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Role of autophagy in the maintenance and function of cancer stem cells

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ABSTRACT Recent advances in experimental technologies and cancer models have made possible to demonstrate that the tumor is a dynamic system comprising heterogeneous populations of cancer cells organized in a hierarchical fashion with cancer stem cells (CSCs) at the apex. CSCs are immature cells characterized by self-renewal property and long-term repopulation potential. CSCs have been causally linked to cancer initiation, propagation, spreading, recurrence and relapse as well as to resistance to anticancer therapy. A growing body of evidence suggests that the function and physiology of CSCs may be influenced by genetic/epigenetic factors and tumor environment. In this context, macroautophagy is a lysosomal degradative process (herein referred to as autophagy) critical for the adaptive response to stress and the preservation of cellular and tissue homeostasis in all eukaryotes that may have a crucial role of in the origin, maintenance and invasiveness of CSCs. The activation of the autophagic machinery is also considered as an adaptive response of CSCs to perturbation of tumor microenvironment, caused for instance by anticancer therapy. Nevertheless, compelling preclinical and clinical evidence on the cytoprotective role of autophagy for CSCs is still missing. Here, we summarize the results on the contribution of autophagy in CSCs and how it impacts tumorigenesis and tumor progression. We also discuss the therapeutical potential of the modulation of autophagy as a means to eradicate CSCs.

KEY WORDS: *autophagosomes, beclin 1, CD133, chloroquine, lysosomes, tumor initiating cells*

Introduction

It is becoming increasingly evident that solid neoplasms are complex and dynamic entities composed of tumor cells and non-tumor components, including infiltrating endothelial, stromal and immune cells, cancer-associated fibroblasts and constituents of extracellular matrix. The continuous crosstalk between these elements allows for the quick adaptation and rapid response of tumor system to modifications of the environmental conditions such as those provoked for instance by low concentration of oxygen (hypoxia) or nutrient (starvation) as well as anticancer therapy(Hanahan and Coussens, 2012).

As additional layers of complexity, there is compelling evidence demonstrating that malignant tissues display: (1) intertumoral heterogeneity (i.e., few alterations shared by tumors of the same histopathologic subtype) and intratumoral heterogeneity (i.e., distinct phenotypes, genotypes, proliferation rates, metabolisms, metastatic potentials and epigenetic status between neoplastic cells of the same tumor)(De Sousa *et al.*, 2013, Gerlinger *et al.*, 2012); (2) a high frequency of aneuploidy and genetic/chromosomal instability(Gordon *et al.*, 2012, Hanahan and Weinberg, 2011, Vitale *et al.*, 2011a, Vitale *et al.*, 2011b); (3) an extensive metabolic rewiring, which is also believed to accompany neoplastic transformation(Galluzzi *et al.*, 2013, Michels *et al.*, 2015, Schulze and Harris, 2012, Vander Heiden *et al.*, 2009); and (4) a hierarchical organization(Nguyen *et al.*, 2012, Reya *et al.*, 2001). In particular, it has been put in evidence the existence of stem

Abbreviations used in this paper: AMPK, AMP-activated protein kinase; ATG, autophagy-related; BCL2, B-cell CLL/lymphoma 2; BECN1, Beclin 1; CML, chronic myeloid leukemia; CQ, chloroquine; CSCs, cancer stem cells; DCIS, ductal carcinoma in situ; DCLK1, doublecortin-like kinase 1; ER, endoplasmic reticulum; LC3, microtubule-associated protein light chain 3; mTOR, mechanistic target of rapamycin (serine/threonine kinase); mTORC1, mTOR complex; PDAC, pancreatic ductal adenocarcinoma; PE, phosphatidylethanolamine; PI3K, phosphatidylinositol-3 kinase; PI3P, phosphatidylinositol-3-phosphate; RB1CC1, RB1-inducible coiled-coil 1; RCD, regulated cell death; shRNAs, short hairpin RNAs; TNBC, triple negative breast cancer; TSC1, tuberous sclerosis 1; ULK, unc-51 like autophagy activating kinase; VPS34, vacuolar protein sorting 34.

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cell-like niches, distinct compartments generated within the tumor housing a subpopulation of immature cells known as cancer stem cells (CSCs)(Beck and Blanpain, 2013). CSCs share with stem cells some properties, including the maintenance of an undifferentiated state and the capability of self-renewal upon symmetrical and asymmetrical divisions(Beck and Blanpain, 2013, Kreso and Dick, 2014, Nguyen *et al.*, 2012). These features allow for the perpetuation and the potential expansion of the cancer stem pool as well as the preservation of the stemness potential. Besides this, CSCs are also able to generate non-tumorigenic, daughter cells with variable degree of differentiation. Of note, multilineage differentiation potential is not considered a bona fide stemness property(Kreso and Dick, 2014).

CSCs are believed to drive tumor growth and progression by contributing to the proliferative potential and constituting a source for relapse/recurrence of the disease(Beck and Blanpain, 2013, Greaves, 2013, Kreso and Dick, 2014, Nguyen *et al.*, 2012). These cells have been prospectively isolated by means of specific markers first in acute myeloid leukemia(Bonnet and Dick, 1997, Lapidot *et al.*, 1994) and then in a variety of solid tumors, including breast, brain, colon, lung, pancreas and prostate cancer(Al-Hajj *et al.*, 2003, Collins *et al.*, 2005, Eramo *et al.*, 2008, Hemmati *et al.*, 2003, Hermann *et al.*, 2007, Li *et al.*, 2009b, Patrawala *et al.*, 2006, Ricci-Vitiani *et al.*, 2007, Singh *et al.*, 2004). In these settings, purified CSCs were able to maintain stem cell property and initiate/propagate neoplasms representative of the parental tumors from which they were derived upon serial xenotransplantation into immunodeficient mice.

Recent experimental observations suggest, however, the need of an evolution of the CSC model, as the situation appears more complex than previously thought(Kreso and Dick, 2014, Magee *et al.*, 2012, Meacham and Morrison, 2013, Nguyen *et al.*, 2012, Zeuner *et al.*, 2014b). First, high percentages of CSCs have been shown in some cancers such as acute lymphoblastic leukemia and melanoma (Cojoc *et al.*, 2015, Kong *et al.*, 2008, Quintana *et al.*, 2008). Second, self-renewal is often deregulated in CSCs(Kreso and Dick, 2014). Third, CSCs appear as a heterogeneous population, whose functional properties are influenced by genetic, epigenetic and microenvironmental factors(Baccelli and Trumpp, 2012, Chen and Dent, 2014, Curtis *et al.*, 2010, Dieter *et al.*, 2011, Giannoni *et al.*, 2010, Iliopoulos *et al.*, 2011, Sharma *et al.*, 2010, Suva *et al.*, 2013, Todaro *et al.*, 2014, Vermeulen *et al.*, 2010). Fourth, in some cases, CSCs exist as a dormant and/or quiescent pool potentially contributing to tumor repopulation(Francescangeli *et al.*, 2012, Kreso *et al.*, 2013, Pece *et al.*, 2010, Roesch *et al.*, 2010). Fifth, a certain degree of plasticity in stem cell status has been identified within tumors. For instance, glioblastoma stem cells can differentiate towards neural and mesenchymal lineages(Ricci-Vitiani *et al.*, 2008). Moreover, cancer cells may experience phases of transition between stem-like and non-stem-like states(Chaffer *et al.*, 2011, Chaffer *et al.*, 2013, Gupta *et al.*, 2011, Iliopoulos *et al.*, 2011, Jopling *et al.*, 2011). Thus the process of differentiation seems not to be unidirectional, but rather is based on a dynamic equilibrium between stemness and differentiation.

Despite these novel findings and some contradictory reports(Ishizawa *et al.*, 2010, Quintana *et al.*, 2010, Shackleton *et al.*, 2009), it is generally accepted that CSCs casually contribute to tumor generation and progression in the vast majority of hematological and solid tumors(Baccelli and Trumpp, 2012, Bartucci *et al.*, 2015; Driessens *et al.*, 2012, Nguyen *et al.*, 2012, Schepers *et al.*, 2012, Todaro *et al.*, 2014). CSCs have clinical relevance as they are endowed with an intrinsic resistance to radio- and chemotherapy (Chen *et al.*, 2012, Cojoc *et al.*, 2015, Eramo *et al.*, 2006, Maugeri-Sacca *et al.*, 2011, Phillips *et al.*, 2006, Vermeulen *et al.*, 2012, Zeuner *et al.*, 2014b). This is believed to occur through mechanisms encompassing the overexpression of one or more members of the ATP-Binding Cassette (ABC) transporter family, which increases drug efflux(Alison *et al.*, 2012); an augmented activity of aldehyde dehydrogenase, which is believed to contribute to the adaptive response to oxidative stress(Ginestier *et al.*, 2007, Kastan *et al.*, 1990); an enhanced activation of DNA damage response often coupled to the evasion of the process of regulated cell death (RCD)(Bao *et al.*, 2006, Bartucci *et al.*, 2012, Capper *et al.*, 2009, Majeti *et al.*, 2009, Todaro *et al.*, 2007, Zeuner *et al.*, 2014a); and the induction of dormancy(Kreso *et al.*, 2013). Emerging evidence also links therapy resistance to the cytoprotective activation of autophagy in CSCs(Biasoli *et al.*, 2013, Chaterjee and van Golen, 2011, Firat *et al.*, 2012, Kantara *et al.*, 2014, Lomonaco *et al.*, 2009, Mai *et al.*, 2012, Ojha *et al.*, 2014, Rao *et al.*, 2012, Wu *et al.*, 2013, Yue *et al.*, 2013). The importance of this catabolic pathway in the function and maintenance of CSCs is however still debated.

Here, we provide an overview of the mechanism of autophagy and the contribution of this process to tumor initiation and progression. We then focus on the role of the autophagic process in CSCs, also discussing the potentiality of strategies based on the modulation of components of the autophagic machinery as a means to efficiently eradicate CSCs.

Autophagy, a brief overview

Physiological functions

Autophagy (from the ancient Greek αυτο + φαγοσ/φαγεῖν = self-eating) is a highly conserved, catabolic process involved in a variety of physiological processes, including normal development, growth and immunity(Choi *et al.*, 2013, Mizushima and Komatsu, 2011, Mizushima *et al.*, 2008). Autophagy deregulation (e.g., owing to defects in autophagy machinery) has been linked to physio-/pathological disorders such as infection, neurodegenerative diseases, cancer and aging(Choi *et al.*, 2013, Levine and Kroemer, 2008, Nixon, 2013, Rubinsztein *et al.*, 2011).

Macroautophagy - the major autophagic pathway in mammals and commonly (and hereafter in this review) referred to as autophagy - is a lysosome-dependent mechanism responsible for the degradation of intracellular components, including cytotoxic protein aggregates, damaged organelles and invading pathogens(Mizushima and Komatsu, 2011, Yang and Klionsky, 2010). During this degradative process, autophagic substrates are normally captured in a non-selective fashion (bulk autophagy). Nevertheless, recent evidence suggests the existence of specialized subtypes of autophagy that selectively recognize their substrates, such as mitophagy, which operates as a mitochondria quality control by eliminating damaged, superfluous or dysfunctional mitochondria(Green and Levine, 2014, Youle and Narendra, 2011).

Autophagy is kept at low level under normal conditions but becomes up-regulated in response to various perturbations, including starvation, hypoxia, pathogen invasion or treatment with cytotoxic compounds(Galluzzi *et al.*, 2014, Kroemer *et al.*, 2010, Mizushima

and Komatsu, 2011). Under these stressful conditions, autophagy is induced to preserve cellular integrity, promote cellular detoxification and provide sources of metabolic energy.

Although the general consensus is that autophagy constitutes a protective response for the short-term adaptation of cells to intracellular stress(Boya *et al.*, 2005, Galluzzi *et al.*, 2012), in some limited instances, such as during the development of *Caenorhadditis elegans*(Erdelyi *et al.*, 2011) and *Drosophila melanogaster*(Berry and Baehrecke, 2007, Denton *et al.*, 2009, Nezis *et al.*, 2010) or upon exposure to chemotherapeutic agents(Grander *et al.*, 2009, Laane *et al.*, 2009, Lamy *et al.*, 2013), this pathway may also mediate cell death. Of note "autophagic cell death" is considered as a type of RCD only when pharmacological or genetic inhibition of the autophagic machinery delays cellular demise(Denton *et al.*, 2012, Galluzzi *et al.*, 2012).

Molecular mechanisms

Upon its first description, which was possibly made more than 50 years ago when Clark observed the presence of peculiar vesicles containing cytoplasmic organelles by transmission electron microscopy analyses(Clark, 1957), an intense wave of studies have been launched to elucidate the cellular and molecular pathways of autophagy.

The autophagic pathway starts with the sequestration of cytoplasmic material within a double-membraned, non-degradative vesicle known as autophagosome(Lamb *et al.*, 2013). The subsequent fusion of the autophagosome with lysosome generates an autolysosome, the organelle into which the autophagic content is degraded and released into cytoplasm for recycling(Hamasaki *et al.*, 2013, Hayashi-Nishino *et al.*, 2009, Lamb *et al.*, 2013)(Fig. 1).

The biogenesis of autophagosomes proceeds via three main steps: 1) the initiation, a signaling pathway whereby the autophagic machinery is targeted to membrane source sites to assembly a pre-autophagosomal membrane, followed by 2) the nucleation of a lipid-based structure known as isolation membrane or phagophore, which is believed to occur at the contact site between endoplasmic reticulum (ER) and mitochondria(Hamasaki *et al.*, 2013, Hayashi-Nishino *et al.*, 2009), and 3) the elongation and sealing of the vesicle to surround autophagic cargoes (Lamb *et al.*, 2013)(Fig. 1). Although the membrane sources for the generation of autophagosome mostly derive from ER and mitochondria, it has been suggested that lipids may also be mobilized to the isolation membrane from plasma membrane (Ravikumar *et al.*, 2010) and other cytoplasmic organelles, including the Golgi (Takahashi *et al.*, 2011).

The molecular core of the autophagic machinery consists of the products of several autophagy-related (*ATG*) genes (Mizushima *et al.*, 2011). Atg proteins were first identified in yeast as essential for their survival on nutrient stress and starvation (Thumm *et al.*, 1994, Tsukada and Ohsumi, 1993). The homologues of *ATG* genes have been subsequently identified in almost all organisms, including mammals. So far, at least 19 mammalian ATG proteins have been involved in the autophagic process (Choi *et al.*, 2013).

At molecular level autophagosome generation is driven by two main multiprotein complexes, the unc-51 like autophagy activating kinase (ULK) complex and the phosphatidylinositol 3-kinase, catalytic subunit type 3 (PK3C3 best known as vacuolar protein sorting 34, VPS34)-Beclin 1(BECN1) complex(Wirth *et al.*, 2013). The ULK complex, known as pre-initiation complex, consists of ULK1, ULK2, RB1-inducible coiled-coil 1 (RB1CC1, best known as FIP200), ATG13 and ATG101(Ganley *et al.*, 2009, Mizushima, 2010). Both negative and positive regulators of ULK1 complex have been identified. Thus, depending on the energy status and/or nutrient resources of the cells ULK complex may be inhibited or activated by mechanistic target of rapamycin (serine/threonine kinase) (mTOR) complex 1 (mTORC1) or by AMP-activated protein kinase (AMPK), respectively(Hardie *et al.*, 2012, Laplante and Sabatini, 2012). ULK1 associates to the negative regulator mTORC1 under fed condition, while on nutrient depletion mTORC1 becomes inactivated, leading to ULK1 release and autophagy engagement(Hosokawa *et al.*, 2009, Jung *et al.*, 2009). Starvation also results in the activation of AMPK, which triggers autophagy by inhibiting mTORC1 - via AMPK-mediated phosphorylation of the mTORC1 component regulatory associated protein of mTORC1 (RPTOR)(Gwinn *et al.*, 2008) or of tuberous sclerosis 1 (TSC1)-TSC2-TBC1 domain family, member 7 (TBC1D7) (TSC-TBC) complex(Inoki *et al.*, 2003) – or by directly activating ULK1(Egan *et al.*, 2011, Kim *et al.*, 2011b)(Fig. 1).

The VPS34-BECN1 complex [also known as class III phosphatidylinositol-3 kinase (PI3K) complex)] is a regulative platform composed by VPS34, BECN1, autophagy/beclin-1 regulator 1 (AMBRA1) and ATG14L(Funderburk *et al.*, 2010, Yang and Klionsky, 2010). The crucial pro-autophagic event promoted by this complex is the conversion of phosphatidylinositol to phosphatidylinositol-3-phosphate (PI3P) at the site of phagophore nucleation(Wirth *et al.*, 2013). PI3P generation, which is catalyzed by the class III PI3K VPS34(Meijer and Klionsky, 2011), triggers phagophore nucleation(Axe *et al.*, 2008, Polson *et al.*, 2010). Under normal conditions the anti-apoptotic proteins of the Bcl-2 family B-cell CLL/lymphoma 2 (BCL2) and BCL2-like 1 (BCL2L1, best known as BCLXL) inhibit the VPS34-BECN1 complex by interacting with BECN1, the co-activator of VPS34(Furuya *et al.*, 2005, Pattingre *et al.*, 2005) (Fig. 1). Such inhibitory association must thus be blocked to promote the PI3K activity of VPS34. The interaction

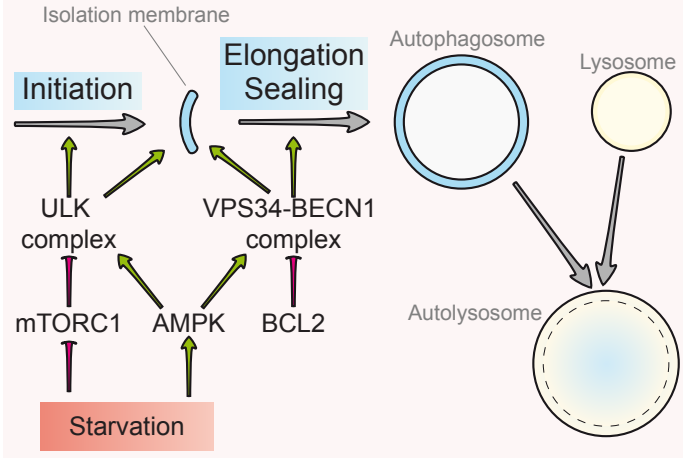

Fig. 1. The autophagic pathway. *At cellular level, the two principal steps of autophagy are the generation of an autophagosome from the isolation membrane and its fusion with a lysosome to generate an autolysosome. At molecular level, autophagy relies on the ULK complex and the VPS34-BECN1complex. The activity of these two macromolecular complexes is finely tuned by both positive (mTORC1 complex) and negative (AMPK and BCL2) regulators.*

between BECN1 and BCL2/BCLXL supports the notion of the existence of a complex interplay between autophagy and apoptosis, which appears relevant in the context of cancer and cancer therapy(Marino *et al.*, 2014). On starvation the activation of the VPS34-BECN1 complex is also promoted by AMPK(Kim *et al.*, 2013) or ULK1(Russell *et al.*, 2013).

Among other molecular components mediating autophagosome generation are included ATG9, a transmembrane protein that is believed to contribute to the recruitment of lipids to the isolation membrane(Mari *et al.*, 2010), and the phosphatidylethanolamine(PE)-conjugated form of microtubule-associated protein light chain 3 (LC3) known as LC3-PE or LC3-II(Weidberg *et al.*, 2010). LC3-II is generated by an ubiquitin-like (UBL) conjugation system via the cleavage of LC3 by the protease ATG4, which produces the cytosolic form LC3-I, followed by the sequential action of the E1-like enzyme ATG7, the E2-like enzyme ATG3 and the E3-ligase complex ATG5-ATG12-ATG16L1, which catalyzes the covalent conjugation of PE to LC3-I(Noda *et al.*, 2008, Ohsumi, 2001). Once generated LC3-II relocates to the nucleation membranes where it exerts a role in the elongation/closure stage(Nakatogawa *et al.*, 2007, Weidberg *et al.*, 2011). Of note, LC3-II remains associated to mature autophagosomal membrane during the autophagic process.

Concerning the last steps of autophagy: the deliver and uptake of the cargoes during autophagosome maturation is mediated by specific adaptors, including the autophagy cargo receptor and substrate p62/sequestosome 1(SQSTM1, best known as p62), which first associates to proteins or organelles (often tagged by ubiquitin modification, e.g., Lys63 ubiquitination) and then interacts with LC3-like proteins through the LC3-interacting region (LIR) (Birgisdottir *et al.*, 2013, Johansen and Lamark, 2011); the fusion between autophagosome and lysosome is promoted by multiple soluble N-ethylmaleimide-sensitive fusion (NSF) attachment protein receptors-(SNARE-)like proteins(Fader *et al.*, 2009, Nair *et al.*, 2011). The hydrolysis of the autophagic cargo, including the adaptors, relies on lysosomal enzymes (i.e., acidic hydrolases), whose catabolic activity is triggered by the activation of H^+ pumps, which lowers the pH of the lysosomal lumen(Kroemer *et al.*, 2010) Lysosomal permeases eventually promote the cytosolic export of the product of this degradation for potential reuse(Kuma and Mizushima, 2010).

Autophagy and cancer

The role of the autophagic machinery in cancer initiation and progression is complex. This catabolic pathway may indeed act either as an oncosuppressive or as a prosurvival mechanism depending on tumor stage and type(White, 2012).

Autophagy limits the early phases of tumorigenesis

Accumulating evidence suggests that autophagy may act as an antioncogenic barrier by limiting, or even suppressing, cancer initiation. Autophagy is indeed stimulated by tumor suppressors, including phosphatase and tensin homolog (PTEN) or serine/threonine kinase 11 (STK11, also known as LKB1), while being abolished by oncogenic signals, including the overactivation of the PI3K-AKT pathway and the overexpression of the antiapoptotic member of the Bcl-2 family(Morselli *et al.*, 2011). Moreover, an autophagy-dependent anticancer immune response has been recently reported to control tumorigenesis(Michaud *et al.*, 2011). In addition, mice bearing monoallelic loss of *Becn1* (*Becn1*$^{+/-}$) spontaneously develop tumors(Qu *et al.*, 2003, Yue *et al.*, 2003), while the whole-body absence of ATG4C increases the incidence of chemically-induced tumorigenesis(Marino *et al.*, 2007) and the deletion of *Atg5* or *Atg7* favors the development of liver hepatomas(Takamura *et al.*, 2011). Along similar lines, the monoallelic deletion of *BECN1* has been described in a large fraction of human neoplasms, including breast, ovarian and prostate cancer(Aita *et al.*, 1999, Choi *et al.*, 2013, Liang *et al.*, 1999), while mutations of other autophagic proteins (e.g., *ATG5*, *ATG12*) have been found in colorectal cancers(Kang *et al.*, 2009). Altered expression of autophagic proteins has also been detected in some tumors. For instance low BECN1 levels correlated with poor prognosis in multiple human malignancies including, colorectal, lung, esophageal and pancreatic cancer(Chen *et al.*, 2009, Jiang *et al.*, 2012, Kim *et al.*, 2011a, Li *et al.*, 2009a), while high BECN1 levels have been associated with improved survival in patients affected by high-grade gliomas, hepatocellular carcinoma or large B-cell lymphoma(Ding *et al.*, 2008, Huang *et al.*, 2011, Pirtoli *et al.*, 2009). In addition, both ATG5 and LC3 levels were found decreased in patients with melanomas as compared with those with benign nevi(Liu *et al.*, 2013).

Despite some contradictory observations(Kim *et al.*, 2011c, Laddha *et al.*, 2014, Takamura *et al.*, 2011, Wan *et al.*, 2010, Wei *et al.*, 2011) the current hypothesis pleads in favor of an oncosuppressive role for autophagy. According to this model, the suppression of this catabolic process would promote oncogenesis by increasing the level of oxidative stress(White, 2012, Yang *et al.*, 2011a); inducing genomic instability (due to accumulation of genomic alteration and/or micronuclei) (Mathew *et al.*, 2007, Rello-Varona *et al.*, 2012, Xie *et al.*, 2011); rewiring bioenergetic metabolism (due to a defective mitochondria turnover) (Karantza-Wadsworth *et al.*, 2007) counteracting oncogene-induced senescence(Iannello and Raulet, 2014, Iannello *et al.*, 2013, Liu *et al.*, 2013, Xue *et al.*, 2007, Young *et al.*, 2009); favoring the accumulation of p62, which triggers a pro-tumorigenic signal via the antioxidant transcription factor nuclear factor, erythroid 2-like 2 (NFE2L2, best known as NRF2)(Inami *et al.*, 2011, Jain *et al.*, 2010); and affecting the mechanism of antitumor immunosurveillance (due to perturbations in the tumor microenvironment)(Michaud *et al.*, 2011, Rao *et al.*, 2014).

Autophagy promotes the survival of cancer cells

A growing body of evidence suggests that once established tumors retain and/or activate autophagy (or reinstate it if the autophagic machinery has been disabled in the early phase of oncogenesis) for sustaining their progression. Human tumor cell lines, and in particular RAS-driven pancreatic cancer cells, display indeed an upregulated basal level of autophagy as compared to normal cells, and this has been associated to poor outcome and increased tumor survival(Guo *et al.*, 2011, Lazova *et al.*, 2012, Wang *et al.*, 2012, Yang *et al.*, 2011a). In addition, autophagy is induced as a cancer pro-survival pathway in response to multiple stress conditions in tumor microenvironment including starvation and hypoxia(Amaravadi *et al.*, 2011, Kimmelman, 2011, White, 2012).

Some experimental evidence supports the notion of a pro-oncogenic function of autophagy in established cancer. First, the hemizygous deletion of *Becn1* hampers tumor formation in ataxia

telangiectasia mutated (*Atm)*$^{-/-}$ and *Tsc2*$^{+/-}$ mice(Parkhitko *et al.*, 2011). Apparently at odds with this, enhanced BECN1 inactivation augments tumor growth of established non-small-cell lung cancer with active mutation of epidermial growth factor (EGFR)(Wei *et al.*, 2013b). Second, the ablation of *Rb1cc1* limits tumor progression in a mouse model of breast cancer driven by the polyoma middle T oncogene (PyMT) oncogene(Wei *et al.*, 2011). Third, the hepatic-specific deletion of *Atg5* or *Atg7* arrests the transition from hepatoma to hepatocellular carcinoma in genetically modified mouse models(Takamura *et al.*, 2011). Fourth, disabling autophagy limits tumor growth in KRAS-transformed cells by interfering with the metabolic functions and the turnover of mitochondria, and consequently affecting both the level of metabolic/biosynthetic substrates and the energy charge(Guo *et al.*, 2011). Fifth, autophagy is reported to facilitate mammary tumorigenesis and promote tumor growth in a model of partner and localizer of breast cancer 2, early onset (BRCA2)-(PALB2-)associated hereditary breast cancer(Huo *et al.*, 2013). Sixth, studies performed on genetically engineered mouse models of KRAS^{G12D}- or BRAF^{V600E}-driven lung cancer suggest that autophagy dictates lung tumor fate(Chen and Guan, 2013, Guo *et al.*, 2013, Rao *et al.*, 2014). Thus, tissue-specific inactivation of ATG5 or ATG7 respectively reduced the progression from adenomas to adenocarcinomas(Rao *et al.*, 2014) or diverted it to more benign tumor oncocytomas(Chen and Guan, 2013, Guo *et al.*, 2013). Of note, the negative impact of autophagy inhibition on tumor progression could be in part reverted by the deletion of the transformation related protein 53 (Tpr53, best known as p53). Seventh, in a humanized genetically-modified mouse model of KRAS^{G12D}-driven pancreatic ductal adenocarcinoma (PDAC), the absence of either ATG5 or ATG7 blocks tumor progression to PDAC(Rosenfeldt *et al.*, 2013). Intriguingly, in this study the combination of autophagy loss and p53 deficiency accelerated tumor initiation and progression, suggesting that p53 status may dictate the role of autophagy in pancreatic tumor development.

Altogether these observations suggest that autophagy promotes the survival of cancer cell by favoring their adaptation and tolerance to environmental stress such as hypoxia, by maintaining mitochondrial homeostasis and functions (e.g., by providing substrates for mitochondrial metabolisms via nutrient recycling or limiting the accumulation of ROS), and by affecting p53 tumor suppressor pathway.

Autophagy in the physiology of CSCs

An increasing number of experimental observations suggest that autophagy may be crucial in the maintenance and function of distinct types of normal stem cells. This catabolic process is indeed involved in the preservation of stem cell homeostasis and the maintenance of stemness property (reviewed in(Guan *et al.*, 2013, Phadwal *et al.*, 2013, Vessoni *et al.*, 2012)). Of note, the protein/organelle quality control operated by autophagy appears of particular relevance during periods of quiescence and/or differentiation(Guan *et al.*, 2013). The exact contribution of autophagy for the biology of CSCs is not yet elucidated(Lin *et al.*, 2015). Given the analogy between CSCs and stem cells, one could expect that autophagy may play a cytoprotective role for CSCs. Nevertheless, as autophagy suppresses the early phases of tumorigenesis (see above), a deregulation of this catabolic process in CSCs could also be plausible. To further complicate this "autophagy paradox", the mechanism of autophagic cell death may be relevant for the effectiveness of specific antineoplastic therapies. In the next paragraphs we summarize the experimental evidence available on the contribution of autophagy in CSCs.

Autophagy promotes CSCs survival

Some components of autophagic machinery are essential for the survival of CSCs and their adaptation to changes of the tumor microenvironment. This applies to: 1) ATG4A, which has been identified as a pivotal factor for CSC maintenance in a high-throughput screening based on mammosphere formation upon transfection with a pool of short hairpin RNAs (shRNAs)(Wolf *et al.*, 2013); 2) BECN1, which has been shown to promote the survival of breast CSCs (but not bulk cancer cells) and to contribute to their *in vivo* tumorigenicity (Gong *et al.*, 2013). In this experimental setting, the expression level of BECN1, and consequently the autophagic flux, were indeed higher in CSC-enriched than in non-CSC-enriched breast tumor cells; 3) DRAM1 and p62, which have been found overexpressed in adult glioblastoma tumors belonging to the mesenchymal subtype(Galavotti *et al.*, 2013). In this study, by using specific siRNAs directed against DRAM1 and p62 both proteins were mechanistically linked to the regulation of bioenergetic metabolism, migration and invasion of glioblastoma CSCs.

Further confirming the crucial role of autophagy in CSC maintenance, the stable or specific knockdown of LC3 and ATG12 (by means of lentiviral-delivered shRNAs) decreased the fraction of tumor cells expressing high levels of CD44 and low levels of CD24 antigens (CD44^{+}/CD24$^{-/low}$), which are believed to include the population of breast CSCs(Cufi *et al.*, 2011). Accordingly, an elevated autophagic flux was found in mammospheres derived from triple negative breast cancer (TNBC) as compared to parental cells(Rao *et al.*, 2012). Moreover, it has been recently demonstrated that a subpopulation of human pancreatic CSCs resistant to KRAS ablation and responsible for tumor relapse relied on autophagy and other catabolic processes for its survival (Viale *et al.*, 2014). Chronic myeloid leukemia (CML) is a disease of hematopoietic stem cells harboring the chimeric gene *BCR-ABL*. Both the survival of CML cells and leukemogenesis was shown to strictly rely on autophagy, as demonstrated in experiments performed by using the conditional knockout of *ATG3*(Altman *et al.*, 2011). The survival of CML stem/progenitor cells was also impaired by the depletion of ATG4B(Rothe *et al.*, 2014). Along similar lines, the inhibition of autophagy via the administration of chloroquine (CQ, a lysosomotropic agent arresting the fusion between autophagosomes and lysosomes(Firat *et al.*, 2012, O'Donovan *et al.*, 2011, Sasaki *et al.*, 2010, Selvakumaran *et al.*, 2013)) depleted the CD44^{+}/CD24$^{-/low}$ population in TNBC, both in preclinical and clinical settings(Choi *et al.*, 2014). CQ also abolished the propagation, invasion and growth of fresh spheroid-forming cells derived from human ductal carcinoma *in situ* (DCIS), the most common non-invasive, pre-malignant condition often progressing to invasive breast cancer(Espina *et al.*, 2010). It should be noted, however, that CQ may modulate signaling pathways other than autophagy, including the permeabilization of lysosomal membrane and the subsequent activation of the mitochondrial pathway of apoptosis(Maycotte *et al.*, 2012, Rubinsztein *et al.*, 2012). Moreover, CQ is reported to exert anti-CSC activity through autophagy-unrelated mechanisms, including the inhibition of Janus kinase 2 (JAK2)(Choi *et al.*, 2014) or the inactivation of the CXCR4 and Hedgehog signaling(Balic *et al.*, 2014).

CD133 (also known as prominin-1) was one of the first surface markers used to prospectively purify and thus enrich CSCs(Singh *et al.*, 2004). Despite some contentions(Clement *et al.*, 2009, Wang *et al.*, 2008), CD133 has been proposed as a marker of a number of solid tumors, and its expression is predictive of glioma patient outcome(Pallini *et al.*, 2008). CD133 has also been described as a marker of bioenergetic stress(Griguer *et al.*, 2008). In line with this, two recent reports of the same group described a prosurvival role of CD133 in hepatoma cancer exerted through CD133-mediated activation of autophagy(Chen *et al.*, 2013a, Chen *et al.*, 2013b). In the first study, the authors showed that a CD133 antibody inhibited spheroids and xenograft tumor formation in NOD/SCID mice, and induced cancer cell death by suppressing autophagy and promoting necrotic cell death(Chen *et al.*, 2013b). In the second study, they reported that on glucose deprivation CD133 translocated to cytoplasm where it promotes autophagosome formation, glucose uptake and ATP synthesis by a mechanism not fully elucidated(Chen *et al.*, 2013a). In this latter experimental setting, the depletion of CD133 also reduced xenograft tumor formation. Apparently at odd with these observations, CD133 is described to activate the PI3K/AKT/mTOR pathway (which among other functions also inhibits autophagy, see above) by interacting with PI3K, and its depletion reduced the self-renewal and tumorigenicity of glioblastoma CSCs(Wei *et al.*, 2013a). This is in line with a role of the PI3K/AKT/mTOR signaling in the origin, survival and maintenance of CSCs from both hematological and solid tumors(Chang *et al.*, 2013, Dubrovska *et al.*, 2009, Francipane and Lagasse, 2013, Martelli *et al.*, 2011, Zhou *et al.*, 2007). Moreover, Cho and colleagues reported that the expression of sex determining region Y(SRY)-box 2 (*SOX2*), a transcription factor involved in the regulation of embryonic development with essential roles for stem-cell maintenance and pluripotency(Boyer *et al.*, 2005), triggered the activation of either autophagy (by inducing the overexpression of *ATG10*) or cellular senescence in colon cancer cells, in turn resulting in the arrest of tumor growth, both *in vitro* and in vivo(Cho *et al.*, 2013).

The current view is that autophagy acts as a pro-survival mechanism whereby CSCs face changes in the tumor microenvironment. Reportedly, extrinsic perturbations, including hypoxic, metabolic and oxidative stress, can promote self-renewal and plasticity. For instance, in hypoxic conditions (such as those encountered in the hypoxic niche(Mohyeldin *et al.*, 2010)) hypoxia inducible factor (HIF) mediates the activation of stem cell markers/factors as well as prosurvival pathways, including autophagy(Heddleston *et al.*, 2009, Lin and Yun, 2010, Ma *et al.*, 2011, Mathieu *et al.*, 2011, Qiang *et al.*, 2012). Accordingly, a high number of autophagosome have been reported in poorly vascularized, hypoxic tumor regions(Degenhardt *et al.*, 2006). Of note, HIF-inducing autophagy has been recently shown to promote the metastatic ability of CD133+ pancreatic cancer stem cells(Zhu *et al.*, 2014). In addition, antiangiogenic agents increased the population of CSCs on hypoxia and this phenomenon could limit their anticancer activity(Conley *et al.*, 2012). In line with the hypothesis of a role of autophagy in the adaptation of CSCs to environmental perturbations, interfering with autophagic flux decreased the survival of either pancreatic cancer cells with stem-like properties under hypoxic conditions(Rausch *et al.*, 2012) or $CD133^+$ liver CSCs on oxygen- and nutrient-deprivation(Song *et al.*, 2013). Importantly, a co-expression between hypoxia, stemness and autophagy markers was found in immunohistochemical analyses performed on tissue samples from PDAC patients(Rausch *et al.*, 2012).

Finally, intriguing evidence also ascribes to autophagy a role in CSC plasticity. Thus, the inhibition of mTOR (and the subsequent induction of autophagy, Fig. 1) increased the $CD133^+$ fraction in liver tumor cells by arresting the differentiation and inducing the conversion of $CD133^-$ to $CD133^+$ cells and also promoted *in vivo* tumor growth(Yang *et al.*, 2011b) Similar results were found by Zhu and collaborators, who demonstrated that the activation of autophagy under hypoxic conditions promoted the dedifferentiation of non-stem pancreatic cancer cell population into stem-like cells(Zhu *et al.*, 2013).

Taken together, these observations suggest that the cytoprotective role of autophagy for CSCs has a crucial impact on tumor initiation, progression and spreading (Fig. 2). Nevertheless, compelling clinical evidence on the exact contribution of autophagy to CSC physiology is required, as in some settings autophagy activation (for instance via the inhibition of the PI3K/AKT/mTOR pathway) may also exert antineoplastic activity by targeting CSCs.

Autophagy activation in CSCs affects therapy response

Contrasting results link the autophagic process to either the resistance of CSCs to, or the fully execution of cell death induced by, radio or chemotherapy.

On the one hand, the depletion of autophagy-related genes and/or the pharmacological inhibition of autophagy increased the sensitivity of glioma CSCs to radiation therapy(Firat *et al.*, 2012, Lomonaco *et al.*, 2009). In one of these two studies, the simultaneous administration of the inhibitor of the PI3K/AKT pathway boosted the antitumor efficacy and allowed for CQ dose reduction(Firat *et al.*, 2012). Salinomycin is a compound identi-

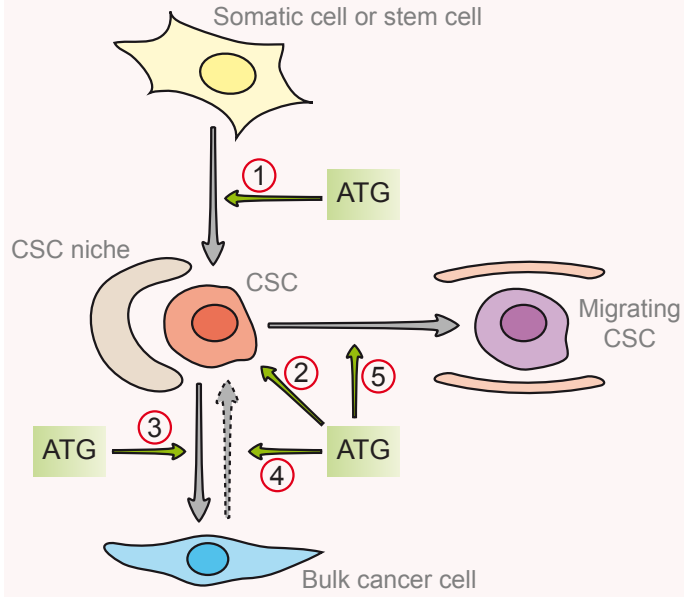

Fig. 2. Contribution of autophagy to cancer stem cell (CSC) biology. *Recent evidence suggests that the activation of the autophagy (ATG) contributes to the generation* **(1)**, *survival* **(2)**, *differentiation* **(3)**, *plasticity* **(4)** *and migrating/invasion property* **(5)** *of CSCs. The impact of the autophagy process on CSC biology may thus have a direct role on tumor initiation, progression and metastasis.*

fied in a screening for novel agents specifically killing epithelial CSCs(Gupta *et al.*, 2009). The molecular mechanism underlying this anti-CSCs effect has been recently characterized to involve the direct abrogation of autophagy by salynomicin(Yue *et al.*, 2013). In addition, the knockdown of ATG7 potentiated the inhibitory effect of salinomycin on breast CSC survival and expansion(Yue *et al.*, 2013). Along similar lines, the inhibition of the autophagic flux increased the killing effect of etopoxide in glioblastoma cell lines and CSCs(Biasoli *et al.*, 2013), sensitized breast CSCs to ginsenoside F2(Mai *et al.*, 2012), and inhibited mammosphere formation of TNBC cells, while enhancing the *in vitro* and *in vivo* anticancer efficacy of panobinostat (Rao *et al.*, 2012). Moreover, autophagy promoted the resistance of colon CSCs to paclitaxel by inhibiting apoptosis and upregulating caudal type homeobox 1 (CDX1)(Wu *et al.*, 2013), induced dormancy in breast CSCs exposed to farnesyl-transferase inhibitors(Chaterjee and van Golen, 2011), and protected bladder cancer side population (SP(Golebiewska *et al.*, 2011)) cells from chemotherapeutic agents such as gemcitabine, mitomycin and cisplatin(Ojha *et al.*, 2014). In addition, curcumin enhanced the proliferation and autophagic survival of doublecortin-like kinase 1(DCLK1)-positive colon CSCs(Kantara *et al.*, 2014). This finding supports the use of DCLK1 as a potential target to sensitize tumors to curcumin. Finally, the depletion of autophagy by different approaches increased the cytotoxicity of the tyrosine kinase inhibitor imatinib or the AKT inhibitor perifosine (two drugs reported to activate autophagy) in CML cell lines (Bellodi *et al.*, 2009, Elzinga *et al.*, 2013, Rothe *et al.*, 2014, Tong *et al.*, 2012, Yu *et al.*, 2012).

On the other hand, the cytotoxic effect of some therapeutic agents is mediated by (and strictly requires) the molecular machinery of autophagy. This applies to 1) temozolomide, whose effect on glioblastoma CSCs involves the activation of autophagic cell death, suggesting that the down-regulation of autophagy-related proteins may be a mechanism to evade temozolomide-induced cytotoxicity (Fu *et al.*, 2009); 2) resveratrol, which eliminates breast CSCs by inducing autophagy via the suppression of the Wnt/-β catenin signaling pathway (Fu *et al.*, 2014) or upstream of the activation of apoptosis (Filippi-Chiela *et al.*, 2011) and 3) metformin (a pharmacological agent currently employed for the treatment of type 2 diabetes and known to selectively kill breast CSCs(Hirsch *et al.*, 2009)), which seems to exert its anti-CSC effect by modulating the mTOR signaling pathway(Mohammed *et al.*, 2013). Along similar line, the depletion of DNA-PKcs radiosensitized glioma CSCs by inducing autophagic cell death(Zhuang *et al.*, 2011b), while autophagy activation by the modulation of mTOR activity promoted neuroblastoma and glioma stem cell differentiation and abrogated resistance of glioma stem cells to radiation(Zeng and Zhou, 2008, Zhao *et al.*, 2010, Zhuang *et al.*, 2011a, Zhuang *et al.*, 2011c). Finally, brain CSCs succumbed to adenovirus-mediated cell death via autophagy, both *in vitro* and in vivo(Jiang *et al.*, 2007)

Reportedly, some drugs may simultaneously trigger distinct pathways of RCD (Galluzzi *et al.*, 2015, Galluzzi *et al.*, 2012). For instance rotterin promotes autophagy followed by cell death in breast, pancreatic or prostate CSCs by acting on the PI3K/AKT/mTOR cascade(Francipane and Lagasse, 2013, Kumar *et al.*, 2013, Kumar *et al.*, 2014, Singh *et al.*, 2012). Rotterin-induced autophagy may thus act as a survival mechanism limiting apoptosis or, alternatively, contribute to the activation of this RCD subroutine. Finally, in one report, no significant difference was observed in the induction of apoptosis and autophagic cell death between $CD44^{+}/CD24^{-/low}$ breast CSCs and parental cells (Yenigun *et al.*, 2013). Altogether, these findings suggest that autophagy inhibition and autophagy activation may be both considered as promising strategies for sensitizing CSCs to anticancer therapy.

Concluding remarks

The contribution of autophagy in the physiology of CSCs appears complex and is not yet fully elucidated. Accumulating evidence suggests that the autophagic process actively contributes to the generation, maintenance, plasticity, distribution and migratory/invasion potential of CSCs (Fig. 2). Moreover this catabolic process takes part to the adaptive stress response mounted by CSCs to cope with perturbations of tumor microenvironment (e.g., hypoxia or therapy).

The inhibition of prosurvival pathways preferentially activated in (and presumably strictly required for the survival of) CSCs, including Notch(McAuliffe *et al.*, 2012, Takebe *et al.*, 2011, Ulasov *et al.*, 2011), Sonic Hedgehog(Song *et al.*, 2011, Takebe *et al.*, 2011, Ulasov *et al.*, 2011), Wnt/-β catenin(Kendziorra *et al.*, 2011, Takebe *et al.*, 2011) and NF-kB(Garner *et al.*, 2013, Sun *et al.*, 2013) signaling cascades, is considered as an efficient antineoplastic strategy. By using a similar approach, the components of the autophagic machinery may thus be employed as a promising target to selectively eradicate CSCs thereby arresting tumor growth/progression/spreading and improving the effectiveness of radio- and chemotherapy (which both are reported to enrich CSCs in tumors)(Bao *et al.*, 2006, De Sousa *et al.*, 2013, Phillips *et al.*, 2006).

Nevertheless, some evidence suggests caution in the use of autophagy inhibitors for cancer therapy: (1) autophagy has a physiological role in the preservation of tissue homeostasis, is involved in innate/adaptive immunity and also acts as a barrier against tumorigenesis and neurodegenerative diseases, implying that its whole-body inhibition may have adverse effects(Choi *et al.*, 2013); (2) CQ and hydroxychloroquine (i.e., the drugs currently used for inhibiting autophagy in multiple ongoing clinical trials launched on cancer patients, source: http://clinicaltrials.gov/) (Manic *et al.,* 2014) have activities on lysosomal (and possibly non-lysosomal) processes distinct from autophagy(Balic *et al.*, 2014, Choi *et al.*, 2014, Maycotte *et al.*, 2012, Rubinsztein *et al.*, 2012) suggesting the need to develop novel, specific pharmacological inhibitors of autophagy; (3) autophagic proteins have autophagy-independent roles (for instance BECN1 contributes to vesicular trafficking pathways (Cao and Klionsky, 2007, Shravage *et al.*, 2013); (4) in some cases autophagy drives the anticancer and anti-CSC activity of specific antitumor agents(Filippi-Chiela *et al.*, 2011, Fu *et al.*, 2009, Fu *et al.*, 2014, Mohammed *et al.*, 2013, Zhuang *et al.*, 2011b), meaning that autophagy inhibition may lead to therapeutic failure; and (5) some signaling pathways that de facto inhibit the molecular machinery of autophagy (e.g. the PI3K/AKT/mTOR cascade) are described as cytoprotective for CSCs(Chang *et al.*, 2013, Dubrovska *et al.*, 2009, Martelli *et al.*, 2011), suggesting that the activation of the autophagic process may also be a potential means to kill cancer cells including CSCs. The real impact of autophagy in CSCs may thus depend on the type of tumor, stage of tumorigenesis, tumor microenvironment

as well as the genetic, epigenetic and metabolic context.

Uncovering the exact contribution of autophagy in tumor system and CSC biology, and the specific role of this catabolic pathway in CSCs may be crucial for the development of novel antineoplastic therapy aiming at tumor eradication.

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