Histol Histopathol (2016) 31: 1291-1301

http://www.hh.um.es

Histology and Histopathology

From Cell Biology to Tissue Engineering

Review

Cancer stem cell as therapeutic target for melanoma treatment

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Summary. Human malignant melanoma is a highly aggressive skin tumor that is characterized by its extraordinary heterogeneity, propensity for dissemination to distant organs and resistance to cytotoxic agents. Although chemo- and immune-based therapies have been evaluated in clinical trials, most of these therapeutics do not show significant benefit for patients with advanced disease. Treatment failure in melanoma patients is attributed mainly to the development of tumor heterogeneity resulting from the formation of genetically divergent subpopulations. These subpopulations are composed of cancer stem-like cells (CSCs) as a small fraction and non-cancer stem cells that form the majority of the tumor mass. In recent years, CSCs gained more attention and suggested as valuable experimental model system for tumor study. In melanoma, intratumoral heterogeneity, progression and drug resistance result from the unique characteristics of melanoma stem cells (MSCs). These MSCs are characterized by their distinct protein signature and tumor growth-driving pathways, whose activation is mediated by driver mutation-dependent signal. The molecular features of MSCs are either in a causal or consequential relationship to melanoma progression, drug resistance and relapse. Here, we review the current scientific evidence that supports CSC hypothesis and the validity of MSCs-dependent pathways and their key molecules as potential therapeutic target for melanoma treatment.

Key words: Melanoma, Cancer stem-like cells, Melanoma stem cells, Signaling pathways, Therapy

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DOI: 10.14670/HH-11-791

Introduction

Human malignant melanoma is a highly aggressive skin tumor that is characterized by its extraordinary heterogeneity, propensity for dissemination to distant organs and resistance to cytotoxic agents. Despite improved treatment options, patients with advanced malignant melanoma still have poor prognosis as measured by progression-free and overall survival (Weiss et al., 2015). Traditional therapeutics can primarily target tumor bulk leaving tumor-initiating cells behind (Czyz et al., 2013). In recent years, cancer stem-like cells gained more attention and suggested as valuable experimental model system for tumor study. The hypothesis of cancer stem-like cells (CSC) suggested that neoplastic clones are maintained by a small fraction of CSCs, whose unique features are essential for tumor initiation, progression, drug resistance and relapse (Reya et al., 2001). Understanding the properties of CSCs and their functional role in the modulation of the primary and acquired resistance

mechanisms may have a therapeutic significance for melanoma treatment. Malignant melanoma are composed of genetically divergent subpopulations, including melanoma stem cells (MSCs) and non-melanoma stem cells. MSCs, as a minor fraction of tumor cells are responsible for tumor maintenance, progression, resistance and relapse (EL-Khattouti et al., 2014, 2015; Leikam et al., 2015; Li et al., 2015). MSCs are characterized by their unique protein pattern and aberrant signaling pathways, which are either in a causal or consequential relationship to melanoma progression and resistance (Liu et al., 2006; Hardesty et al., 2013; Jazirehi et al., 2014; Paulitschke et al., 2015). In advanced stages of melanoma, current treatments rarely result in long-term survival. Treatment failure is common among melanoma patients and the most available therapies fail to eradicate CSCs that, in turn, can initiate and relocate a new tumor leading to tumor relapse (EL-Khattouti et al., 2014, 2015). Accordingly, patients with initial treatment response are infamous for tumor relapse, even if only a small number of CSCs are able to escape drug toxicity. To that end, the development of therapeutic strategy, based on the elimination of CSCs, may help to block tumor regression and relapse. Here, we review the current scientific evidence that supports CSC hypothesis and the aberrant signaling pathways of MSCs together with their key molecules that may function as a potential therapeutic target for melanoma treatment.

Cancer stem cells

CSCs are in many aspects similar to normal stem cells. They are hierarchically organized at cellular level besides being located in their origin tissues, where they form a small fraction of genetically divergent subpopulations (Kasai et al., 2014). In addition to their tissue-specificity and unique properties, CSCs have the capability to self-renew and differentiate into different cell types (Jang et al., 2014; Rasheed et al., 2015). The occurrence of CSCs is the consequence of intrinsically asymmetric cell divisions of stem cell lineage into two daughter cells that substantially differ in their morphology and biological behavior (Pine and Liu, 2014; Wang et al., 2014). During progression of the asymmetric cell divisions, the stemness properties are inherited by only one of the two daughter cells, so that one of them, CSC, that is genetically programmed for the maintenance of stemness properties, while the other one, the non-cancer stem cell, represents the majority of the formed tumor mass (Gomez-Lopez et al., 2014). Asymmetric cell division is a type of cell division that is characteristic to cells with stemness properties leading to the generation of intratumoral heterogeneity (Boesch et al., 2014). In addition to their ability to undergo asymmetric cell division, CSCs are able to grow continuously and to divide indefinitely (Gomez-Lopez et al., 2014; Ma et al., 2014). Although the mechanisms of asymmetric division of CSCs and conventional symmetric cell division are similar, the mechanisms regulating the differentiation of CSCs into different ways is quite different and determined mainly by CSCs-specific mechanisms (Harandi and Ambros, 2015). While the larger daughter cell can self-renew and divide indefinitely, the smaller daughter cells can only undergo limited cell division before entering into the differentiation processes (Sell, 2004; Rajaraman et al., 2005).

Although the hypothesis of CSCs suggests that cancer stem cells are derived from normal stem cells rather than from progenitor cells based on the functional analysis of leukemia initiating cells (Blair et al., 1998), the rise of cancer from normal stem cells (Mao et al., 2001), or progenitor stem cells (Haeno et al., 2009; Kim et al., 2012), have been reported. Aside from their origin and unique properties, CSCs offer extraordinary model for the elucidation of the mechanisms regulating tumor initiation, progression, drug resistance and relapse. To that point, CSCs can serve as a relevant experimental model for the development of more efficient anti-cancer agents. The possible ways leading to the development of cancer stem cells are outlined in detail (Fig. 1).

Signaling pathways regulating cancer stem-like cells progression and resistance

Activation of aberrant signaling pathways by genetic and epigenetic-dependent mechanisms are characteristic to CSCs survival and maintenance (Sunayama et al., 2010; Pulvirenti et al., 2011; Augustin et al., 2012; Swartling et al., 2014). The most common mechanisms of CSCs progression and resistance are attributed to mutation-driving signals that can trigger the activation of tumor growth-driving pathways (Pappalardo et al., 2016). Signaling pathways, such as Wnt/β-catenin, TGF-β, Notch1, EGFR/HER2, SMO and EpCAM have been discussed as targets for CSCs progression (Cheng et al., 2013; Singh et al., 2013; Wang et al., 2013; Bao et al., 2014; Ordonez et al., 2014). In accordance, the functional analysis of pathways, such as Wnt, Hedgehog and Notch signaling pathways in CSCs derived from solid and non-solid tumors confirmed their central role in the regulation of self-renewal activity of CSCs (Perry et al., 2011; Lee et al., 2014; Madeja et al., 2015). The ability of activated β-catenin to increase the self-renewal capacity of the epidermal stem cell *in vitro* (Zhu and Watt, 1999), as well as in the formation of epithelial cancers *in vivo* (Gat et al., 1998), has been reported.

In addition to its role in the regulation of variable cellular processes, including proliferation, differentiation, motility, and survival and/or apoptosis, Wnt signaling pathways are implicated in the regulation of CSCs progression and maintenance (Mohammed et al., 2016). The most prevalent Wnt signaling pathways include the canonical pathway that is known as Wnt/β-catenin pathway and the noncanonical pathways, such as the planar cell polarity pathway, the Wnt/Ca^{2+} pathway and the protein kinase A pathway (Boutros et al., 1998;

Bradley and Drissi, 2011). Once extracellular Wnt ligands have been ligated to the frizzled cell receptor, the Axin-1 protein starts to translocate to the plasma surface, where it prevents the degradation of β-catenin. As a consequence, β-catenin can then translocate to the nucleus where it enhances the transcription of Wnt target genes, such as Cyc lD, MYC and CD44 which, in turn, potentiates the self-renewal capacity of CSCs (Cheng et al., 2013; Cai and Zhu, 2012).

Like the Wnt pathway, the importance of the Notch signaling pathway in the regulation of CSCs maintenance and self-renewal has been reported (Wang et al., 2012). Notch receptors have been identified as a single-pass transmembrane proteins that function as receptors for Delta Like (Dll) 1, 3 and 4, and Jagged 1 and 2 ligands (Yoon et al., 2012). Activation of Notch receptors by their corresponding ligands stimulates their cleavage by γ-secretase to generate a Notch intracellular domain (NICD). The translocation of NICD to the nucleus, where it interacts with C promoter binding factor 1(CBF1)/ recombination signal binding protein for immunoglobulin kappa J region (RBPJ-k). The RBPJ-k is a protein that is essential for the transcriptional activation of Notch target genes (Panaccione et al., 2016). The most recognized Notch signaling target genes include the Hairy/Enhancer of Split (Hes) as well as the Herp/Hesr/Hrt/CHF/gridlock (Hey) families of basic helix-loop-helix (bHLH)-type transcriptional repressors (Iso et al., 2003). The activation of Hes/Hey transcription by Notch/RBP-J results in the repression of tissue-specific transcriptional activators leading to the prevention of cell differentiation (Wang et al., 2009; Borggrefe and Oswald, 2013). The involvement of Hes genes in the regulation of self-renewal and maintenance of melanoma stem cells has been suggested (Malbrain et al., 1996; Katoh and Katoh, 2007). For example, the inhibition of Notch signaling and subsequently its target genes results in the suppression of melanoma stem cell self-renewal (Kaushik et al., 2015). In accordance, the therapeutic impact of the inhibition of Notch signaling has been demonstrated in glioblastoma stem cells (Takebe et al., 2015). The role of Notch1 signaling

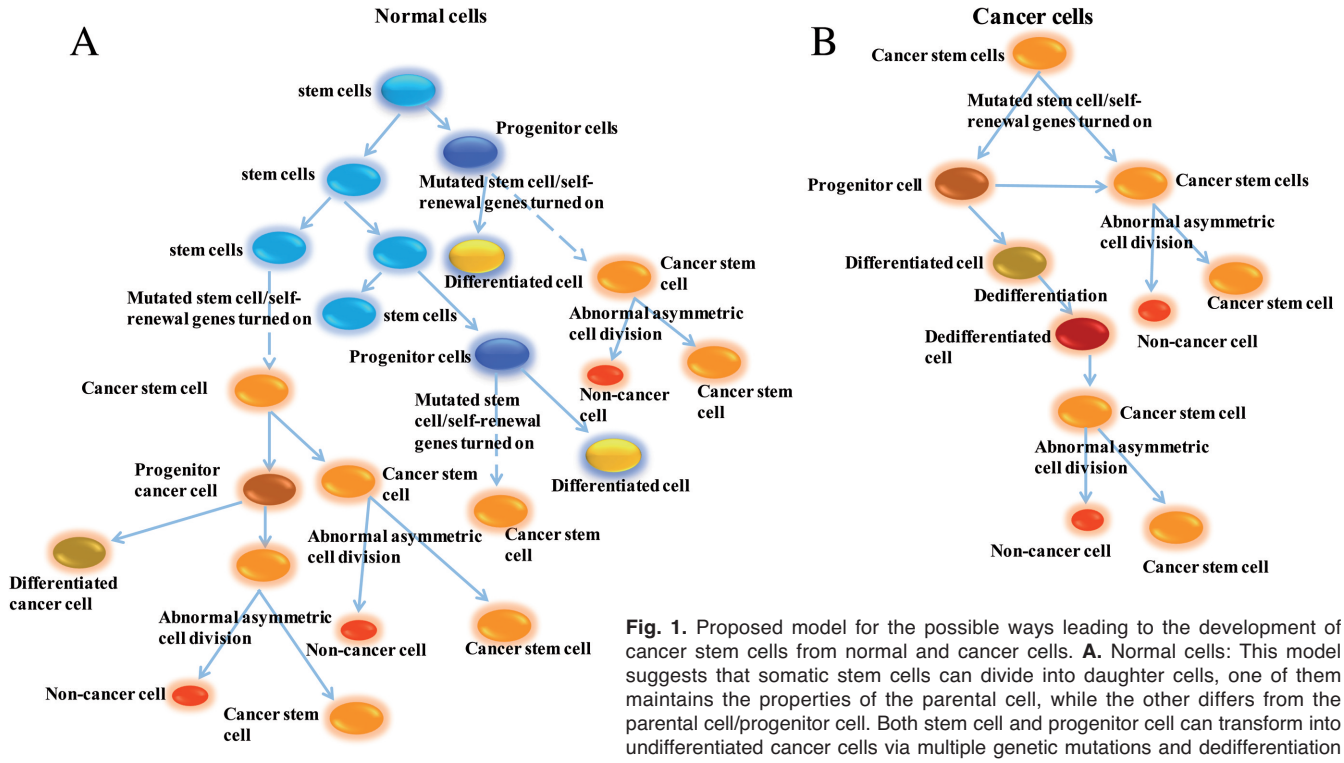

Fig. 1. Proposed model for the possible ways leading to the development of cancer stem cells from normal and cancer cells. **A.** Normal cells: This model suggests that somatic stem cells can divide into daughter cells, one of them maintains the properties of the parental cell, while the other differs from the parental cell/progenitor cell. Both stem cell and progenitor cell can transform into undifferentiated cancer cells via multiple genetic mutations and dedifferentiation processes. Cancer stem cells can undergo abnormal asymmetric cell division resulting in the formation of two daughter cells: one is the cancer stem cell that is responsible for tumor maintenance and progression based on its self-renewal potency and ability to divide indefinitely, while the other daughter cell (non-cancer stem cell) is responsible for the formation of tumor mass. Similar to cancer stem cells, the transformed progenitor cancer cells can undergo abnormal asymmetric cell division resulting in the formation of daughter cancer stem cells with limited cell division potency and one daughter non-cancer stem cell. **B.** Cancer cells: This model suggests that under the activation of aberrant signaling pathways via driver mutation of tumor growth signal and turning on self-renewal genes, then the cancer cell becomes able to divide into cancer progenitor cell and cancer stem cell. The division of cancer progenitor cell can divide into two daughter cells, one daughter cell as a differentiated cell and one as a cancer stem cell. Once the differentiated cell undergoes the dedifferentiation processes, it becomes able to transform into a cancer stem cell that, in turn, can undergo abnormal asymmetric cell division resulting in the production of one cancer stem cell and one non-cancer stem cell.

pathway has been suggested as a critical regulator of stemness in head and neck squamous cell carcinoma (HNSCC) (Lee et al., 2016a). Also, the impact of both Notch and Wnt/β-catenin signaling pathways in activation of liver cancer stem cells has been reported (Wang et al., 2016). Thus, suppression of Notch and/or Wnt/β-catenin signaling pathway is expected to impair melanoma stem cell survival and maintenance.

The hedgehog (Hh) pathway is one of the fundamental signal transduction pathways in tissue development, besides being involved in the self-renewal, maintenance and carcinogenesis of stem cells (Santini et al., 2012). Hh is a secreted molecule that can influence cell growth and differentiation via the binding patched (Ptch), a membrane transporter that is essential for the regulation of smoothened receptor (SMO) (Shahi et al., 2010). The main function of SMO is to mediate the signal transduction in the Hh pathway (Chen et al., 2010). Thus, in the absence of Hh ligand, Ptch is able to inhibit the signal transducer SMO and its downstream signaling pathways (Ecke et al., 2008). As a consequence, Gli 2 can be phosphorylated and cleaved into a truncated peptide that function as repressor for the transcription of secreted Hh (sHh) target genes such as Cycl D, MYC and Jag2 (Jiang et al., 2014). In accordance, the sonic hedgehog pathway has been reported to be essential for the maintenance of cancer stem-like cells in human gastric cancer (Song et al.,

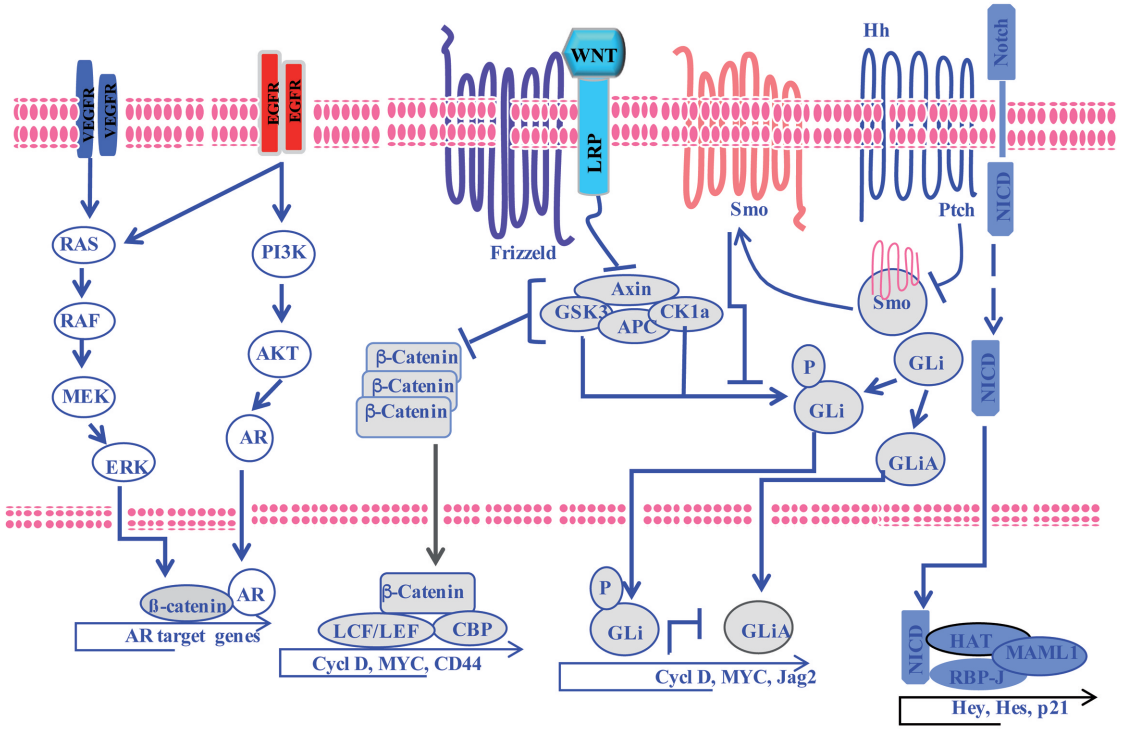

Fig. 2. The possible pathways which are involved in the regulation of self renewal and maintenance of melanoma stem cells. Notch signaling pathway. The legation of Notch ligands (Jag/Delta) to the Notch receptor mediates the cleavage of the intracellular domain (NICD) that, in turn, translocates to the nucleus where together with the co-activators it enhances the transcriptional activation of its targets, including Hairy/Enhancer of Split related with YRPW motif (Hey), Hairy/enhancer of Split (Hes) and cyclin-dependent kinase inhibitor 1 (p21). Hedgehog signaling (Hh). The activation of Patched (Ptch) receptor in response to the ligation to Hh legand results in the inhibition of the Smoothened (SMO) receptor allowing the SMO receptor to localize to the cell membrane to block the inhibitory effect of SMO on the phosphorylation and cleavage of Gli that can be induced by GSK3, CK1α, and PKA via mechanism mediated by preventing the formation of repressive Gli (GliR) and enhancing the formation of activated Gli (GliA). The activated GliA then can translocate into the nucleus, where it initiates transcription of target genes such as Cycl D, MYC encoding for Myc proto-oncogene protein and jagged-2 (Jag2). Wnt signaling pathway. Wnt signal can be initiated through the stabilization and nuclear accumulation of β-catenin via a mechanism mediated by the ligation of Wnt ligands to the frizzled receptor and the LRP co-receptor. As a consequence, the activation of the frizzled receptor together with its co-receptor LRP hold off Axin degradation complex to prevent the degradation of β-catenin. As a consequence, the β-catenin can be translocated to the nucleus, where it associates with the transcription factors LCF/ LEF and co-activators (e.g., CBP) to initiate transcriptional activation of the target genes, including Cycl D, MYC and CD44. Endothelial growth factor pathway: The activation of the endothelial growth factor (EGF) signal can be mediated through the EGFR-ligand complex formation. The activation of EGFR is mostly associated with the enhancement of two signaling Ras/Raf-MEK and PI3 kinase/Akt pathways leading to the transcriptionl activation of target genes regulating tumor progression and resistance. Vascular endothelial growth factor pathway: The stimulation of the vascular endothelial growth factor (VEGF) signal is the consequence of the ligation of VEGF ligand to the corresponding receptor that, in turn, can trigger cell growth and migration via the activation of the Raf-MEK-ERK cascade and a PKC-dependent ERK-independent pathway, respectively.

2011). Also, the inhibition of this signal pathway by sulforaphane confirmed the importance of sHh role in the regulation of self-renewal of pancreatic cancer stem cell (Rodova et al., 2012). However, the dysregulation of Hh pathway in cancer has been reported to result from the occurrence of a frequent mutation in Ptch and/or SMO (Saldanha, 2001). Interestingly, the functional analysis of many human tumors types addressed a central role for the Hh pathway in the regulation of CSC self-renewal and maintenance (Cochrane et al., 2015; Wang et al., 2016; Zhou et al., 2016). To that end, the inactivation of the Hh signaling pathway has been reported to be essential for the loss of the tumorigenic potential of CSCs (Sahebjam et al., 2012). Also, the elevated activation of the Hh signaling in CSCs that phenotypically resemble normal memory B cells has been noted in multiple myeloma (Agarwal and Matsui, 2010). The Hh signaling plays an essential role in the regulation of self-renewal and differentiation of CSCs (Huang et al., 2012). In addition to its potential role in tumor formation, the Hh signaling is thought to play a critical role in tumor progression and metastasis. Although all the entire mechanisms of Hh-mediated carcinogenesis are not described in detail, the role of the Hh pathway in the prevention of cell cycle arrest of epithelial cells has been reported (Mukherjee et al., 2006). Similar to its mechanistic role in the regulation of normal stem cells, the Hh signaling pathway is expected

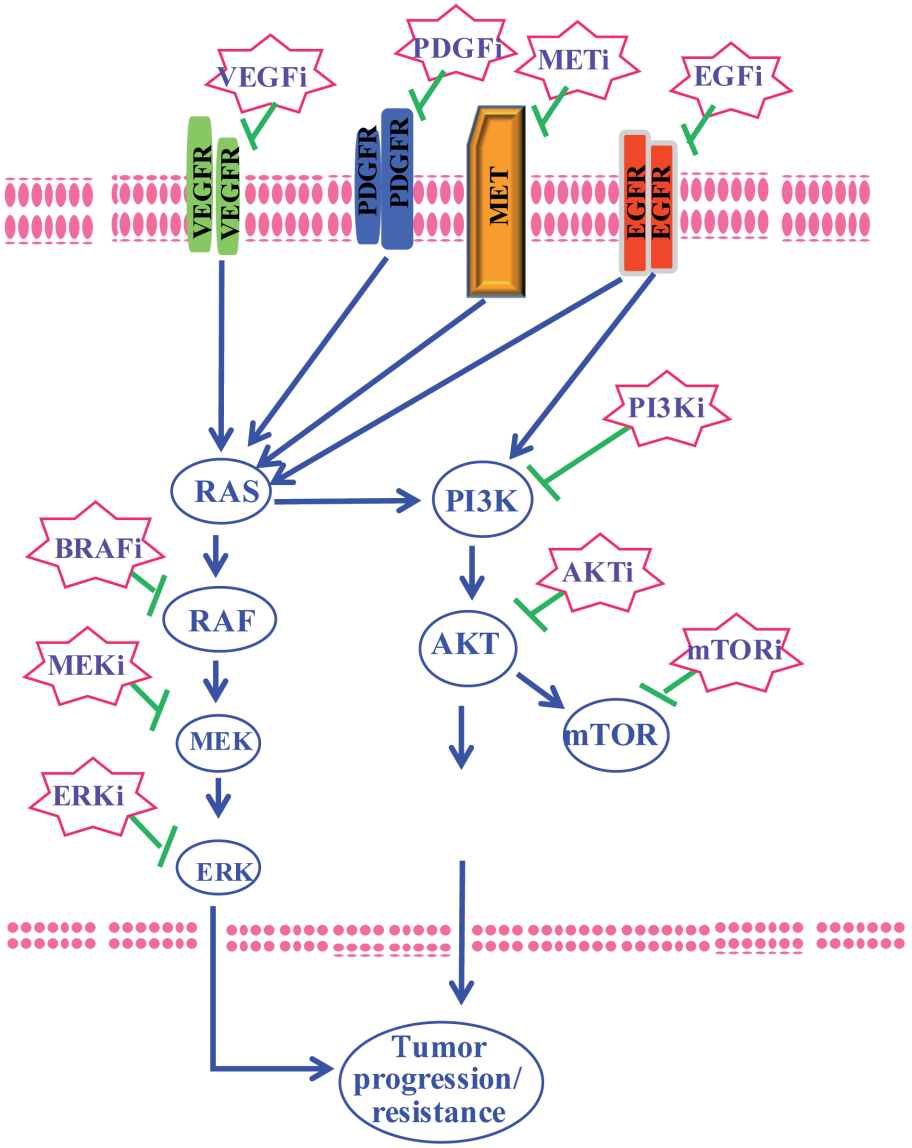

Fig. 3. Signaling pathways and their key molecules, which are thought to function as potential therapeutic targets in melanoma treatment. BRAF inhibitors (BRAFi) that are already approved for melanoma treatment include dabrafenib. MEK inhibitors (MEKi) that are already approved for melanoma treatment include trametinib. ERK inhibitors include PD98059 and U0126. Phosphoinositide 3-kinase (PI3K) inhibitors, include NVP-BEZ235, BKM120, BAY 80-6946, GDC-0941 and GDC-0980. AKT inhibitors (AKTi) include AZD5363, MK-2206 and KRX-0401. mTOR inhibitors include AP23573/MK-8669, and CCI-779. Vascular endothelial growth factor inhibitors (VEGFi) include ranibizumab, pegaptanib sodium, aflibercept and bevacizumab. PDGF inhibitors (PDGFi) include imatinib, sunitinib, sorafenib, pazopanib, nilotinib and cediranib.

to contribute to the promotion of the tumorigenic potential and self-renewal of CSCs (Nanta et al., 2013). Thus, the inhibition of the Hh signaling pathway may be an effective strategy to overcome CSCs resistance to current therapies. A proposed model describing the signaling pathways, which are thought to be responsible for CSCs progression and resistance is outlined in Fig. 2.

Signaling pathways as therapeutic target in melanoma treatment

The development of an efficient therapeutic protocol for melanoma treatment still remains a challenge for researchers and clinicians. Although targeted (BRAF) inhibitor-based therapy has greatly improved the clinical

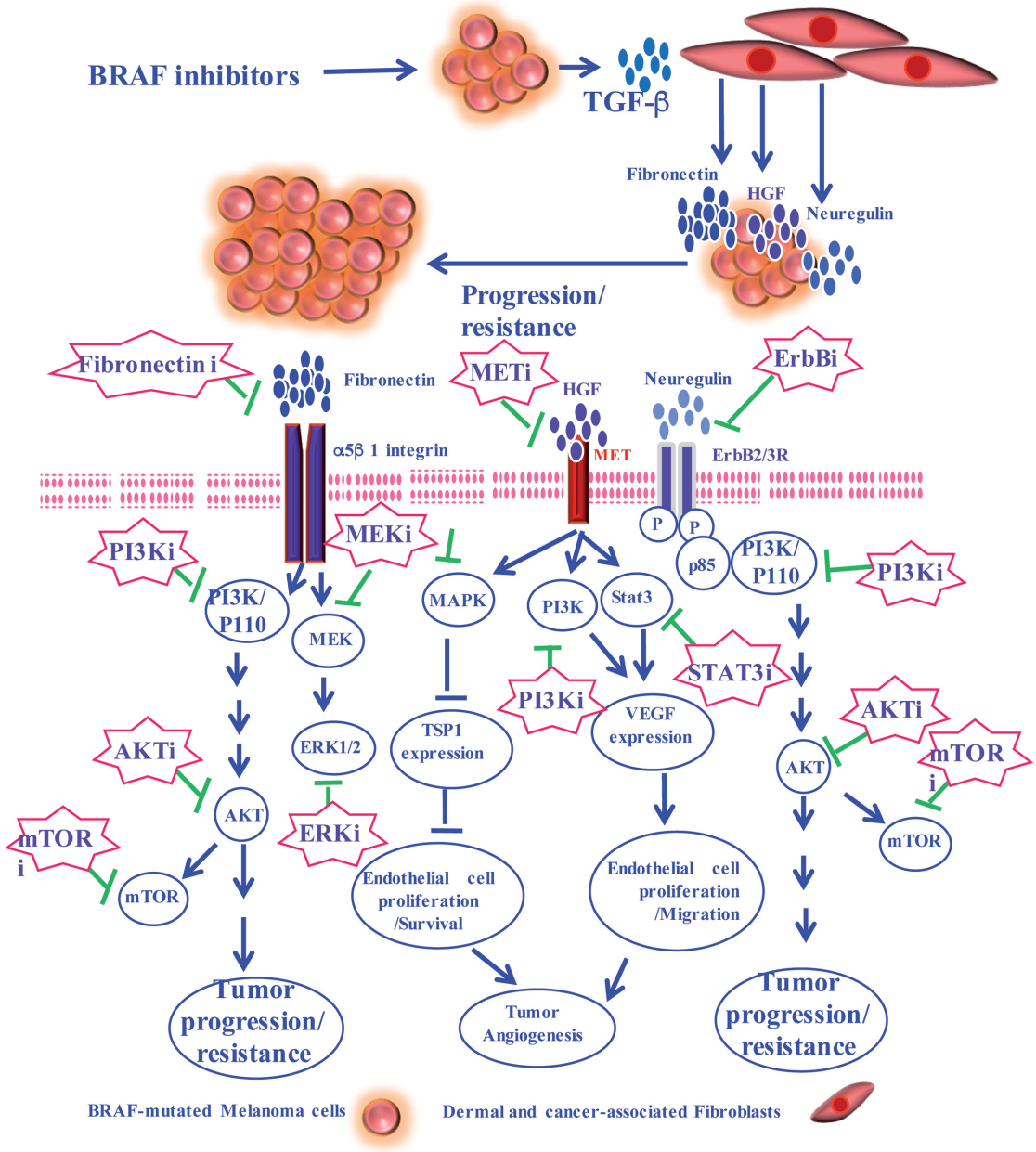

Fig. 4. Signaling pathways involved in the regulation of tumor microenvironment-mediated tumor progression and resistance, and the key molecules that are thought to function as potential therapeutic targets in melanoma treatment. BRAF inhibitors (BRAFi) that are already approved for melanoma treatment include dabrafenib. MEK inhibitors (MEKi) that are already approved for melanoma treatment include trametinib. ERK inhibitors include PD98059 and U0126. PI3K inhibitors include NVP-BEZ235, BKM120, BAY 80-6946, GDC-0941 and GDC-0980. AKT inhibitors (AKTi) include AZD5363, MK-2206 and KRX-0401. mTOR inhibitors include AP23573/MK-8669, and CCI-779.

outcome of $BRAF^{V600E}$ metastatic melanoma, both primary and acquired resistance mechanisms of CSC represent an immense problem in melanoma treatment. Although standard therapeutics can kill only tumor mass leaving cancer-initiating cells behind, tumor cells evading drug toxicity are able to repopulate tumors and disseminate to distant organs (Luanpitpong et al., 2014). Accordingly, the development of efficient therapeutic strategies to eliminate CSCs is a good choice for melanoma treatment. Therapeutic strategies dealing with disruption of aberrant signaling pathways such as Notch, particularly, which are implicated in CSCs self-renewal processes have been reported (Prokopi et al., 2014). However, other strategies dealing with the inactivation of anti-apoptotic pathways and/or activation of pro-apoptotic pathways may be a good option for melanoma treatment.

The inhibition of Notch signaling is a relevant prospect for melanoma treatment. For example, the inhibition of Notch1 is associated with the reduction of CD44C/ CD24/ low phenotype cancer stem cells in MCF7 and Hep-2 cell lines (Yan et al., 2013). Moreover, the combination of Notch inhibitor GSI was found to enhance the killing efficiency of cisplatin in CSCs-derived human lung adenocarcinoma cell lines (Lopez-Ayllon et al., 2014). Also, the antagonist of Wnt signaling, sFRP4, has been demonstrated to sensitize CSCs to doxorubicin and cisplatin (Warrier et al., 2013). Furthermore, the reliability of Wnt signaling pathway as therapeutic target for the eradication of CSCs derived from different tumor types has been reported (Sun et al., 2014; Cui et al., 2015). Also, the suppression of the self-renewal potency of pancreatic CSCs in response to the inhibition of the Hh signaling pathway by Sulforaphane has been reported in several studies (Rodova et al., 2012). To that end, the disruption of the aberrant signaling pathways by targeting their key components may be an attractive therapeutic strategy for melanoma treatment.

Tumor growth-driving PI3K/AKT pathway has been suggested as a therapeutic target for melanoma treatment. This suggestion is based on the potential role of PI3K/AKT pathway in the regulation of stem cell growth and maintenance (Kim et al., 2005). In accordance, the inhibition of PI3K-AKT-mTOR by a small molecule inhibitor can block the expansion of CSCs *in vivo* (Chang et al., 2015).

Mutation of B-Raf or its upstream activator N-Ras in cutaneous melanoma presents the early mechanistic event of melanoma progression (Thomas et al., 2015). However, in non-B-Raf mutated tumors, wild type B-Raf and C-Raf proteins can substitute the function of the mutated B-Raf leading to tumor progression (Lin et al., 2010). However, apart from the key B-Raf and C-Raf in melanoma development and progression, the role of Raf/extracellular regulating kinase (ERK) pathway is considered. The Raf/ ERK pathway has been shown to function as a link between microphthalmia-associated transcription factor (Mitf) and the stem cell growth factor receptor c-Kit (Lee et al., 2016b), an essential mechanism for tumor development and progression. Accordingly, targeting Raf/ERK pathway by multiple therapies using a small molecule inhibitor is expected to be an efficient therapeutic option for melanoma treatment.

Therapeutic strategy based on the inactivation of anti-apoptotic pathways parallel to the activation of pro-apoptotic pathways may be a relevant strategy to overcome the resistance of CSCs to conventional therapies. Recently, we demonstrated the reliability of NF-κB pathway inhibition to sensitize melanoma cells to imiquimod (El-khattouti et al, 2016). Also, targeting NF-κB by specific inhibitors can mediate anti-tumor responses and enhance the sensitivity of tumor cells to anti-cancer drugs such as vinblastine (Selimovic et al., 2013), doxorubicin and paclitaxel (Pushkarev et al., 2015). Also, based on its role in the regulation of drug resistance, ceramide pathway has been functionally analysed as a therapeutic target for melanoma treatment (Yu et al., 2012). For example, the inhibition of the activity of ceramide-metabolizing enzymes by tamoxifen is expected to decrease the level of ceramide that, in turn, results in the activation of the apoptotic machinery, particularly in cancer stem cells (Senchenkov et al., 2001). Also, the combination of paclitaxel with tamoxifen can overcome melanoma resistance to chemotherapy (Nathan et al., 2000). A proposed model for the signaling pathways and their key molecules that may function as therapeutic targets for melanoma treatment is outlined in Fig. 3.

Tumor microenvironment as a therapeutic target in melanoma treatment

Accumulated evidence suggests a potential role for the tumor microenvironment in the regulation of all phases of tumorigenesis, including initiation, progression, maintenance and metastasis (Quail and Joyce, 2013). The ability of the microenvironment to create a niche for CSCs maintenance and protection from drug-induced cytotoxicity is the main cause for treatment failure and tumor relapse (Whiteside, 2008). The ability of melanoma-initiating cells to interact with both endothelial cells as well as with stromal fibroblasts is determined by the components of the micro-environment of the melanoma niche (Li et al., 2001; Smalley et al., 2005). Thus, targeting the components of tumor microenvironment may decrease tumor resistance and prevent the possible tumor relapse. The crosstalk between tumor and its microenvironment is tightly regulated via a mechanism mediated by stromal cell-derived factor-1 (SDF-1/CXCL12) (Kollmar et al., 2007). Also, the activation of chemokine receptor 4 (CXCR4) by SDF-1 is responsible for tumor cell growth, trafficking and migration (Lee et al., 2013). The role of the SDF-1-CXCR4 axis in the regulation of metastatic dissemination of melanoma to lymphatic and blood vessels is widely documented (Kim et al., 2010). The

inhibition of the interaction between SDF-1 and CXCR4 by CXCR4 antagonists, such as Plerixa (AMD3100) and T14003 analogs, exhibited therapeutic impact on melanoma treatment (O'Boyle et al., 2013). The mechanisms regulating tumor microenvironement-mediated tumor progression and resistance, and the reliability of tumor microenvironment as a therapeutic target for melanoma treatment are outlined in Figure 4.

Conclusions

CSCs exist in various malignancies besides being characterized by their potency for self-renewal and differentiation, the common mechanisms which are critical for tumor initiation, progression, metastasis and recurrence. The rise of the CSC hypothesis expands our overview of how to prevent and eradicate malignancies. Although the investigated CSC markers are not common among tumor types, the identification of CSC populations has been continuously improved by the identification of new markers. Apart from the management protocol of their identification, increasing evidence has suggested CSCs as a relevant model for the development and design of therapeutic approaches with minimized side-effects. Thus, the identification of therapeutic strategies that exploit the unique characteristics of the aberrant signaling pathways requires further and specific studies. Such studies will help to determine more specifically and powerfully therapeutic targets for melanoma treatment.

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Accepted June 15, 2016