clinical practice. As already discussed, the close resemblance between CSCs and normal/tissue-resident stem cells, the plasticity of the CSCs, and the lack of universal markers still represent obstacles for effective and safe CSC-targeting therapies.

As for UM, research aimed at targeting CSCs is still an emerging branch. Indeed, new specific features and markers of UM stem-like cells are being discovered and placed alongside those identified in other tumor types. For instance, both intrinsic and extrinsic mechanisms of CSC resistance and survival have been reported in UM, thus opening the possibility to target the stem-like component as well as the supporting microenvironment. In this frame, the preclinical and clinical studies are aimed at developing combination strategies in order to simultaneously strike both CSC and non-CSC subpopulations, and to target the cross-talk between TME and CSCs.

Most of the research efforts are now concentrating on the so-called big killer solid tumors, and this represents an additional limitation for rare cancers, like UM, that lacks high number of samples and studies. In this frame, the application of last generation single cell and spatial techniques, which are revolutionizing the comprehension of tumor complexity and heterogeneity, could provide a major contribution [30,111,112]. Indeed, a more extensive enforcement of these molecular approaches in UM would allow for a better understanding of the interconnection between different cell populations within the tumor mass. Moreover, these techniques are of fundamental importance to study rare tumors, precisely due to their low frequency which limits the amount of material available to perform in-depth characterizations.

In conclusion, despite the questions that remain to be addressed, the promising results of the preclinical studies described in this review demonstrate both relevance and feasibility of CSC targeting to improve UM management. Further studies focused on unravelling the biological mechanisms involved in CSC sustenance, on the identification of new molecular targets, and on the application of novel technologies will widen the spectrum of potential therapies to be applied in the clinical practice, with the goal of improve the management of UM and the survival expectancy of patients.

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CRediT authorship contribution statement

Alessandra Loda: Conceptualization, Visualization, Writing – original draft, Writing – review & editing. **Francesco Semeraro:** Writing – review & editing. **Silvia Parolini:** Writing – review & editing. **Roberto Ronca:** Writing – original draft, Writing – review & editing, Supervision. **Sara Rezzola:** Conceptualization, Visualization, Writing – original draft, Writing – review & editing, Supervision.

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.

Data availability

No data was used for the research described in the article.

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