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LETTER TO THE EDITOR

Autophagy is required for self-renewal and differentiation of adult human stem cells

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Dear Editor,

Patients receiving anticancer therapy usually suffer from side effects, such as loss of hair, epithelial barrier defects, and myelosuppression, which, however, are usually reversible when the therapy is discontinued. Although this clinical experience suggests that adult stem cells may survive such therapeutic interventions, the underlying molecular regulatory mechanisms are poorly understood. Such regulatory mechanisms also seem to protect adult stem cells from acquiring mutations, which could lead to additional tumorigenesis.

Autophagy is a conserved proteolytic mechanism that degrades cytoplasmic material, including cell organelles. It is required for maintaining cellular homeostasis, which also includes cell differentiation processes such as erythropoiesis, lymphopoiesis, and adipogenesis. Increased autophagic activity is seen when cells are exposed to stress, such as lack of oxygen or food. Under such conditions, superfluous cytoplasmic macromolecules are sequestered into double-membrane structures, called autophagosomes. In contrast, under physiological conditions, autophagosomes are only rarely seen in cells.

A very recent work suggested that autophagic mechanisms are active in hematopoietic stem cells (HSC). For instance, FIP200 (200 kDa focal adhesion kinase family interacting protein) was shown to be essential not only for the induction of autophagy, but also for the maintenance and function of HSC *in vivo* [1]. Moreover, HSC lacking autophagy-related gene (*ATG*) 7, another essential autophagy protein, were also unable to survive under *in vivo* conditions [2]. In this report, we provide evidence that high autophagic activity is a general phenomenon of adult stem cells and extend the earlier findings to the human system. Moreover, we demonstrate a role for autophagy in stem cell differentiation and resistance to cytotoxic drugs in these cells.

We were surprised to see numerous vacuoles in epidermal (ESC), dermal (DSC), and HSC, suggesting increased levels of autophagy in these cells (Figure 1A and Supplementary information, Data S1 and Figure S1). The low numbers of vacuoles per cell in control NB4 cells are in agreement with previously reported data obtained in primary promyelocytes and mature neutrophils [3]. Autophagosomes fuse with lysosomes to form autolysosomes where the captured material is degraded. The main elements required for autophagosome and autolysosome formation, respectively, are two ubiquitin-like conjugation systems, which involve several ATGs: (1) the ATG12-ATG5 and (2) the microtubule-associated protein 1 light chain 3 (LC3)-phosphatidylethanolamine (PE) systems [4]. ESC, DSC, and HSC exhibited a punctated immunostaining pattern for LC3 and ATG5 (Figure 1B), confirming the accumulation of autophagosomes. In contrast, LC3 staining was diffuse and relatively weak in control NB4 cells (Figure 1B), as well as in primary immature keratinocytes, fibroblasts, and neutrophils (Supplementary information, Figure S2). These data are supported by biochemical analysis, in which we observed the conversion from cytosolic LC3-I to membrane-bound LC3-II (Figure 1C). Moreover, the lack of p62 protein, a marker protein which serves as a link between LC3 and ubiquitinated substrates [5], in adult stem cells (Figure 1C) suggested that the accumulation of autophagosomes is the result of high autophagic activity and is not due to a functional defect in autophagic catabolism. In contrast, following induced differentiation, immature keratinocytes, fibroblasts, and neutrophils had less LC3-II and expressed LC3-I, as well as detectable amounts of p62, a pattern that is usually seen in cells with basal autophagic activity (Figure 1C) [6]. The downregulation of autophagy in differentiated cells was usually associated with lower levels of ATG5 and Beclin1 (Figure 1C), at least partially due to reduced transcription of these essential ATGs (Supplementary information, Figure S3).

Adult stem cells are characterized by their capacities of self-renewal and differentiation. Single cells from epidermal and dermal spheres, as well as HSC were cultured and analyzed for their capacity to form colonies. Single ESC, DSC, and HSC could be induced to undergo self-renewal. Pharmacological blocking of autophagy by 3-methyladenine (3-MA) for 24 h before analyzing single

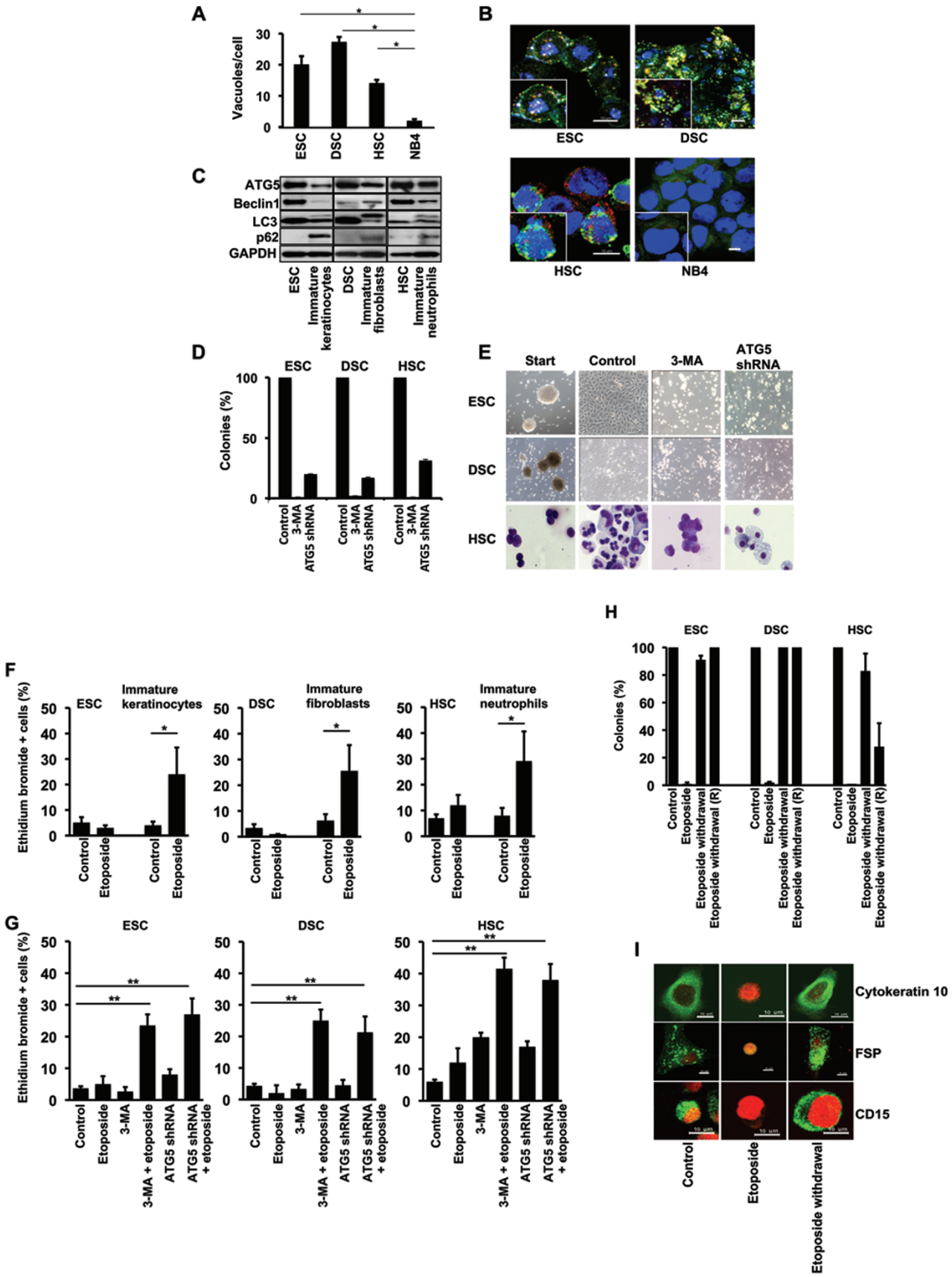
A
Vacuoles/cell
ESC
DSC
HSC
NB4
B
ESC
DSC
HSC
NB4
C
ATG5
Beclin1
LC3
p62
GAPDH
ESC
Immature keratinocytes
DSC
Immature fibroblasts
HSC
Immature neutrophils
D
Colonies (%)
Control
3-MA
ATG5 shRNA
E
Start
Control
3-MA
ATG5 shRNA
F
Ethidium bromide + cells (%)
Immature keratinocytes
Immature fibroblasts
Immature neutrophils
Etoposide
G
3-MA + etoposide
ATG5 shRNA + etoposide
H
Etoposide withdrawal
Etoposide withdrawal (R)
I
Cytokeratin 10
FSP
CD15
Etoposide withdrawal

Figure 1 Autophagy is required for self-renewal and differentiation of adult human stem cells, which are largely cytotoxic drug-resistant, but become sensitive upon differentiation or when autophagy is blocked. **(A)** Transmission electron microscopy (TEM). Epidermal, dermal, and hematopoietic stem cells (ESC, DSC, and HSC) exhibited numerous vacuoles with cytoplasmic content; most of them were clearly identified as being autophagosomes (double membrane visible). The AML cell line NB4 was used for comparison. In each cell population, the numbers of vacuoles per cell (means ± SEM) are presented. Representative images are shown in Figure S1. * $P < 0.05$. **(B)** Confocal microscopy. In control NB4 cells, LC3 (red) and ATG5 (green) showed a weak cytosolic distribution. In contrast, adult stem cells of all three lineages exhibited punctated LC3 and ATG5 staining patterns. Lower left corner: Magnification of one cell. Bars, 10 μm. Additional controls are shown in Supplementary information, Figure S2. **(C)** Immunoblottings. Stem cells expressed almost exclusively LC3-II and no p62. Upon differentiation, however, the resulting immature cells expressed cytosolic LC3-I and p62, as well as less LC3-II. ATG5 and Beclin1 were detectable in all cells and their expression often declined upon induction of differentiation, perhaps due to reduced transcription (Supplementary information, Figure S3). **(D)** Colony-forming cell assays. Stem cells were counted for colony-forming efficiency analysis. Note that 3-MA and ATG5 shRNA treatment occurred within 24 h before and not during the assay. Values are means ± SEM. of at least three independent experiments in each stem cell population. **(E)** Differentiation assays. Stem cells were cultured in differentiation media to obtain keratinocytes, fibroblasts, and neutrophils, respectively. Differentiation was additionally controlled by analyzing lineage-specific markers in each experiment (Supplementary information, Figure S5). 3-MA and ATG5 shRNA pretreatment occurred 24 h before and not during the assay. ATG5 shRNA reduced ATG5 protein levels by 55%-75% (Supplementary information, Figure S4). **(F)** Cell death assays. Cells were cultured in the presence and absence of etoposide (10 μM, 24 h). Values are means ± SEM. of at least three independent experiments in each stem cell population. Concentration-dependent effects are shown in Supplementary information, Figure S6. **(G)** Cell death assays. Cells were pretreated with 3-MA and ATG5 shRNA, respectively, and then cultured in the presence and absence of etoposide (10 μM, 24 h). Values are means ± SEM of at least three independent experiments in each stem cell population. Etoposide-induced cell death was mostly apoptosis (Supplementary information, Figure S7). **(H)** Colony-forming cell assays. Stem cells were counted for colony-forming efficiency analysis in the presence, absence, and following removal of etoposide (10 μM). The colonies after etoposide withdrawal were replated (R). Colony-forming efficiency after replating usually increased when skin stem cells were analyzed, but was somewhat lower in case of HSC. Values are means ± SEM. of at least three independent experiments in each stem cell population. **(I)** Differentiation assays. Stem cells were cultured in differentiation media to obtain immature keratinocytes, fibroblasts, and neutrophils, respectively, in the presence, absence, and following removal of etoposide (10 μM). Cells were investigated regarding the expression of lineage-associated markers (cytokeratin 10, fibroblast specific protein (FSP), and CD15, respectively) by confocal microscopy. The indicated immature cells were able to fully mature as assessed by light microscopy (data not shown). Representative data of at least three independent experiments in each cell population are shown. Bars, 10 μm.

cells for their self-renewal completely abolished this characteristic capacity of stem cells (Figure 1D). ATG5 has been shown to be essential for autophagy. Therefore, to specifically reduce autophagic activity, we blocked ATG5 expression by using ATG5 shRNA (Supplementary information, Figure S4), and again observed lack of self-renewal capacity in all three adult stem cell systems (Figure 1D). For differentiation analysis, ESC were differentiated to keratinocytes, DSC to fibroblasts, and HSC to neutrophils by adding appropriate differentiation factors *in vitro*. Inhibition of autophagy in the adult stem cells resulted in a complete block of differentiation in all systems (Figure 1E and Supplementary information, Figure S5). Taken together, lowering the autophagic activity of adult stem cells of the skin and blood leads to a loss of their self-renewal and differentiation capacities, and, therefore, under such conditions, these cells no longer exhibit their stemness properties.

Independent of the trigger, the high autophagic activity within adult stem cells may ensure their survival under conditions of tissue damage and organ dysfunction, allowing for subsequent repair. To test this hypothesis, we exposed ESC, DSC, and HSC to the cytotoxic agent etoposide (range 1-50 M) and observed death resistance, particularly in ESC and DSC (Figure 1F and Supplementary information, Figure S6). In contrast, as soon as differentiation was induced, cells acquired susceptibility to undergo etoposide-induced death (Figure 1F). Subsequently, we investigated the effect of blocking autophagy. Whereas 3-MA and ATG5 shRNA alone had no effect on the viability of ESC and DSC, HSC were partially susceptible (Figure 1G). Moreover, under conditions of autophagy blockade, ESC, DSC, and HSC underwent significant etoposide-induced apoptotic cell death (Figure 1G and Supplementary information, Figure S7). These data suggest that autophagy promotes survival of adult stem cells, even when they are stressed by a cytotoxic drug. Moreover, etoposide-exposed stem cells were able to self-renew and to differentiate after removal but not in the presence of the stressor (Figure 1H and 1I). Similar results were observed when cells were stressed with

doxorubicin or UV light (data not shown).

Although it was recently shown that autophagy is required for the maintenance of mouse HSC [1, 2], it remained unclear whether other adult stem cells would also depend on intact autophagic pathways. The data reported here point to the possibility that autophagy represents an essential process that ensures the maintenance of adult stem cells in general. Moreover, we demonstrate for the first time that adult human stem cells exhibit a higher autophagic activity compared with cells that were differentiated from them.

Interestingly, the high autophagic activity in adult stem cells largely contributes to cytotoxic drug resistance, a long-known phenomenon of these cells. Moreover, as the results of this study suggest, adult stem cells appear to be not only able to tolerate a certain degree of stress but also to be ready to initiate regeneration and repair of tissues that were damaged as a consequence of cytotoxic treatment. Our observations are in agreement with recent reports demonstrating impaired production of lymphoid and myeloid progenitors in mice with autophagy-deficient HSC [1, 2]. However, in these previous experimental mouse studies, the defect in differentiation might have been due to insufficient stem cell maintenance. We started in our *in vitro* experiments with the same numbers of stem cells before initiating differentiation, directly pointing to the possibility that differentiation depends on an intact autophagic machinery.

The high activity of autophagy in adult stem cells reported here may suggest unfavorable environmental and experimental conditions, respectively. On the other hand, during differentiation, when cells are exposed to additional growth factors, high mTOR activity might lower autophagic activity. While it could be argued that the culture conditions of the epidermal and dermal stem cells were suboptimal, the HSC in this study were freshly isolated and immediately analyzed. Moreover, both ESC and DSC received appropriate growth factors and were part of spheres, which are believed to mimic the stem cell niche [7], and could be cultured over months, suggesting optimal culture conditions. Therefore, our data point to the possibility that the microenvironment within stem cell niches is limited under *in vivo* (and perhaps *in vitro*) conditions, driving the process of autophagy in stem cells.

In conclusion, in contrast to differentiated cells, where autophagy is usually induced as a consequence of stress, high autophagic activity is a general phenomenon of adult skin and blood stem cells under physiological conditions. It may represent an immediately available safety mechanism to ensure cell repair and subsequent survival under stress conditions, including anticancer therapy. Moreover, high levels of autophagy in adult stem cells could help to prevent cancer and may also explain why at least a proportion of adult stem cells can survive during transplantation procedures before they find their niche and engraft.

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(**Supplementary information** is linked to the online version of the paper on the *Cell Research* website.)