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The Role of Autophagy in the Maintenance of Stemness and Differentiation of Mesenchymal Stem Cells

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Abstract Regulated self-consumption, also known as autophagy, is an evolutionary conserved process that degrades cellular components by directing them to the lysosomal compartment of eukaryotic cells. As a major intracellular degradation and recycling pathway, autophagy is crucial for maintaining and remodeling cellular homeostasis during normal cellular and tissue development. Recent studies have demonstrated that autophagy is necessary for the maintenance of cellular stemness and for a number of differentiation processes, including the lineage determination of mesenchymal stem cells. These are multipotent progenitor cells with self-renewal capacities that can give rise to a subset of tissues and thus hold a consistent potential in regenerative medicine. Here, we review the current literature on the complex liaison between autophagy induced by various extra- or intracellular stimuli and the molecular targets that affect mesenchymal stem cells proliferation and differentiation.

Keywords Mesenchymal stem cell · Autophagy · Stemness · Differentiation · Hypoxia · Acidity · Senescence

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Autophagy

The catabolic and degradative process termed autophagy consists of three different forms: microautophagy, which implies the direct uptake of soluble cytosolic substrates in the lysosomes via invagination of the lysosomal membrane [1]; chaperone-mediated autophagy, which degrades specific proteins carrying the peptide motif KFERQ and translocated to lysosomes via chaperone protein Hsc70 (heat shock cognate 70) [2]; and macroautophagy, involving the formation of double-membrane vesicles (autophagosomes) containing an autophagic cargo and their fusion with lysosomes [3]. This review will focus on macroautophagy, from now on referred to as autophagy. Independently of the type of autophagy, the autophagic cargo is degraded by lysosomal acidic hydrolases and cathepsins and the molecules produced are released into the cytoplasm and re-used as building blocks in different anabolic pathways [4]. Basal autophagy allows the removal of redundant or damaged and potentially toxic organelles and protein aggregates, thus representing an important system for quality control in cellular homeostasis. Autophagy can be upregulated by conditions of stress represented by hypoxia, nutrient deprivation, metabolic, oxidative and proteotoxic stress [5]. Although starvation-induced autophagy is a non-selective process degrading bulk cytosolic material to provide nutrients and support cellular metabolism and survival in stress conditions, there are several types of selective-autophagy, where specific organelles and substrates like mitochondria, lipid droplets, protein aggregates and ferritin are targeted to the autophagosome and delivered to lysosomes for degradation [6]. Many types of selective-autophagy rely on the recognition of poly-ubiquitylated targets by specific autophagy receptors, including sequestosome 1 (SQSTM1 or p62), NBR1 and Optineurin [7]. Thus, autophagy is a crucial process that modulates the adaptive response to cellular stress and

contributes to maintain cellular homeostasis in physiological conditions. Because of these important functions, alterations in autophagy are associated with several human conditions, including neurodegenerative, cardiovascular and infectious diseases, as well as cancer [8].

The autophagic process starts with the elongation of membrane precursors and the formation of a double membrane vesicle (autophagosome), which engulfs bulk cytosolic material and targeted cellular organelles. Autophagy-related proteins (ATG) are responsible for the elongation and formation of the autophagosome [9]. Moving along the microtubules network [10], the autophagosome fuses with a lysosome whose acidic lumen activates hydrolytic enzymes that degrade the content of the autolysosome, giving rise to amino acids, fatty acids, nucleosides and other metabolites released into the cytosol and recycled in anabolic cellular metabolism [11]. At the molecular level, autophagy is negatively regulated by the MTORC1 complex, which in the presence of sufficient nutrients and growth factors phosphorylates and inactivates the serine/threonine kinase ULK1/2 [12]. The complex containing ULK1/2 regulates the initiation of the autophagic process and is activated by AMPK-mediated phosphorylation and in presence of inactive MTORC1 due to nutrients starvation and/or growth factors deprivation [13]. The ULK1 complex activates the class III PI3K complex, containing Beclin-1 and the class III phosphatidylinositol 3-kinase (PtdIns3K) protein VSP34 [14]. VPS34 is a lipid kinase producing phosphatidylinositol-3-phosphate (PI3P), essential for autophagosomal membrane elongation and for the recruitment of proteins with PI3P binding domains (e.g. WIPI1/2) involved in vesicle elongation, followed by the activity of two ubiquitin-like conjugation systems. The first is the complex ATG12–ATG5-ATG16L, an E3-like ligase that mediates the lipidation of the LC3 family members LC3, GATE16 and GABARAP, which will be then associated to the autophagosomal membrane. The ATG12–ATG5-ATG16L complex will dissociate from the membrane before closure while lipidated LC3 members (e.g. LC3-II or LC3-PE) will regulate final maturation of the autophagosome, being retained on the inner membrane. In the case of selective autophagy, autophagy receptors like SQSTM1 contain an LC3-interacting region (LIR) and an ubiquitin-associated domain (UBA), which allow the selective recruitment of ubiquitinated organelles or structures to the autophagosome (Table 1).

Table 1 Summary of the different roles of autophagy in the different cell types and species

Cell type/Species/Site of origin /Name of cell line	Role of autophagy	References
Rat MSC from bone marrow	Alleviating glucose-derived cytotoxycity of pancreatic INS-1 cells	[31]
Human MSC from umbilical cord	Promotion of authophagy in the injured cells of wounds under diabetic condition to ameliorate the healing process	[32]
Human and rat MSC from bone marrow, human IMR90 and BJ fibroblasts	Promotion of senescence and inhibition of stemness under stressing conditions, or when exposed to FGFs	[34–36, 38, 79]
Human MSC from bone marrow	Promotion of senescence induced by hyperglicemia	[33]
Human, mouse, and rat MSC from bone marrow, H9 human ESC	Control of osteogenic differentiation	[54, 55, 73, 75, 81, 82]
Human MSC from umbilical cord, mouse mesenchymal stem cell line CH3H10	Maintenance of stemness thorough the reduction of mitochondria to protect from ROS	[45, 46]
Human, mouse and rat MSC from bone marrow	Mediating hypoxia-induced apoptosis	[29, 51, 52]
Rat MSC from bone marrow	Protection from hypoxia-induced apoptosis	[53]
Mouse MSC from bone marrow	Mediating hypoxia-induced proliferation	[54]
MDA-MB-231, HS766T, Me30966, Mel501, WM793, A375, SK-Mel-28 cancer cell lines	Adaptation to extracellular acidosis	[69, 70]
Mouse and human ESC	Maintenance of stemness and induction of the progression of the early stage of embryogenesis	[84, 85]
Mouse hematopoietic stem cells from bone marrow and spleen	Control of myeloproliferation, protection from ROS production and DNA damage	[87–89]
Mouse muscle satellite cells	Maintenance of quiescence, protection from senescence and oxidative stress	[91–93]
Cancer stem cells isolated from fresh ductal carcinoma in situ, breast carcinoma, and pancreatic ductal adenocarcinoma. Cancer stem cells isolated from MDA-MB-231, MDA-MB-468, MCF-7, BxPc-3, MIA-PaCa2, and BT474 cell lines.	Up-regulation of stemness under both normoxia and hypoxia, promotion of cell survival	[94–97, 100, 102]

Mesenchymal Stem Cells

Mesenchymal stem cells (MSC) are multipotent progenitor cells that were originally identified ex vivo in small numbers by plastic adherence from rat whole bone marrow cultures (BM-MSC) [15]; in time, MSC have been identified from

various other sources, such as adipose tissue (ASC), Wharton's jelly umbilical cord and dental pulp (DPSC) [16]. MSC isolated from different sources share many biological characteristics but also show differences in their immunophenotype, differentiation potential, transcriptome and proteome immunomodulatory activity [17], and secretome [18]. For example, the two most common sources of MSC, ASC and BM-MSC, have considerable differences: ASC are genetically and morphologically more stable in a long-term culture, display a lower senescence ratio, show a higher proliferative capacity and retain differentiation potential for a longer period in culture compared with human BM-MSC [19]. Recently, it has been demonstrated that human ASC support hematopoiesis both in vitro and in vivo and, unexpectedly, seem to exert this activity more efficiently than human BM-MSC [20]. Nevertheless, after expansion, ASC and BM-MSC seem to have the same effectiveness [21]. As other stem cell types, MSC have a high capacity for self-renewal while maintaining multipotency; one of the key features of MSC is the overexpression of well-defined stem-cell transcription factors. Among these, Nanog, Sox2 and Oct4 are master-regulators involved in self-renewal and the maintenance of pluripotency of mammalian embryos during organogenesis and stem cell functions. MSC can also be induced to differentiate by the addition of several factors in the culture medium; indeed MSC can form, in vitro, adipocytes, cartilage, bone, tendons, muscle and skin and thus provide a humongous potential in tissue regeneration and repair and in regenerative medicine applications [22–24]. When transplanted in vivo, MSC home to areas of insult, where they promote tissue repair via secretion of soluble factors that enhance tissue regeneration, stimulate proliferation, migration, differentiation and survival of endogenous local progenitor cells found in the microenvironment, as well as by decreasing inflammatory and immune reactions and apoptosis [25, 26].

The Role of Autophagy in Stemness Maintenance of Mesenchymal Stem Cells

Emerging evidence indicates that autophagy plays a consistent role in the modulation of cell proliferation, differentiation and stemness in a wide variety of cell types, including MSC. In human MSC, the autophagic flux is constitutively activated, as evidenced by LC3-I to LC3-II conversion [27, 28] and seems to be dependent on the antiapoptotic protein Bcl-Xl [28]. Bearing this in mind, a great effort has been made trying to evaluate the role of autophagy induced by various extra- or intracellular stimuli in the maintenance of cell stemness and survival [29]. Here, we will summarize some of the modifier stimuli. Figure 1 recapitulates the current knowledge so far.

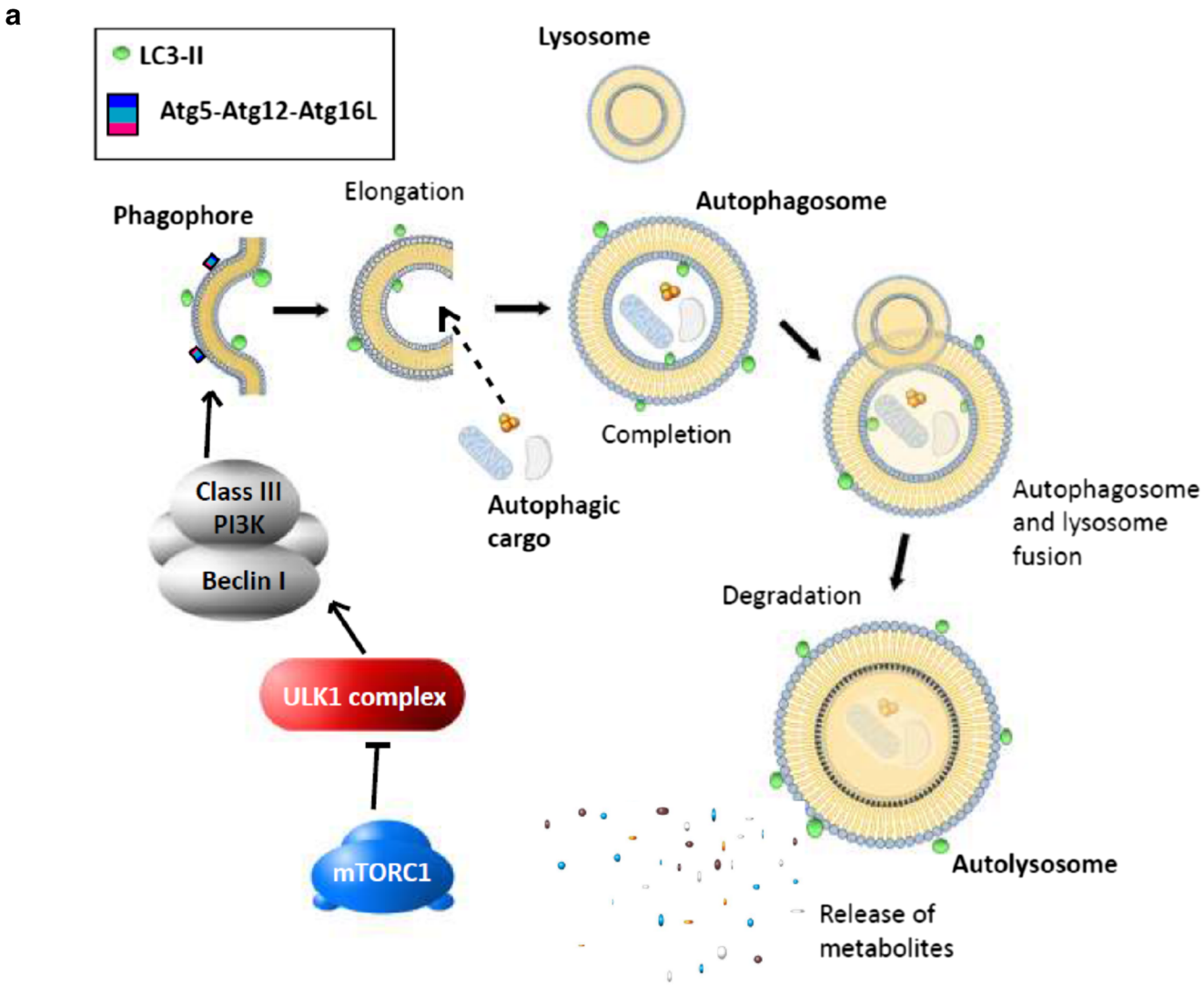
Fig. 1 Schematic representation of autophagy activation in MSC and the function of autophagy in different stem cells. **a)** Basic macroautophagic flux; **b)** A number of extra- or intra- cellular stimuli have been described to be activators of autophagy in MSC. Among these, hyperglycemia, ROS, senescence, or hypoxia lead, eventually, to LC3 conversion and lysosomal organelle degradation. Whether activation of autophagy in response to these stimuli is cytoprotective or leads to cell failure is controversial and seems to be strictly context- and cell type-dependent. For references see Table 1

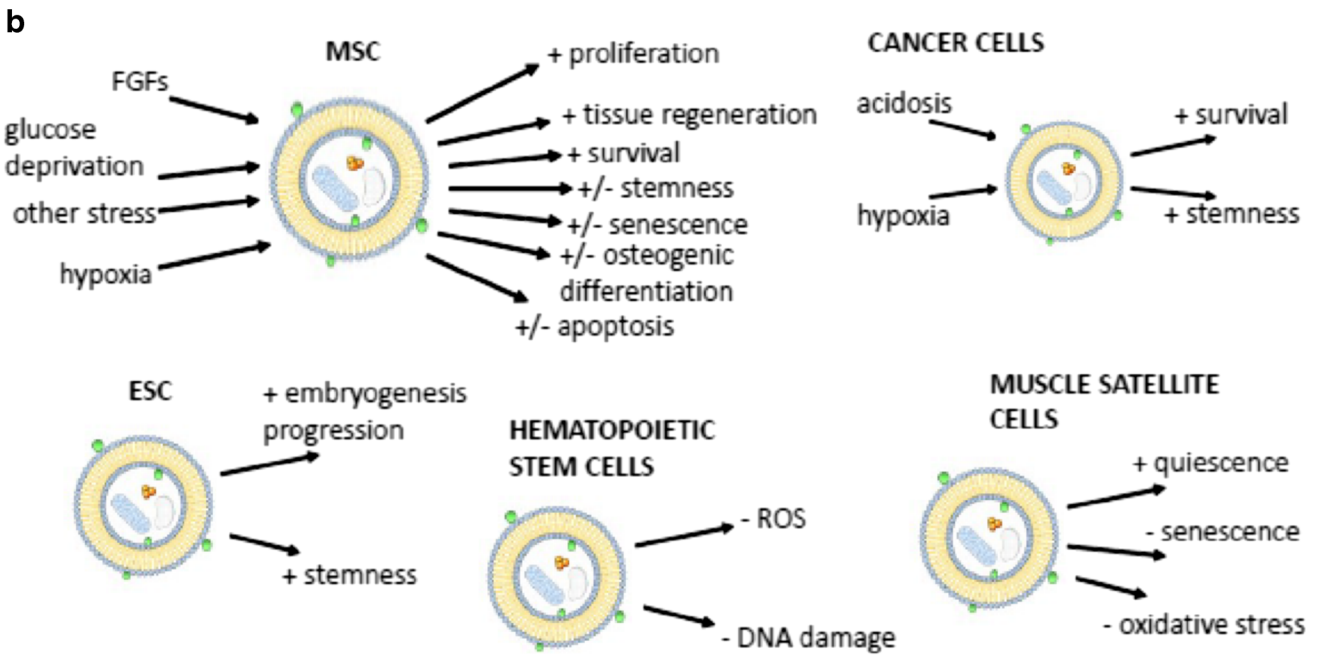
Hyperglycemia

Hyperglycemia due to diabetes mellitus and metabolic syndrome has emerged as a major problem for human health, causing vascular and organ dysfunction. Recent reports indicate that hyperglycemia impairs bone marrow hematopoietic function and alters the hematopoietic niche [30] ultimately leading to pancreatic β-cell failure. Zhao et al. [31] have shown that BM-MSC significantly alleviate the glucose-derived cytotoxicity of pancreatic INS-1 cells and that this mechanism strictly relies on the activation of autophagy in vitro and in vivo, since its inhibition dramatically reduces the protective effects of BM-MSC on β-cells. Also, umbilical cord MSC have a high therapeutic value in the treatment of diabetes, as their induction can ameliorate the wound healing processes in the clinical scenario of diabetic patients, possibly inducing autophagy in the injured cells [32].

Senescence

BM-MSC cultured in medium containing high glucose concentrations exhibit premature senescence, genomic instability and telomere changes; autophagy normally provides a survival effect for cells under stress, and thus the results obtained from Chang et al. were rather unexpected when MSC were cultured in conditions of high glucose concentration [33]. Indeed, it was demonstrated that activation of autophagy, monitored by upregulation of Beclin-1, Atg 5 and 7, and increased LC3-II conversion, correlates with senescence changes in BM-MSC and that, conversely, inhibition of autophagy by 3-methyladenine prevents cellular degeneration. When undergoing senescence, cells develop an enlarged and flat morphology and ultimately stop dividing. Simultaneously, they lose their stem cell characteristics [34] therefore affecting their clinical application. Despite being clear that autophagy is activated during senescence [35, 36], its role in cellular maintenance and stemness is conflicting and poorly understood. It was reported that deletion of autophagy-related genes accelerates cell senescence [37] while other types of stresses, such as oncogenic stress, induce senescence through activation of autophagy [38]. A recent report suggests that autophagy is a stress adaptation response that avoids cell death (and suppresses apoptosis) or constitutes an alternative cell-death pathway depending on the cellular context [39]. It is therefore


a
LC3-II
Atg5-Atg12-Atg16L
Lysosome
Phagophore
Elongation
Autophagosome
Completion
Autophagic cargo
Autophagosome and lysosome fusion
Degradation
Autolysosome
Release of metabolites
Class III PI3K
Beclin I
ULK1 complex
mTORC1


b
MSC
FGFs
glucose deprivation
other stress
hypoxia
+ proliferation
+ tissue regeneration
+ survival
+/- stemness
+/- senescence
+/- osteogenic differentiation
+/- apoptosis
CANCER CELLS
acidosis
hypoxia
+ survival
+ stemness
ESC
+ embryogenesis progression
+ stemness
HEMATOPOIETIC STEM CELLS
- ROS
- DNA damage
MUSCLE SATELLITE CELLS
+ quiescence
- senescence
- oxidative stress

possible that a "housekeeping" level of autophagy is required to prevent cellular senescence while excessive autophagic activation abbreviates cellular lifespan.

The molecular mechanisms underlying senescence, especially those overlapping with autophagy, are still poorly understood. Interestingly, the increase of the cell cycle regulating factor and tumor suppressor p53 has been correlated with aging BM-MSC and shown to be involved in functions controlling the cell-cycle, apoptosis and genomic stability [40]. Knockdown of p53 results in reduced LC3-II conversion and mTOR upregulation, thus demonstrating that it constitutes an essential trigger for autophagy during culture expansion of BM-MSC [41, 42]. Indeed, despite recent progress in the comprehension of cellular senescence, a number of works has yet to be done to clarify the role of autophagy and the molecular mechanisms underlying cellular lifespan.

ROS

In order to maintain their stemness, especially long-lived cells actively reduce senescence by establishing low-reactive oxygen species environments [43]. Primary sources of ROS are damaged mitochondria, which can be efficiently removed in stem cells by autophagy in a process called mitophagy [44]. Recently, it has been demonstrated that autophagy induced by starvation or rapamycin can reduce irradiation-generated ROS and DNA damage, therefore maintaining cell stemness of MSC, whereas inhibition of autophagy leads to ROS accumulation and DNA damage, ultimately resulting in loss of cell stemness [45]. The same has been proven for the treatment of diabetic erectile dysfunction, where ROS induce autophagy to counteract apoptosis in MSC by activation of JNK. Thus, augmentation of autophagy may reduce apoptosis, prolonging MSC survival and improving MSC-based therapeutic efficacy for diabetic erectile dysfunction [46] and, more in general, autophagy may protect from ROS-induced cell death modalities.

Hypoxia

Among the extracellular stimuli that regulate cell survival and stemness, several physical and chemical features of the stem-cell niche, such as oxygen tension, osmolarity and pH have a strong influence on MSC behaviour and differentiation [47]. Hypoxia-inducible factor 1 (HIF-1) is a transcription factor, which functions as a master regulator of adaptive responses to conditions of reduced pO_2 [48]. HIF-1 improves local microcirculation via its effects on vascular growth and functioning, and regulates O_2 utilization by switching oxidative metabolism to glycolytic metabolism [49]. As in the case of cellular senescence, the relationship between autophagy or apoptosis of MSC induced by hypoxic stimuli is not well understood, despite a number of studies confirm autophagy activation in hypoxic conditions [50]. Different studies have recently demonstrated that hypoxic conditions activate BM-MSC autophagic flux through the AMPK/mTOR pathway and that activation of the latter process contributes to hypoxia-induced apoptosis, as demonstrated by the reduction of TUNEL positive cells in the presence of the autophagy inhibitor 3-methyladenine, whereas was aggravated by rapamycin, a positive inducer [29, 51, 52]. In an opposite work from the group of Xu, the levels of hypoxia-induced apoptosis were increased by 3-methyladenine, while decreased by rapamycin, thus suggesting that the "self-eating" process might play a protective role in hypoxia-induced apoptosis of MSC, and that atorvastatin, a commonly prescribed statin, could effectively activate autophagy via AMPK/mTOR pathway to enhance MSC survival during hypoxia [53]. Further complicating this scenario, hypoxia has been shown to promote BM-MSC proliferation, through autophagy and apelin, a neuropeptide with mitogenic effects [54]. Perhaps, the great variability in the above results might depend on the site-specific properties of the MSC. For example, mandible-derived BM-MSC possess stronger expression of the stemness markers Nanog, Oct-4 and Sox2 but also stronger autophagy and anti-aging capacities under normoxia or hypoxia, when compared to tibia-derived BM-MSC [55].

In conclusion, the role of autophagy in the modulation of MSC stemness, proliferation and survival is still controversial. Further in vivo and in vitro studies are therefore necessary before MSC-based cell therapies can be proposed as a new strategy for the amelioration of cell survival rate.

Acidity

It is assessed that among the peculiar features of the stem-cell niche microenvironment, its characteristic pH value influences MSC proliferation, differentiation and paracrine activity [47]. In physiological conditions, peripheral blood pH is approximately 7.4–7.35, while pH of the fluids flowing among cells in tissues is lower, and subject to alterations due to cell metabolism. Extracellular acidosis is caused by an increase in glycolytic and oxidative metabolism of the cells that leads to the production of high amount of carbonic and lactic acids, which are extruded to maintain an intracellular pH near the physiological value. As a consequence, an inverted membrane pH gradient is established: the extracellular pH (pHe) is lower than the intracellular pH (pHi) [56]. This altered acidic microenvironment is commonly associated with damaged tissues, diabetes, chronic renal failure, obesity, osteoporosis, and arthritis [57–60]. Local acidosis can trigger the activation of inflammatory pathways [60], an altered release of metabolites [61, 62], an increased release of exosomes [63] and growth factors involved in inflammation, infection, ischemia, healing of bone fractures, as well as tumors [64–66]. Acidity in tumors has been widely studied, showing that cancer cells use

glucose, rather than oxidative phosphorylation, to produce energy, even in normal oxygen pressure. This peculiar feature is called Warburg effect [65, 67]. Low tumor pHe has been found in several preclinical models of human cancers, showing a pHe between 5.9 and 7.2, depending on the tumor type [68]. Specifically, there are studies reporting that cancer cells use autophagy as a mechanism of adaptation to acidosis [69, 70]. Although it has been proven that extracellular acidosis promotes the stem cell phenotype of cancer stem cells [71, 72], the acidic microenvironment ability to induce stemness in normal cells has not been shown yet. Specifically, the relationship between extracellular acidosis and MSC behavior still needs to be better clarified [73]. In vitro studies suggest that overexpression of mTOR induces the differentiation of MSCs into osteoblasts, adipocytes, and myoblasts [74]. However, the involvement of mTOR in osteoblasts differentiation is still a matter of debate [75–78] and further studies are needed. According to recent works, an acidic microenvironment modulates stemness and regenerative potential of MSC [78]. Similarly, autophagy has been associated with the regulation of MSC stem-like features, as well as with senescence and cell death/survival [79], and can be induced by acidic conditions. Given the lack of reports in the field, to further understand whether acidity can affect or improve MSC regenerative potential through the induction of autophagy, we reasoned to investigate the role of autophagy in MSC cultured in acidic conditions in order to improve the outcome of approaches of regenerative medicine occurring in patients with subclinical acidosis.

The autophagic flux of bone marrow MSC was evaluated by protein expression of typical autophagic markers (LC3-II, p62 and Beclin1) at lower (pH 6.8) or neutral (pH 7.4) pH (Fig. 2a and b). Despite we could observe a detectable autophagic activity of BM-MSC in both culture conditions, as shown by LC3-I to LC3-II conversion, no significant variations between pH conditions could be observed, leading to the speculation that autophagy is not the main mechanism by which MSC face an acidic microenvironment. Ultrastructural analysis was used to confirm our findings: we observed the typical features of autophagy, such as formation of autophagic vacuoles containing cytoplasmic components and organelles (Fig. 2c), but no qualitative differences among different pHe.

The Role of Autophagy in the Differentiation of MSC

MSC that give rise to the osteoblastic lineage are commonly defined by their in vitro ability to differentiate into osteoblasts, chondrocytes, and adipocytes [16]. Commitment of stem cells to different lineages is regulated by many cues in the local tissue microenvironment, such as plating density, cell shape, cytoskeleton tension and adhesive, mechanical or structural cellular properties. Lower cell densities seem to support osteoblastic differentiation of MSC, whereas higher cell densities cause the cells to condense and become adipocytes. Cell shape regulates the adipogenic-osteogenic switch in lineage commitment by modulating endogenous Rho GTPase (RhoA) activity [80]. Expressing dominant-negative RhoA commits MSC to become adipocytes, while constitutively active RhoA causes osteogenesis. Proliferation and differentiation potential of MSC dramatically depend also on a variety of growth factors that might stimulate tissue regeneration in an autocrine fashion. Among these, FGF, EGF and HGF induce proliferation capacity of MSC and, interestingly, HGF has been shown to enhance the adipogenic differentiation potential of the cells [79].

Indeed, autophagy plays a basic role also in the commitment of MSC to different lineages, especially in the osteoblastic lineage. Nuschke et al. have recently demonstrated that undifferentiated MSC have an accumulation of undegraded autophagic vacuoles and little autophagic turnover, whereas stimulation of osteogenic differentiation leads to a consistent increase in turnover [81]. Thus, autophagy seems to be of fundamental importance in the control of osteogenic differentiation and this seems to be related to the early mTOR inhibition and the late activation of the Akt/mTOR signaling axis, as demonstrated by the genetic knockdown approaches of AMPK, mTOR, Akt and autophagy markers in dental pulp MSC [73]. Also SATB2, an AT-rich binding protein, has the capacity of promoting osteogenic differentiation and bone defect regeneration of BM-MSC, and this is sought to occur by upregulation of pluripotency genes and autophagy-related genes which, in turn, activate the mechanistic target of rapamycin signalling pathway [55]. While mTOR signaling may function to affect osteoblastic differentiation, conflicting results have been reported on whether rapamycin decreases or increases osteogenesis, according to the cell type. In rats, rapamycin does not exhibit a spontaneous osteogenic effect on MSC, but inhibits the effect of osteogenic differentiation induced by dexamethasone [82]. On the other hand, rapamycin promotes osteogenic differentiation in human embryonic stem cells (ESC) by blocking mTOR and stimulating BMP/Smad signaling pathway [75], once again suggesting that no general statement can be made on the role of autophagy.

Autophagy in Other Stem Cells

Embryonic Stem Cells

ESC are pluripotent stem cells that derive from the inner mass of the blastocyst, and distinguished by their ability to differentiate into any cell type and to exhibit remarkable long-term proliferative potential. The precise roles of autophagy during early human embryonic development remains largely

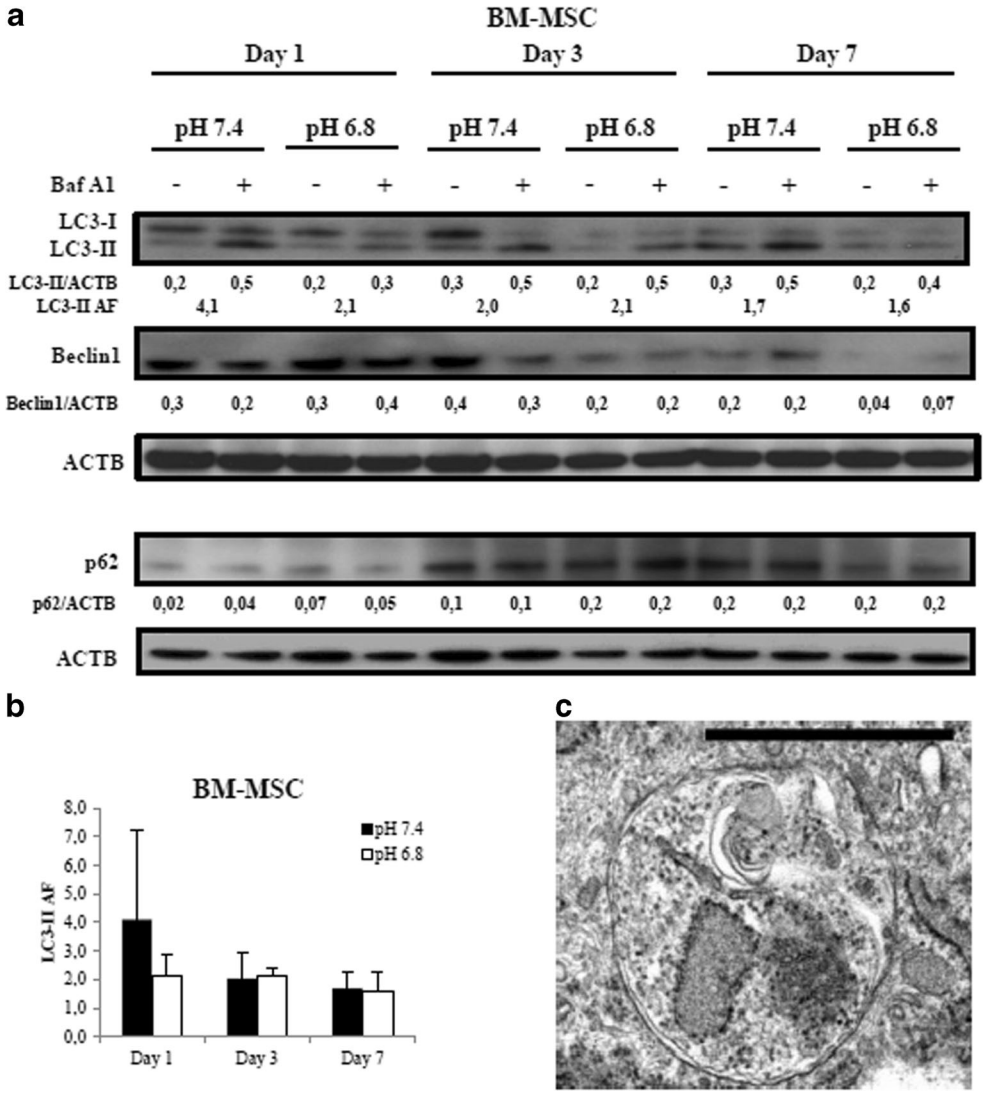


Fig. 2 Autophagic flux in BM-MSC at pH 7.4 and pH 6.8. **a)** Protein expression of typical autophagic markers (LC3-II, p62 and Beclin1) was evaluated by Western blot, at 1, 3 and 7 days of exposure to culture media at different pHs (6.8 and 7.4). pH was maintained in the media during the incubation period by using different $NaHCO_3$ concentrations, according to the Henderson-Hasselbach equation. LC3-II, p62 and Beclin1 protein expression levels were normalized on β-actin (ACTB). The autophagic flux (LC3-II AF) is defined as the ratio between the normalized LC3-II levels in presence and absence of Bafilomycin A1 (BafA1) at saturating concentrations (50 nM). BafA1, the well-known inhibitor of the late phase of autophagy [118], was added during the last 2 h of incubation to emphasize the autophagic process detection. Quantification of LC3-II AF is shown in panel **b)** with data expressed as mean and SE. **c)** Representative transmission electron microscopy image of a double-membrane autophagosome in BM-MSC cells. *Scale bar* corresponds to 1 μm. BM-MSC pellets from healthy donor were fixed with 2.5 % glutaraldehyde in 0.1 M cacodylate buffer, dehydrated in an ethanol series and embedded in Epon resin. Ultrathin sections were stained with lead citrate and uranyl acetate and observed with a Jeol Jem 1011 transmission electron microscope, operated at 100 kV

uncharacterized, despite a number of studies have made a great effort in the attempt to shed some light on this field (for a comprehensive review see [83]). It has been established that autophagy is essential for the very early stages of embryogenesis in vitro and in vivo and is dependent on mTOR and PI-3K pathway. Fertilized mouse oocytes lacking ATG5 (by oocyte-specific conditional knockout of the Atg5 gene) do not proceed beyond the 4- to 8-cell stage if they are fertilized by Atg5-null sperm, and therefore fail to form the blastocysts and the inner cell mass [84]; moreover similarly to other cell lines, Atg−/− ESC display impaired LC3-II conversion. Presumably, autophagy is required for controlling levels of key regulatory protein complexes or perhaps to provide substrates for cellular energy homeostasis prior to pre-implantation, after which cells have access to trans-placental nutrients. Like in the case of MSC, the pluripotency of ESC is maintained by the stemness genes Sox2, Oct4 and Nanog. Cho et al. have recently shown that autophagy acts together with the ubiquitin-proteasome system to modulate the levels of the stemness genes proteins in human ESC and that its inhibition

impaired the pluripotency despite increment of Sox2, Oct4 and Nanog [85]. The role of autophagy in ESC function in later stages of embryo development is less clear, suggesting that autophagy does not appear to play a pivotal role in the timing and coordination of differentiation in the developing embryo. Interestingly, autophagy was upregulated in ESC induced to undergo differentiation by treatment with type I TGF-beta receptor inhibitor or removal of secreted maintenance factors [86].

Hematopoietic Stem Cells

Recent evidence suggests that autophagy may also be important for the long-term health of progenitors as well as fully differentiated long-lived cell types. Autophagy inhibition was shown to manifest in functional defects in hematopoietic stem cells (HSC) and cause severe myeloproliferation [87]. In the absence of Atg7, hematopoietic stem and progenitor cell compartment displayed an accumulation of mitochondria and reactive oxygen species, as well as increased proliferation and DNA damage [87, 88]. These results led to the conclusion that autophagy is required for maintenance of the HSC compartment in adult mice and that Atg7 is an essential regulator of adult HSC maintenance. Importantly, FoxO3 is critical for the expression of autophagy genes and the induction of autophagy in response to stress in HSC. Thus, genes encoding the machinery for the autophagic response are important targets for FoxOs in HSC and other cell types [89].

Muscle Satellite Cells

Muscle stem cells, also called satellite cells, are essential for skeletal muscle formation and regeneration. In physiological conditions, the major challenge for these cells is maintenance of the quiescent state to preserve their number and functions throughout life [90]. Quiescent cells are characterized by protective gene programs against environmental stresses to maintain homeostasis; yet, little is known about the regulation of the quiescent stem cell state. An elegant, recently published work has shown that basal autophagy is essential to maintain the stem-cell quiescent state in mice [91]. Failure of autophagy in physiologically aged satellite cells or genetic impairment of autophagy in young cells causes entry into senescence by loss of proteostasis, increased mitochondrial dysfunction and oxidative stress, resulting in a decline in the function and number of satellite cells. Re-establishment of autophagy reverses senescence and restores regenerative functions in geriatric satellite cells [92] and this is sought to be dependent on the nutrient sensor SIRT1 [93]. Thus, autophagy is required also for muscle stem cell homeostasis maintenance.

Cancer Stem Cells

Autophagy has been reported to play an important role for the maintenance and survival of cancer stem cells or tumor-initiating cells in breast and pancreatic carcinomas. Espina and colleagues have shown that in ductal carcinoma in-situ (DCIS) of the breast the expression of beclin-1 is upregulated in the hypoxic niche [94]. Also, a population of stem-like cells cytogenetically abnormal and with tumorigenic capacity isolated from DCIS is characterized by upregulated expression of autophagy proteins and increased autophagic flux. Treatment with chloroquine inhibits many of the phenotypic properties of the tumor-initiating cells present in pre-malignant lesions, indicating that these cells rely on autophagy for survival [94, 95]. Similarly, autophagy was shown to positively regulate the stem-like population of CD44+ CD24−/low cells in breast cancer [96]. In line with this observation, using both patients derived breast cancer stem cells and in vitro generated stem-like breast cancer cells the group of Mehrpur has reported that Beclin 1 and autophagy are needed for the maintenance and expansion of breast CSC [97] and that inhibition of the autophagic flux using salinomycin inhibits the maintenance of breast CSC [98], thus providing a mechanism for the ability of Sal to target CSCs. Interestingly, salinomycin has been identified as a compound with selective activity towards breast cancer stem cells [99] and it has been recently reported that acidic culture conditions enhance the activity of salinomycin towards CD24low HMLER cells and breast cancer stem cells isolated from cancer patients (Pellegrini et al., Oncotarget 2016 in press). It has been suggested that autophagy regulates human breast CSC maintenance by modulating IL6 secretion [100] and that in murine models autophagy regulates breast CSC EGFR/Stat3 and TGFβ/Smad signaling [101]. A recent study has reported that markers of autophagy, hypoxia, and autophagy are present in patient-derived pancreatic cancer lesions [102]. Using a cell line with increased CSC properties, these authors suggest that autophagy enables survival of CSC under conditions of nutrients starvation and hypoxia.

For a quick look of all the references mentioned in this review and related to the role of autophagy in different cell types, species or site of origin refer to Table 1.

Autophagy-Related microRNAs in Stem Cell Functions

MicroRNAs (miRNAs) are a class of endogenously expressed, short non-coding RNAs, which post-transcriptionally regulate gene expression. They coordinate the binding of the RNA-induced silencing complex to partial complementary regions located mainly within 3′untranslated regions (UTRs) of target messenger RNA (mRNA) molecules. The final result is mRNA translational inhibition and/

or degradation [103]. Notably, miRNAs play a pivotal role in a consistent number of biological processes including proliferation, differentiation, apoptosis and stress response, connecting them to several human diseases [104]. Recent studies have identified an important role for microRNAs in the regulation of autophagy and its cross-talk with cell death [105]. Specifically, miRNAs can interfere with common regulators of both autophagy and apoptosis, such as BECN1, which is the gene encoding Beclin-1. Actually, the first studies suggesting a regulatory role of miRNAs on autophagy identified BECN1 as a direct target for miR-30a [106]. Overexpression of miR-30a could reduce rapamycin-induced autophagy, and endogenous miR-30a levels were affected by autophagy induction, suggesting a physiological role for this miRNA in autophagy regulation. The identification of BECN1 as a direct target indicates that this regulation probably occurs at the level of vesicle nucleation. A second miRNA regulating BECN1 is miR-376b, identified in a screen based on miRNA overexpression in MCF-7 cells using GFP-LC3 [107]. Finally, miR-519a was also found to regulate BECN1 in a 3′UTR reporter–based assay [108]. This same study showed direct regulation of UVRAG, the Beclin-1 binding partner, by both miR-630 and miR-374a, suggesting regulatory effects for these miRNAs at the nucleation step, which remains to be further characterized. Furthermore, Xiao et al. identified miR-204 as a regulator of the vesicle elongation process [109]. Its role in autophagy regulation was initially detected in cardiomyocytes and then confirmed in renal clear cell carcinoma [110]. Interestingly, Kovaleva et al. identified ATG2B as a direct target of miR-130a which effectively inhibited autophagic flux and induced cell death [111]. miR-130a seems to interfere with ATG9-ATG2-ATG18 complex formation recovering lipids and proteins from the growing phagophore. However, the functional importance of ATG2B as a miR-130a target remains to be further understood. Another potential regulator of the retrieval step is miR-34a, which has recently been identified as an inhibitor of autophagic flux and a direct regulator of ATG9A in mammalian cells [112]. To date, there are no identified miRNAs directly affecting the fusion process. However, a computational systems biology approach identified a set of miRNAs with potential functional involvement in the autophagy-lysosomal pathway including miR-130, 98, 124, 204 and 142 [113]. Proteins important for autophagosome–lysosome fusion, like LAMP1, LAMP2 and VAMP7 are also possible targets for these miRNAs, suggesting their role in the fusion process [114].

Studying stem cells is important to develop novel strategies to cure multiple disorders that can affect the nervous system, cardiovascular system, immune system, metabolism, and cancer. The main goal of these studies is the protection and maintenance of stem cell populations. Notably, miRNA involvement in stem cell renewal and differentiation is of great interest. For example, Maiese K. studied miRNAs ability to regulate stem cell proliferation by targeting the proliferative pathways of silent mating type information regulation 2 homolog 1 (SIRT1), which can be increased by a loss of mTOR activity, thus inducing autophagy and making critical conditions for stem cell proliferation [115]. Recently, Zhai et al. identified Smad2 as the target of hsa-miR-140-5p, which is involved in autophagy [116]. Smad2 is a key element downstream of the TGF-β signaling pathway to regulate cancer metastasis by promoting epithelial to mesenchymal transition and maintaining the cancer stem cell (CSC) phenotype. Hsa-miR-140-5p directly targets Smad2 and its overexpression in colorectal cancer cell lines decreases Smad2 expression levels, leading to a lower cell invasion and proliferation, and increasing cell cycle arrest. Ectopic expression of hsa-miR-140-5p in colorectal CSCs inhibited CSC growth and sphere formation in vitro by interfering with autophagy. Another study about the correlation between stem cell behavior, autophagy and processes involving miRNAs shows that a specific miRNA expression (miR-34a) is markedly downregulated during neurogenesis in mouse neural stem cell (NSC) [117]. Autophagy seems to have a critical role during neuronal differentiation, as a response-survival mechanism to limit oxidative stress and regulate synaptogenesis associated with this process.

Importantly, regulation of autophagy by non-coding RNAs, and in particular by miRNAs, represents a new post-transcriptional regulatory step, which needs to be further explored. Since miRNA expression is altered during conditions of stress and disease, the complexity and dynamics of this regulation and its potential consequences for disease pathogenesis are widespread. Several aspects of this regulation remain to be better analyzed.

Conclusions

The implications of autophagy in the control of cell stemness add a new layer in the understanding and maintenance of cell activity. In regenerative medicine this might be of pivotal importance, as the amelioration of growth and differentiation techniques provides a humongous potential in the regeneration and repair of tissues such as adipocytes, cartilage, bone, tendons, muscle and skin. Nevertheless, the great confusion that still dominates the field and that emerges from this review suggests that great caution has to be taken when considering the transplant of MSC in donor patients.

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Compliance with Ethical Standards

Conflict of Interest The authors declare NO potential conflict of interest.

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