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Targeted deletion of Atg5 reveals differential roles of autophagy in keratin K5-expressing epithelia

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ABSTRACT

Autophagy contributes to the homeostasis of many tissues, yet its role in epithelia is incompletely understood. A recent report proposed that Atg5-dependent autophagy in thymic epithelial cells is essential for their function in the negative selection of self-reactive T-cells and, thus, for the suppression of tissue inflammation. Here we crossed mice carrying floxed alleles of the Atg5 gene with mice expressing the Cre recombinase under the control of the keratin K5 promoter to suppress autophagy in all K5-positive epithelia. The efficiency of autophagy abrogation was confirmed by immunoanalyses of LC3, which was converted to the autophagy-associated LC3-II form in normal but not Atg5-deficient cells, and of p62, which accumulated in Atg5-deficient cells. Mice carrying the epithelium-specific deletion of Atg5 showed normal weight gain, absence of tissue inflammation, and a normal morphology of the thymic epithelium. By contrast, autophagy-deficient epithelial cells of the preputial gland showed aberrant eosinophilic staining in histology and premature degradation of nuclear DNA during terminal differentiation. Taken together, the results of this study suggest that autophagy is dispensable for the suppression of autoimmunity by thymic epithelial cells but essential for normal differentiation of the preputial gland in mice.

1. Introduction

Autophagy is the main mechanism by which cells degrade organelles and parts of their cytoplasm. The predominant mode of autophagy in mammalian tissues is macroautophagy [1] which we will refer to by using the term "autophagy" here. The sequestration of cytoplasmic components in vesicles (autophagosomes) and their delivery to lysosomes is controlled by autophagy-related genes (Atg). In the early phase of autophagy an isolation membrane is formed and microtubule-associated protein light chain 3 (LC3) is lipidated, which can be monitored by a shift in electrophoretic mobility from the LC3-I to the LC3-II band on Western blots [2]. p62/sequestosome 1 functions as an adaptor for the delivery of cytoplasmic proteins to the autophagosome. Together with its cargo, p62 is degraded in lysosomes. Inactivation of autophagy-related genes such as Atg5 blocks autophagy and leads to the accumulation of LC3-I and p62 [2–4].

The physiological roles of autophagy have been studied, amongst other approaches, by deletion of Atg5 in the mouse. Constitutive deletion of Atg5 is lethal on the first day after birth, presumably due to lack of amino acids in the neonatal starvation period [5]. The generation of mice carrying floxed (flanked by loxP sites) Atg5 alleles, which can be deleted by the introduction of the Cre recombinase under the control of tissue-specific promoters, has facilitated the investigation of autophagy functions in distinct tissues [6]. For example, deletion of Atg5 by Mx1-Cre and nestin-Cre caused severe liver injury and nerve degeneration, respectively [6].

A novel role of autophagy was recently suggested on the basis of a study in which the thymus of Atg5-deficient embryos was transplanted under the kidney capsule of wildtype adult mice [7]. Although thymus grafts from autophagy-deficient embryos were smaller than those from wildtype embryos, they showed normal morphology with distinct cortical (K8-positive) and medullary (K5-positive) epithelia. Transplantation of Atg5-null thymi into athymic mice resulted in the aberrant activation of T cells, severe

Abbreviations: GFP, green fluorescence protein; LC3, microtubule-associated protein light chain 3; RT-PCR, reverse-transcription polymerase chain reaction; H&E, hematoxylin and eosin; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labeling.

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inflammation of multiple organs and massive weight loss [7]. Ultimately, the recipients of an Atg5-deficient thymus but not those of a wildtype thymus had to be sacrificed due to the deleterious symptoms of autoimmune disease. Klein and colleagues proposed that autophagy contributes to the delivery of self antigens to the MHC class II compartments in the thymic epithelium and that this process is essential for the negative selection of T cells and for the suppression of autoimmunity [8,9]. This concept was challenged by our recent report that suppression of autophagy by deletion of the essential autophagy-related gene Atg7 in the thymic epithelium does not cause autoimmunity-mediated tissue inflammation [10].

Here we have tested the function of Atg5-dependent autophagy in the epithelia of the thymus and other organs by deleting Atg5 using the Cre-loxP system. The Cre recombinase was expressed under the control of the keratin K5 promoter which is active in the epithelium of the thymic medulla, i.e. the site of presentation of self antigens to T cells [9] as well as in other epithelia. In contrast to the experimental system of Nedjic et al. [7], our epithelium-specific approach of deleting Atg5 did not result in a phenotype indicative of aberrant autoimmunity. Rather, our study reveals a previously unknown crucial role of autophagy in the differentiation process of K5-positive cells within the murine preputial glands.

2. Materials and methods

2.1. Mice and cells

ATG5-floxed mice have been described previously [6]. These mice were crossed with mice expressing the Cre recombinase under the control of the K5 promoter [11] to yield Atg5 f/f and Atg5 f/f K5-Cre mice. The mice were maintained in a regular 12 h light–dark cycle on a normal diet with free access to drinking water. Keratinocytes were isolated from the tail of mice according to a published protocol [12] and cultured in 12-well plates coated with collagen IV (Vitrogen) using keratinocyte growth medium (Lonza).

2.2. Reverse-transcription polymerase chain reaction (RT-PCR)

RNA was prepared from cells using the TRIZOL reagent (Invitrogen) and reverse-transcribed with iScript cDNA Synthesis Kit (Bio-rad). The resulting cDNAs were subjected to PCRs with the primer pairs mKrt5-s, 5′-GAAGGAGTTGGACCAGTCAAC-3′ and mKrt5-a, 5′-AGGGTCTTGCAGCAGGCTCT-3′ to amplify keratin 5, Cre-s, 5′-ATGCTTCTGTCCGTTTGCCG-3′ and Cre-a, 5′-CCTGTTTTGCACGTTCA CCG-3′ to amplify Cre recombinase [13] and mAtg5-s, 5′-GCCTATA TGTACTGCTTCATCCA-3′ and mAtg5-a, 5′-CATTTCAGGGGTGTGC CTTCA-3′ to amplify Atg5.

2.3. Weight of body and organs

Immediately after the mice were sacrificed, the body weight was measured. Thymus, spleen and preputial glands were prepared and weighed. The weights and the ratios of organ weight to body weight were compared between groups of Atg5 f/f and Atg5 f/f K5-Cre mice of the same sex and age. The *t*-test was used for statistics, and p-values smaller than 0.05 were considered significant.

2.4. Western blot analysis

Tissue samples were lysed in a buffer containing 50 mM Tris (pH 7.4), 2% SDS and complete protease inhibitor cocktail (Sigma). After sonication and removal of insoluble debris, the protein concentration was determined by the BCA method (Pierce). Equal amounts of soluble protein were electrophoresed through SDS–polyacrylamide gels and analyzed by Western blot using first step antibodies against p62 (rabbit polyclonal antibody, Enzo Life-science, 1:2000) and LC3 (rabbit polyclonal antibody, GeneTex, 1:2000) was performed as described previously [10]. Equal loading of the lanes was confirmed by Ponceau staining of the membranes.

2.5. Immunofluorescence analysis

Tissues were fixed in phosphate-buffered 7.5% formaldehyde for 24 h, embedded in paraffin and thin-sectioned. Immunofluorescence labeling was performed as described previously [10] using first step antibodies against keratin K5 (rabbit polyclonal antiserum, Covance, 1:1000), keratin K8 (rat antibody TROMA-1, Developmental Studies Hybridoma Bank, 1:800), and p62 (affinity-purified rabbit antiserum PM045, MBL, 1:2000). Isotpye controls confirmed the specifity of the first step antibodies. Nuclear DNA was labeled with Hoechst 33258 (Molecular Probes).

2.6. Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL)

DNA fragments with free 3′-OH ends were detected using the in situ cell death detection kit (Roche) according to the manufacturer's instructions [14]. In addition, DNA was labeled with the fluorescent dye Hoechst 33258 on thin sections of tissues. For determining the number of TUNEL-positive nuclei per area, images were analyzed with the ImageJ software.

2.7. Histopathology

Inflammatory changes of tissues were assessed and scored as described previously for Atg7 f/f K14-Cre mice [10]. In brief, organs were fixed in phosphate-buffered 7.5% formaldehyde, embedded in paraffin, thin-sectioned at a thickness of 5 μm and stained with hematoxylin and eosin (H&E). Blinded scoring of tissue inflammation [15] was performed by an experienced pathologist. Scoring by a second investigator yielded essentially the same results. Scores 0, 1, and 2 indicated the absence of inflammatory infiltrates, the presence of small inflammatory infiltrates, and the presence of large inflammatory infiltrates, respectively. Score 3 indicated "manifest signs of tissue destruction" [7], however, this phenotype was not observed.

3. Results

3.1. Deletion of ATG5 leads to suppression of autophagy in keratin K5-expressing cells

Atg5-floxed mice [6] and K5-Cre mice were crossed to delete Atg5 in K5-expressing cells and their progeny. In contrast to mice with a systemic deletion of Atg5 [5], Atg5 f/f K5-Cre mice survived the perinatal period and had a macroscopically normal appearance. To test the efficiency of the gene deletion system, keratinocytes were isolated from Atg5 f/f K5-Cre mice and, for comparison, from Atg5 f/f mice lacking the K5-Cre transgene. RT-PCR confirmed that cells from both sources expressed K5 and only cells from Atg5 f/f K5-Cre mice expressed the Cre recombinase (Fig. 1). Atg5 was expressed in the Atg5 f/f cells whereas it was completely abolished in cells expressing Cre (Fig. 1). Western blot analysis of protein lysates from these cells showed that Atg5 f/f cells contained both LC3-I and LC3-II, indicative of constitutively active autophagy (Fig. 1). By contrast, Atg5 f/f K5-Cre keratinocytes lacked the autophagy-associated LC3-II form and contained a strongly

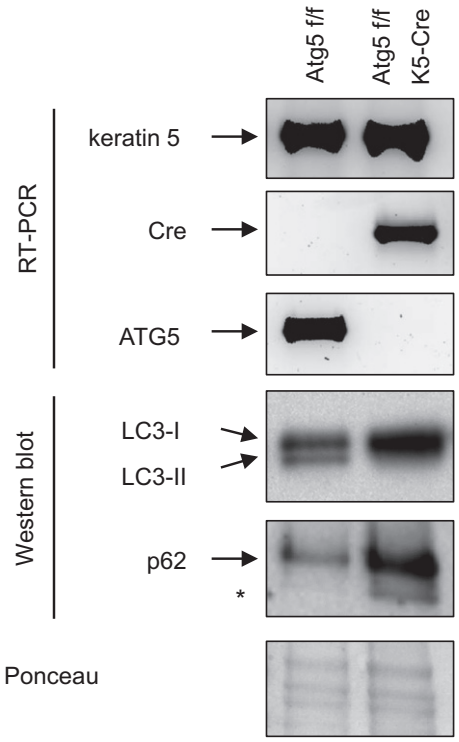

Fig. 1. Suppression of autophagy by deletion of ATG5 in keratin K5-expressing cells. Keratinocytes were isolated from the tail epidermis of ATG5 f/f and ATG5 f/f K5-Cre mice. RT-PCR analysis was performed with primers specific for keratin K5, Cre recombinase and ATG5. Protein lysates were electrophoresed through polyacrylamide gels and subjected to Western blot analysis using first step antibodies against LC3 and p62. An asterisk marks a band of unknown identity that is frequently detected in Western blot analysis using p62 antibodies [24]. Ponceau staining of the blotting membrane was used to confirm equal loading in the different lanes.

increased amount of LC3-I. Likewise, p62 accumulated in Atg5 f/f K5-Cre cells, suggesting that the autolysosomal degradation of both LC3 and p62 was prevented by deletion of Atg5.

3.2. Atg5 deletion in K5-positive epithelia does not disturb the morphology of the thymus nor body weight gain nor is it associated with inflammation in tissues

As the primary aim of the study was to explore the role of Atg5-dependent autophagy in the thymic epithelium, we next determined LC3 processing and the abundance of p62 in the thymus. In agreement with previous reports stating that autophagy is constitutively active in the thymic epithelium [16,10], LC3-II was readily detectable in protein lysates of Atg5 f/f thymi (Fig. 2A). By contrast, LC3-II was absent and increased amounts of LC3-I were present in Atg5 f/f K5-Cre thymus lysates, showing effective suppression of LC3 conversion in epithelial cells. These results are also in agreement with other articles in which little, if any, contribution of T cells to overall autophagic activity in the thymus was reported [16,10]. The abundance of p62 was increased in the thymus of Atg5 f/f K5-Cre mice relative to that of control mice. Together, these results confirmed that, like in isolated keratinocytes, autophagy was suppressed by the Cre-loxP system in the thymic epithelium.

Histological investigation showed that the thymus of Atg5 f/f K5-Cre had a normal organization in cortex and medulla (Fig. 2B), and immunofluorescence labeling confirmed normal expression of keratins K5 and K8 in thymic epithelial cells (Supplementary Fig. S1). H&E staining of other organs revealed absence or low abundance of infiltrating inflammatory cells in both Atg5 f/f and Atg5 f/f K5-Cre mice (Supplementary Fig. S2 and data not shown). Following the approach of Nedjic et al. [7], tissue inflammation was scored by H&E-stained sections of various organs. As shown in Fig. 2C, low inflammation scores were detected in liver, colon, lung, skin, harderian glands, and preputial glands of both Atg5 f/f and Atg5 f/f K5-Cre mice. Interestingly, the inflammation scores of the colon showed a tendency to be even lower in Atg5 f/f K5-Cre mice than in Atg5 f/f mice, but the significance of this difference needs further investigation. Consistently, the inflammatory infiltrates were much less abundant than those reported for mice receiving thymus transplants from Atg5-deficient embryos [7].

Similar to Atg7 f/f K14-Cre mice reported previously [10] and in contrast to mice receiving Atg5-deficient thymic transplants [7], female Atg5 f/f K5-Cre mice had fat pads of normal size and the diameter of the colon appeared normal in all mice investigated (Supplementary Fig. S2 and data not shown). The body weight (Fig. 2D) as well as the weights of the thymus and the spleen, both in absolute terms (not shown) and in relation to the body weight (Fig. 2E and F), did not differ significantly between Atg5 f/f and Atg5 f/f K5-Cre mice.

3.3. Atg5 deletion leads to aberrant differentiation of preputial gland cells

During H&E screening of organs, we noted apparently inflammation-independent changes in the preputial glands of male Atg5 f/f K5-Cre mice which prompted us to perform a more detailed investigation of these organs. Preputial glands of male mice are located next to the penis, show a similar structure as sebaceous glands and produce a sebum-like secretion supposedly containing pheromones [15,17]. Immunofluorescence labeling showed that the cells in the basal layer of preputial gland acini express K5 (Fig. 3A), suggesting that the presence of a K5-Cre transgene leads to the deletion of the floxed Atg5 gene in these cells. When cells start terminal differentiation in suprabasal positions, K5 immunoreactivity is largely lost (Fig. 3A). p62/sequestosome 1 was detected by immunofluorescence labeling in all epithelial cells of the preputial gland (Fig. 3B). Distinct areas of Atg5 f/f K5-Cre preputial glands showed elevated abundance of p62 (arrowheads in Fig. 3B).

H&E staining showed progressive differentiation from the basal cell layer towards the site of holocrine secretion into the ducts of the glands (Fig. 3C). In Atg5 f/f glands the cells retained a distinct affinity to hematoxylin (blue) and showed pyknotic nuclei over multiple layers of differentiating cells (Fig. 3C, left panel). By contrast, sections of Atg5 f/f K5-Cre preputial glands were much more strongly stained red by eosin, and cells containing hematoxylin-stained pyknotic nuclei were much less abundant (Fig. 3C, right panel). These features were quantitively assessed in preputial gland acini of 6 mice of each genotype. 44 ± 7% (mean ± standard deviation) of the area of longitudinally sectioned Atg5 f/f K5-Cre acini lacked the normal blue hematoxylin staining and showed aberrantly strong red eosin staining whereas none of the Atg5 f/f acini showed this staining pattern. In Atg5 f/f K5-Cre preputial gland acini, 53 ± 3% of the acinus area lacked a blue nuclear staining whereas only 20 ± 3% of the area of Atg f/f preputial gland acini were free of nuclear remnants. This difference was highly significant ($p < 0.001$, t-test). The total area of the investigated acini was slightly but not significantly larger in sections of Atg5 f/f K5-Cre preputial glands than their Atg5 f/f counterparts (not shown). The weights of preputial glands did not differ between Atg5 f/f and Atg5 f/f K5-Cre mice.

As H&E staining revealed an increase of anucleate cell remnants in the preputial glands of Atg5 f/f K5-Cre mice, we next investigated the process of nuclear DNA degradation in situ. The TUNEL assay was used to detect DNA fragments with free 3′-OH ends.

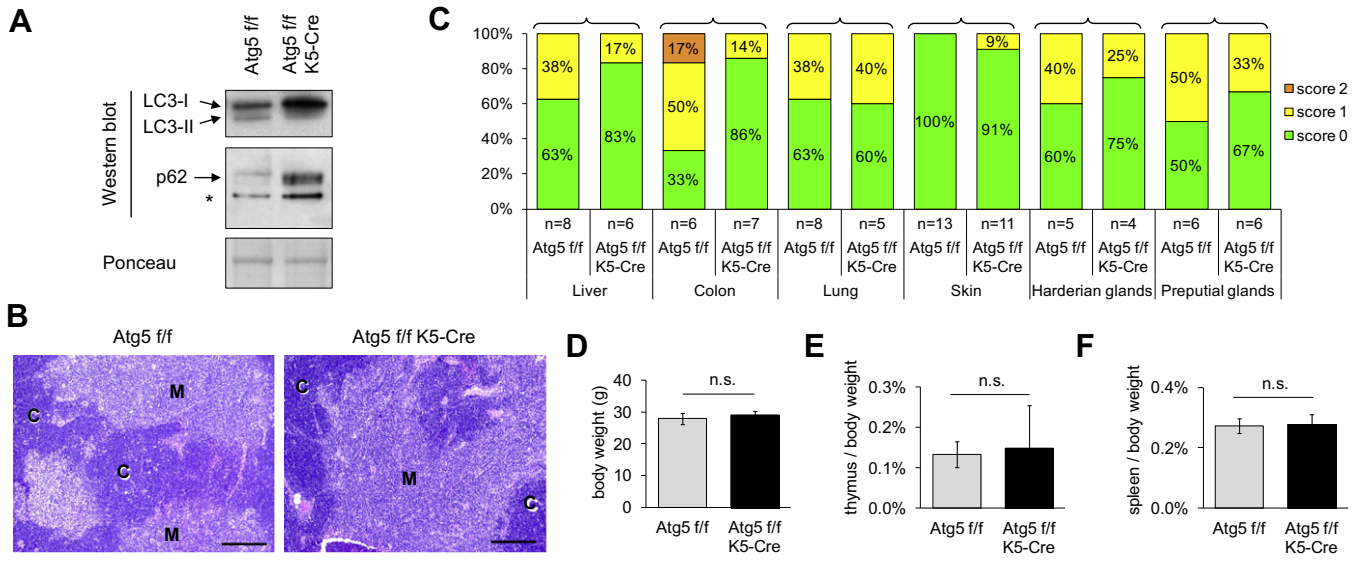

Fig. 2. ATG5 deletion in K5-positive epithelia does not affect the morphology of the thymus and is not associated with aberrant inflammation of other organs. (A) Western blot analysis of protein lysates from the thymus of ATG5 f/f and ATG5 f/f K5-Cre mice. Antibodies against LC3 and p62 were used as first step reagents. An asterisk marks a band of unknown identity that is frequently detected in Western blot analysis using p62 antibodies [24]. Ponceau staining of the membrane is shown to confirm equal loading of each lane. (B) H&E staining of representative thin sections of the thymus. Bars = 200 μm. (C) H&E-stained sections of organs of mice expressing ATG5 (ATG5 f/f) or lacking ATG5 in the thymic epithelium were scored as described in Section 2. The green, yellow, and orange portions of the bars show the percentage of mice without inflammation, with mild and with moderate inflammation, respectively. The number of mice investigated is shown below each bar. (D–F) The weights of the body (D), the thymus and the spleen were determined for male mice in the age range from 6 to 12 months. Weights of the thymus and the spleen are shown in percent of the body weight (E, F). n.s., non-significant difference (*t*-test). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Such fragments are generated, for example, by caspase-activated DNase during apoptosis [18] and by DNase1L2 during terminal differentiation of keratinocytes [19]. However, DNA can also be degraded without the appearance of TUNEL-positive intermediates [20]. When autophagy-competent preputial glands were subjected to TUNEL, distinct signals were obtained in differentiating cells (Fig. 3D, left panel). Both the intensities of the DNA fragment-specific TUNEL signal and of Hoechst dye labeling of total DNA decreased progressively during terminal differentiation. By contrast, only few TUNEL-positive nuclei were detected in Atg5 f/f K5-Cre preputial glands and, in line with the H&E stainings, more distal cells in longitudinally sectioned acini lacked Hoechst-labeled DNA (Fig. 3D, left panel). Quantitative analysis confirmed that the deletion of Atg5 led to a significant decrease in the frequency of TUNEL-positive nuclei relative to the area of the preputial gland acinus (Fig. 3E).

4. Discussion

This study sheds new light on the roles of autophagy in epithelia. Previous studies of autophagy in the thymic epithelium have yielded contradictory results. The transplantation of the thymus from Atg5-deficient embryos to adult athmyic mice caused severe autoimmunity which led the authors of that study to conclude that autophagy in the thymic epithelium is essential for the presentation of self antigens and suppression of autoimmunity [7]. Recently, we have shown that abrogation of autophagy in the thymic epithelium by targeted deletion of Atg7 using K14 promoter-driven Cre recombinase does not cause signs of autoimmunity, suggesting that autophagy in the thymic epithelium is dispensable for the development of self-tolerance [10]. One of the differences between these studies was the choice of the autophagy-related gene to be deleted, namely Atg5 or Atg7. In the present study we have suppressed epithelial autophagy by deletion of the same gene, i.e. Atg5, as in the study of Nedjic et al. [7], yet this approach also did not lead to increased tissue inflammation in mutant animals, so that the results of the present study are in full agreement with those obtained by deletion of Atg7 in the thymic epithelium [10]. Thus, the apparently essential role of Atg5 in the experimental system of Nedjic et al. [7] may have been caused by transplantation of non-epithelial thymus cells, by autophagy deficiency together with the environment of the kidney capsule into which the thymus was grafted, or by stress associated with the transplantation procedure. Considering these potential confounding factors in the study by Nedjic et al. [7], the effects observed by the cell type-specific abrogation of autophagy by deletion of either Atg7 [10] or Atg5 (this study) suggest that the thymic epithelium does not require autophagy for its function in the suppression of autoimmunity.

Another main finding of this study is the disturbance of preputial gland cell differentiation by the suppression of autophagy. Like terminal differentiation of epidermal keratinocytes [21,22], the differentiation of preputial gland cells represents a mode of programmed cell death in which nuclear DNA is degraded. While cornification-associated cell death was apparently normal in the epidermis of Atg5 f/f K5-Cre mice (our unpublished data), the preputial glands of these mice had a reduced frequency of differentiating cells containing nuclear remnants but retained more anucleate cells in the distal portion of preputial gland acini. The zone of cells undergoing nuclear breakdown was narrower and DNA fragments with TUNEL-positive 3′-OH ends were less abundant in Atg5 f/f K5-Cre mice. These findings indicate that the nuclear breakdown of preputial gland cells is initiated earlier and completed more rapidly in the absence of autophagy. In contrast to the concept of an effector role of autophagy in cell death [23], our data thus argue for a role of autophagy in preventing premature cell demise during differentiation. Our additional finding of aberrant H&E staining of the cytoplasm of terminally differentiated preputial gland cells in Atg5 f/f K5-Cre mice indicates the existence of cytoplasmic alterations that are caused by the lack of Atg5-dependent autophagy. It is conceivable, that these changes are associated with the retention of more anucleate cells in acini of Atg5 f/f K5-Cre mice. Further studies will

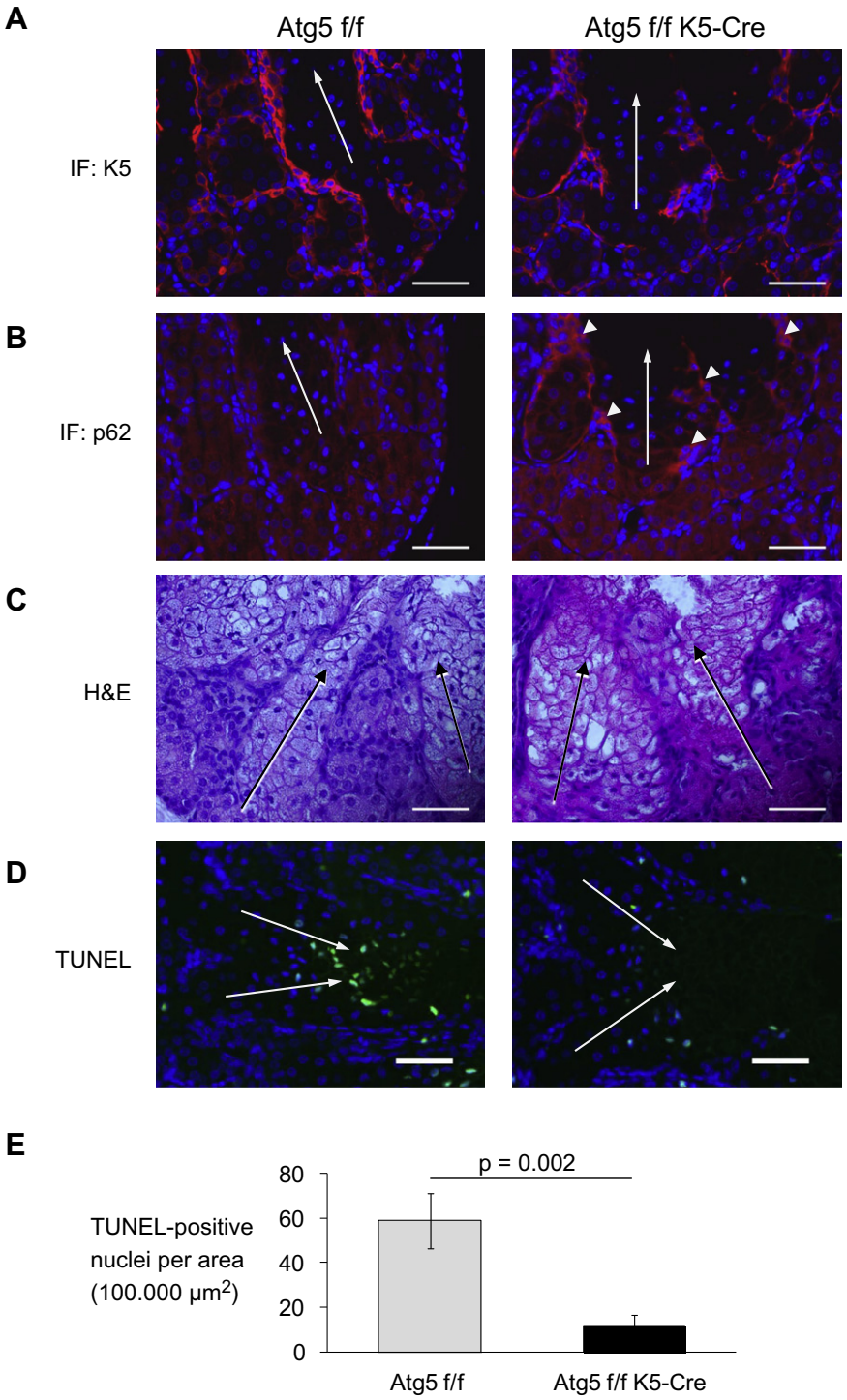

Fig. 3. Deletion of ATG5 leads to aberrant differentiation of preputial gland cells. Preputial glands of ATG5 f/f and ATG5 f/f K5-Cre mice were subjected to immunofluoresence (IF) analysis for keratin K5 (red) (A) and p62 (red) (B) (A and B shows serial sections for each genotype), stained with H&E (C), and subjected to TUNEL (green) (D). DNA was labeled with Hoechst 33258 (blue) in A, B, and D. Bars = 50 μm. Arrows indicate the direction of cell differentiation. Arrowheads in B indicate sites of p62 accumulation. (E) TUNEL-positive nuclei and nuclear remnants were counted in longitudinally sectioned acini from four mice of each genotype. The columns show the number of TUNEL-positive nuclei per 10^5 square micrometers of acinar area. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

address the molecular links between autophagy and cell differentiation in the preputial gland.

In summary, the results of this study demonstrate that autophagy is, at least under non-stressed conditions, dispensable for the immunomodulatory function of the K5-positive thymic epithelium but essential for normal preputial gland differentiation.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2012.11.090.

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