

FGF7/KGF regulates autophagy in keratinocytes

A novel dual role in the induction of both assembly and turnover of autophagosomes

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Abbreviations: 3-MA, 3-methyladenine; ACTB, actin, beta; AKTi, AKT inhibitor; ATG5, autophagy-related 5; BECN1, Beclin 1, autophagy-related; DAPI, 4',6-diamidino-2-phenylindole; EGF, epidermal growth factor; EGFP, enhanced green fluorescent protein; ER, endoplasmic reticulum; FGF2, fibroblast growth factor 2 (basic); FGFR2/KGFR, fibroblast growth factor receptor 2; FGF7/KGF, fibroblast growth factor 7; HaCaT, human adult skin keratinocytes propagated under low calcium conditions and elevated temperature; HKs, human keratinocytes; IGF1, insulin-like growth factor 1 (somatomedin C); KRT1/K1, keratin 1; LAMP2, lysosomal-associated membrane protein 2; MAP1LC3/LC3, microtubule-associated protein 1 light chain 3; MAPK/ERK, mitogen-activated protein kinase; MAP2K/MEK, mitogen-activated protein kinase kinase; MTOR, mechanistic target of rapamycin; NS, not significant; pAKT, phospho-AKT; PDGF, platelet-derived growth factor; PI3K, class I phosphoinositide 3 kinase; PtdIns3K, class III phosphatidylinositol 3-kinase; RAP, rapamycin; SD, standard deviation; SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis; SE, standard error; siRNA, small interfering RNA; SQSTM1, sequestosome 1; TG, thapsigargin

Autophagy is a degradative pathway through which cells overcome stressful conditions and rapidly change their phenotype during differentiation. Despite its protective role, when exacerbated, autophagy may lead to cell death. Several growth factors involved in cell survival and in preventing differentiation are able to inhibit autophagy. Here we investigated the autophagic role of FGF7/KGF, an important player in epithelial cell protection and differentiation. Biochemical and quantitative fluorescence approaches showed that FGF7 and its signaling induce autophagy in human keratinocytes and the use of specific inhibitors indicated that this effect is independent of the PI3K-AKT-MTOR pathway. The selective block of autophagosome-to-lysosome fusion clarified that FGF7 induces autophagy stimulating autophagosome formation. However, quantitative fluorescence approaches also indicated that, upon a prolonged autophagic stimulus, FGF7 is able to accelerate autophagosome turnover. Moreover, in differentiating keratinocytes, the use of the autophagic inhibitor 3-MA as well as the depletion of BECN1 and ATG5, 2 essential regulators of the process, counteracted the FGF7-induced increase of the differentiation marker KRT1/K1, suggesting that autophagy is required for the FGF7-mediated early differentiation. These results provide the first evidence of a role of FGF7 in the regulation of sequential steps of the autophagic process and strengthen the hypothesis of a direct interplay between autophagy and differentiation. On the other hand, the ability of FGF7 to accelerate autophagosome turnover, preventing their dangerous accumulation, is consistent with the well-established protective role played by the growth factor in epithelial cells.

Introduction

Macroautophagy is a finely regulated “self-digestion” pathway which mediates protein, macromolecule, and organelle turnover, inducing their lysosomal degradation. The process is initiated by vesicle fusion that generates closed double-membrane organelles, the autophagosomes, in which cytoplasmic components remain sequestered. Subsequently, the autophagosomes fuse with lysosomes to form autolysosomes in which all contents

are enzymatically digested and the resulting biomolecules are recycled back to the cytoplasm.¹⁻³ Autophagy constitutively occurs at very low levels, but it can be enhanced by endogenous as well as exogenous cellular stresses, such as nutrient starvation, allowing cell survival thanks to a rapid adaptation to the microenvironmental changes.⁴ However, even if autophagy is a predominantly cytoprotective process, a nonphysiological exacerbated autophagic stimulus may trigger autophagosomal clustering and accumulation leading to an autophagy-dependent

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cell death.^{3,5} Many growth factors involved in cell survival, such as FGF2,^{6,7} IGF1 and EGF,^{8,9} or PDGF¹⁰ negatively regulate autophagy prevalently through the activation of the PI3K-AKT-MTOR signaling pathway. Interestingly, FGF2 prevents premature differentiation of cardiomyocyte progenitors blocking autophagy,⁷ providing the important evidence that autophagy is directly correlated with cell differentiation and that a growth factor can affect cellular differentiation by affecting autophagy. In the past few years, the use of several inhibitors of the fusion of autophagosomes with lysosomes has made it possible to demonstrate that autophagy can be regulated at different steps. Using this approach, it has been demonstrated that FGF2 inhibits the autophagic process blocking autophagosome formation,⁶ while the inhibitory effect of IGF1 is essentially due to its ability to accelerate autophagic turnover.⁹ However, since the physiological effect of growth factors can be affected by the endocytic trafficking of their activated receptors, the use of nonspecific inhibitors blocking both endosomal and autophagosomal trafficking, such as bafilomycin A₁, was found to be not suitable. Recently a specific inhibition of the autophagic flux was obtained with thapsigargin (TG), an inhibitor of endoplasmic reticulum (ER) Ca²⁺-ATPase pump family,¹¹ which irreversibly and selectively blocks autophagosome-lysosome fusion without interfering with the endocytic pathway.¹² Ganley and coworkers demonstrated that the accumulation of clustered autophagosomes upon TG treatment, previously interpreted as an exclusive consequence of the ER stress-dependent induction of autophagy,¹³⁻¹⁶ was indeed prevalently due to the inhibition of the autophagosome confluence in lysosomes.¹² Therefore, inhibition with TG can represent a powerful tool to investigate the effects of growth factors on the different autophagic steps.

The keratinocyte growth factor FGF7/KGF, secreted by mesenchymal cells, regulates epithelial cell differentiation,¹⁷ survival,¹⁸⁻²⁰ and motility.^{21,22} FGF7 specifically binds and activates the FGFR2/KGFR (fibroblast growth factor receptor 2) isoform b,²³ a splicing transcript variant exclusively expressed on epithelial cells.²⁴ The possible crucial role of FGF7 and its receptor in epithelial cell homeostasis has been widely proposed.²⁵⁻²⁷ In particular, we have recently demonstrated that the FGF7-mediated activation of FGFR2 triggers early differentiation in keratinocytes during the switch from undifferentiated to differentiating cells,²⁸ the same step in which it has been recently proposed that autophagy might represent a

crucial event for cell commitment to differentiation.²⁹ Moreover, FGFR2 expression and signaling regulate the phagocytic process in keratinocytes.³⁰ Based on these findings, we wondered if FGF7 may modulate the autophagic process in keratinocytes. Using TG as a selective, irreversible inhibitor of autophagosome flux toward lysosomes, we found that FGF7 is able to induce the formation of autophagosomes and accelerate their fusion with lysosomes. In addition, we demonstrated that the inductive effect of FGF7 on autophagy is required for the growth factor ability to trigger early differentiation. These results provide further support for the existence of a close relationship between autophagy and cell differentiation and demonstrate for the first time that FGF7 controls keratinocyte differentiation and survival also through a fine modulation of the autophagic process.

Results

FGF7 induces autophagy in keratinocytes

To determine whether FGF7 and its receptor might be involved in the regulation of autophagy in keratinocytes, the autophagic effect of serum deprivation was first analyzed in the human keratinocyte HaCaT cell line, spontaneously immortalized from a primary culture of keratinocytes.³¹ To this aim cells were subjected to a serum-starvation time course (4, 8, 24, and 48 h) and the levels of the 16-kDa membrane-associated microtubule associated protein 1 light chain 3-II (LC3-II) were analyzed by western blot and compared with those detected in control cells grown in complete medium. LC3-II is derived from the conversion of the cytosolic form of LC3, also called LC3-I, and represents a well-established marker for phagophores and autophagosomes.^{32,33} Equal loading was assessed using anti-ACTB (actin β) antibody and the densitometric analysis was performed as described in Materials and Methods. In agreement with previous evidence obtained in other epithelial cells,⁸ a significant increase of LC3-II levels was clearly evident only after 48 h of serum deficiency (Fig. 1A), suggesting that shorter times of serum-starvation are inefficient to induce autophagy; alternatively, autophagosome turnover could be so rapid in these cells, that it could make changes in LC3-II levels undetectable until 48 h. To more carefully check the autophagic flux in our cellular model, the levels of the well-known autophagy substrate SQSTM1/p62 (sequestosome 1) during the serum-starvation time course were analyzed by western blot: the specific band of

Figure 1 (See opposite page). Induction of the autophagic flux by serum deprivation in HaCaT cells. **(A)** HaCaT cells were left in complete medium or serum-starved for different times (4, 8, 24, and 48 h). Western blot analysis using anti-LC3 polyclonal antibodies showed a significant increase of the band at the molecular weight of 16 kDa corresponding to LC3-II after 48 h of serum deprivation. Equal loading was assessed with anti-ACTB antibody. For densitometric analysis the values from 3 independent experiments were normalized, expressed as fold increase and reported in graph as mean values ± standard deviation (SD). The Student *t* test was performed and significance levels have been defined as *P* < 0.05: **P* < 0.05 vs. the corresponding serum-cultured cells. **(B)** Western blot analysis using anti-SQSTM1 monoclonal antibody shows that the band at the level of 62 kDa corresponding to SQSTM1 significantly decreased upon 24 h and 48 h of serum-starvation; no significant changes were visible at shorter time points. The densitometric analysis and Student *t* test were performed and significance levels have been defined as above: **P* < 0.05 vs. the corresponding serum-cultured cells; ***P* < 0.01 vs. the corresponding serum-cultured cells. **(C)** HaCaT cells were transiently transfected with EGFP-LC3 (HaCaT EGFP-LC3) and then left in complete medium or serum-starved for different times (0.5, 1, 2, 4, 8, 24, and 48 h). Cells were then fixed, permeabilized, and nuclei were stained with DAPI. Quantitative fluorescence analysis showed that a significant increase of LC3-positive fluorescent dots was detectable at 24 h and 48 h of serum deprivation. The quantitative analysis was assessed as reported in Materials and Methods and results are expressed as mean values ± standard errors (SE). Student *t* test was performed and significance level has been defined as *P* < 0.05: *, ***P* < 0.001 vs. the corresponding serum-cultured cells; NS vs. the corresponding serum-cultured cells. Scale bar: 10 μm.

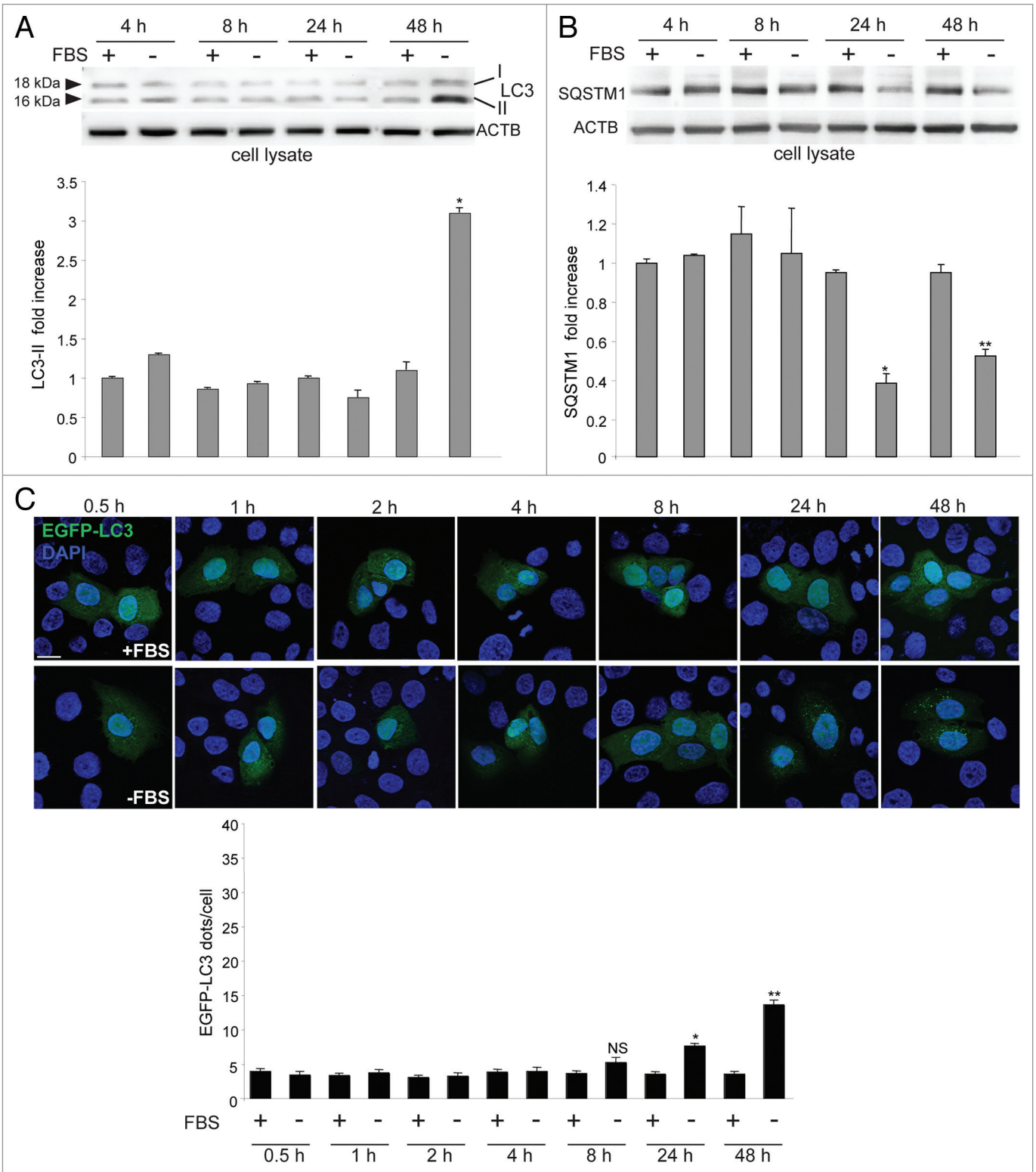


Figure 1. For figure legend, see page 804.

62 kDa corresponding to SQSTM1 was significantly decreased at 24 h and 48 h of starvation (Fig. 1B), while at shorter time points no significant changes were observed (Fig. 1B). Our results are in agreement with a previous study³⁴ reporting that the serum deprivation in human epithelial cell lines induces a slower autophagic response relative to other cell types.

Because the measurement of LC3-II protein levels by western blot analysis is not always the most sensitive system to follow autophagic flux,⁹ another widely accepted method was applied. HaCaT cells were transiently transfected with EGFP-LC3 (HaCaT EGFP-LC3) and then serum-starved for different time points, including less than 4 h (0.5, 1, 2, 4, 8, 24, and 48 h). Cells were then fixed, permeabilized, and nuclei were stained with DAPI. Autophagosome formation was assessed by quantitative fluorescence analysis as reported in Materials and Methods. A significant increase of fluorescent EGFP-LC3-positive dots was evident only after 24 h of serum deprivation, with a further increase after 48 h (Fig. 1C), confirming that in HaCaT cells the induction of autophagic flux is a quite slow phenomenon.

To investigate whether FGF7 treatment may affect autophagy, a serum-starvation time course was performed as above in the presence of saturating doses of FGF7 (100 ng/ml) and the LC3-II protein levels were compared by western blot analysis. The results showed that the addition of the growth factor induced a significant increase of LC3-II amount after 24 h (Fig. 2A); in contrast, the LC3-II levels appeared very high and comparable in FGF7-stimulated and unstimulated cells at 48 h (Fig. 2A), suggesting that the autophagic stimulus induced by serum deprivation could be so intense that it could make undetectable any possible additive effects due to FGF7. Consistent with these findings, the SQSTM1 levels appeared drastically decreased upon FGF7 treatment at both 24 h and 48 h (Fig. 2B). Thus, differently from other growth factors, such as FGF2, EGF, and IGF1, which have been shown to inhibit autophagy in various cellular contexts,^{6,8,9} FGF7 is able to induce the autophagic process in keratinocytes.

To visualize and to finely quantify the autophagy induced by FGF7, HaCaT EGFP-LC3 cells were serum-starved for 24 h or 48 h in the presence or absence of FGF7 as above. Quantitative fluorescence analysis showed that the addition of FGF7 significantly enhanced the number of LC3-positive dots per cell after either 24 h or 48 h (Fig. 2C, left and middle panels). To confirm the physiological role of FGF7 as an inducer of autophagy, parallel experiments were performed using primary cultures of normal human keratinocytes (HKs) grown in low Ca²⁺ medium (0.03 mM). HKs were transiently transfected with EGFP-LC3 and then serum-starved for 24 h in the absence or in the presence of FGF7 as above. Quantitative fluorescence analysis showed that FGF7 addition significantly increased the number of EGFP-LC3-positive dots per cell also in these primary cultures (Fig. 2C right panels), indicating that the role of FGF7 in the regulation of autophagy represents a physiological general phenomenon in keratinocytes.

It is well known that the autophagic process is inhibited by the PI3K-AKT signaling pathway, which induces the activation of MTOR, the main negative regulator of autophagy.² Alternatively,

MTOR may be also activated through PI3K-AKT-independent pathways.² Since PI3K-AKT is one of the multiple signaling pathways triggered by activated FGFR2,³⁵ we determined whether this pathway or the direct activation of MTOR could affect FGF7-mediated induction of autophagy. To this aim, HaCaT cells were stimulated with FGF7 in the presence or absence of a specific AKT inhibitor (AKTi) or the MTOR inhibitor rapamycin (RAP). Western blot analysis demonstrated that, while the inhibition of AKT phosphorylation was evident in cells treated with the specific inhibitor, but not in untreated or in rapamycin-treated cells (Fig. 3, right panel), none of these drugs were able to affect the increase of LC3-II protein triggered by FGF7 (Fig. 3, right panel). As a control, treatment with the inhibitors alone did not significantly change LC3-II levels (Fig. 3, left panel). These results indicate that FGF7 induction of autophagy is not significantly affected by PI3K-AKT signaling and MTOR activation.

FGF7 triggers the formation of autophagosomes

The increase of LC3-II protein levels usually depends on increased formation of autophagosomes or on their reduced turnover,^{36,37} but it may also occur when both processes are simultaneously active.³⁸ To investigate if FGF7 induces the assembly of the autophagosomes or it decreases their fusion with lysosomes, the degradation of the autophagosomes was selectively blocked by treating cells with high doses of thapsigargin (TG). In fact, even if TG has been considered an ER stressor able to induce autophagosome formation, very recently it has been found that this drug is primarily able to specifically block autophagosome-lysosome fusion without interfering with endocytosis.¹² Again, to evaluate serum deprivation-induced autophagic flux in the presence of TG, HaCaT EGFP-LC3 were pretreated for 1 h at 37 °C with TG and then serum-starved for different time points as above. Quantitative fluorescence analysis showed that after 8 h, the number of LC3-positive dots was significantly increased by TG (Fig. 4) and this increase became more evident after 24 h and 48 h (Fig. 4). Then, in order to determine which step of the autophagic process is affected by FGF7, HaCaT cells were pretreated for 1 h at 37 °C with TG and then serum-starved in the presence or absence of FGF7 for 24 h or 48 h. Western blot analysis clearly indicated that, independently from FGF7 stimulation, the treatment with TG caused an increase of the LC3-II protein levels (Fig. 5A). However, FGF7 addition significantly increased the LC3-II amount either in TG-treated or TG-untreated cells (Fig. 5A and B) indicating that FGF7 triggers the autophagosome formation. Again, as observed in the absence of TG treatment (Fig. 2A), a prolonged serum deprivation up to 48 h resulted in the induction of very high levels of LC3-II, which might hide any possible additive effect of FGF7 (Fig. 5B). Interestingly, the SQSTM1 protein levels revealed that, independently of FGF7 stimulation, the pretreatment with TG strongly increased the amount of this autophagic substrate (Fig. 5A) as a result of the irreversible block of the autophagosome-lysosome fusion and consequent inhibition of SQSTM1 degradation.

The nascent autophagosomes are small vesicles that become large vacuoles when they accumulate and fuse with each other in clusters upon a massive autophagic stimulus or, more

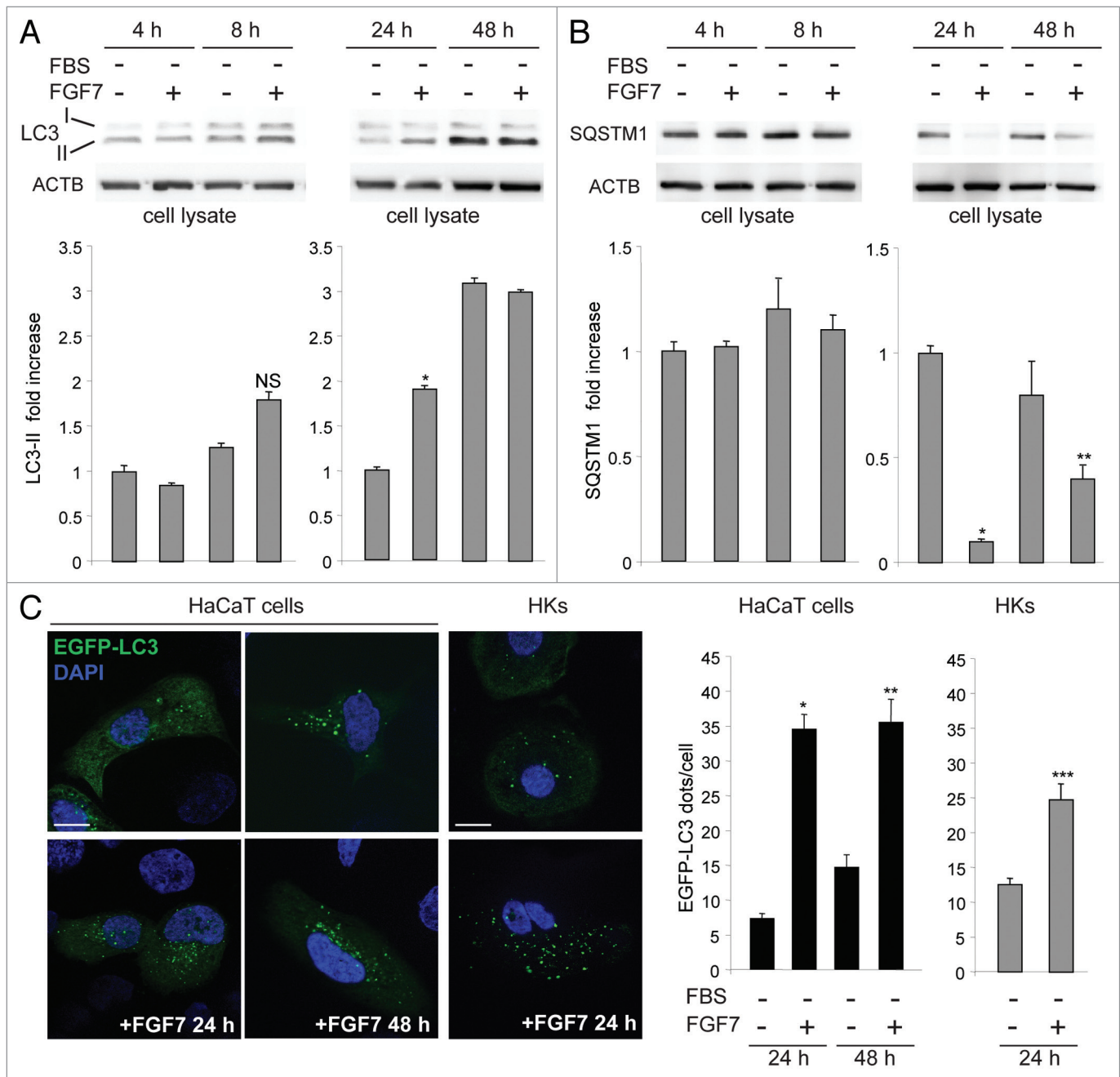


Figure 2. FGF7 induces autophagy in human keratinocytes. (A) HaCaT cells were serum-starved for different times (4, 8, 24, and 48 h) in the presence or absence of FGF7 (100 ng/ml). Western blot analysis showed that LC3-II levels were significantly increased by FGF7 after 24 h, while they appeared very high and comparable in FGF7-stimulated and FGF7-unstimulated cells after 48 h. The densitometric analysis and Student *t* test were performed and significance levels have been defined as above: NS vs. the corresponding serum-starved cells; **P* < 0.05 vs. the corresponding serum-starved cells. (B) Western blot analysis using anti-SQSTM1 monoclonal antibody showed that FGF7 decreased the level of SQSTM1 at 24 h and 48 h. The densitometric analysis and Student *t* test were performed and significance levels have been defined as above: **P* < 0.05 vs. the corresponding serum-starved cells. (C) HaCaT cells and normal human keratinocytes (HKs) grown in low calcium were transiently transfected with EGFP-LC3 and then serum-starved (24 h or 48 h) in the presence or absence of FGF7 as above. Cells were then fixed, permeabilized and nuclei were stained with DAPI. Quantitative fluorescence analysis showed that, after either 24 h or 48 h, FGF7 increased the number of EGFP-LC3-positive dots per cell in both HaCaT and HKs. The quantitative analysis was assessed as reported in Materials and Methods and results are expressed as mean values \pm standard errors (SE). Student *t* test was performed and significance level has been defined as *P* < 0.05; **P* < 0.001 vs. the corresponding unstimulated cells; ****P* < 0.005 vs. the corresponding unstimulated cells. Scale bar: 10 μ m.

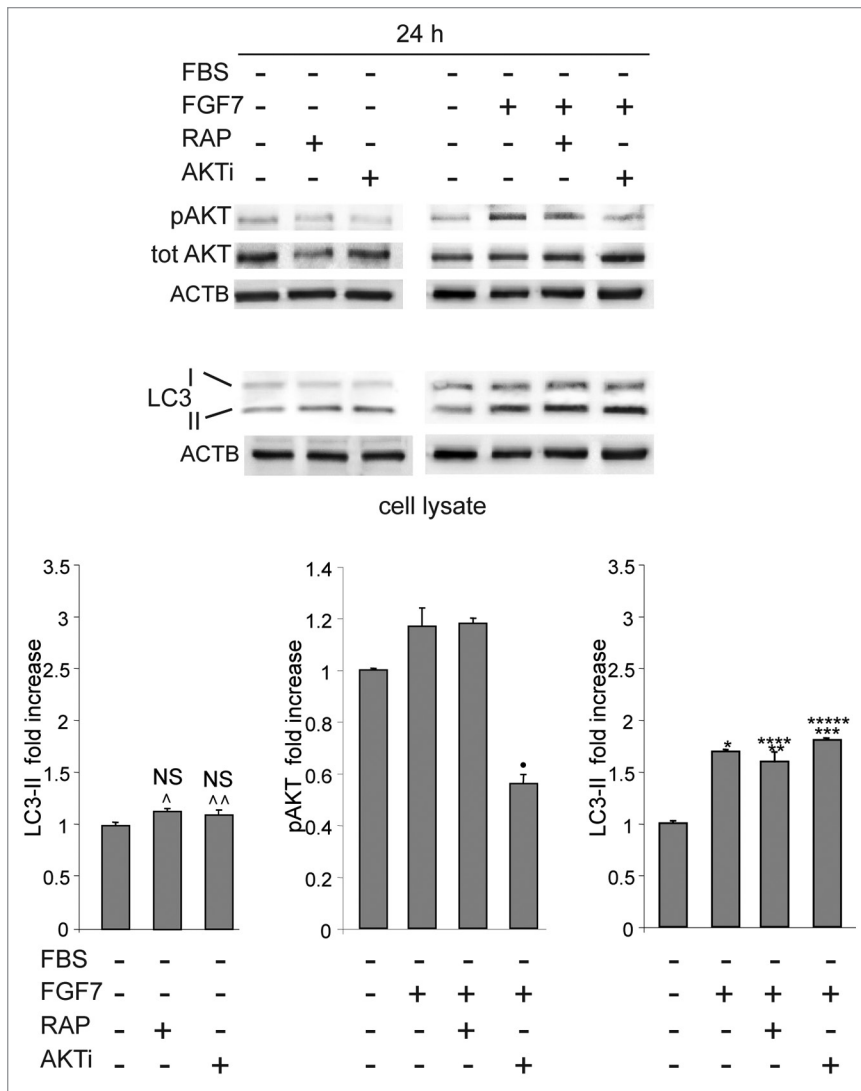


Figure 3. FGF7-mediated autophagy is not dependent on PI3K-AKT signaling or MTOR activation. HaCaT cells were stimulated with or without FGF7 in presence or absence of a specific AKT inhibitor (AKT i) or of the direct MTOR inhibitor rapamycin (RAP) as reported in Materials and Methods. Western blot analysis performed using anti-LC3, anti-phospho-AKT (pAKT) and anti-AKT polyclonal antibodies showed that either AKTi or RAP did not trigger LC3-I conversion to LC3-II in unstimulated cells and they did not affect the increase of LC3-II protein observed in FGF7-stimulated cells; the inhibition of AKT phosphorylation was evident only in cells treated with AKTi. The equal loading was assessed with anti-ACTB antibody. The densitometric analysis and Student *t* test were performed and significance levels have been defined as above: **P* < 0.05 vs. the corresponding FGF7-stimulated cells; *, **, *****P* < 0.05 vs. the corresponding serum-starved cells; ****, *****NS vs. the corresponding FGF7-stimulated cells; ^, ^^NS vs. the corresponding AKTi and RAP-untreated cells.

prominently, upon a specific block of their turnover.^{9,12} Based on these assumptions, to confirm that FGF7 is able to induce autophagosome formation, we assessed whether FGF7 could increase the number of small LC3-positive dots (<0.7 μm diameter) representing nascent autophagic vesicles in serum-starved HaCaT EGFP-LC3 cells in which the autophagosome turnover has been previously blocked by TG treatment. Results showed that, in the absence of the TG block, serum-starved cells showed very few small dots (Fig. 5C); as expected, the pretreatment with

TG, which blocks the LC3-II degradation, induced an increase of total LC3-positive dots which is due to a significant increase of clustered, larger vacuoles (> 0.7 μm diameter) (Fig. 5C, arrowheads). The stimulation with FGF7 of these TG-treated cells resulted in a further rise of the total LC3-positive dots, which was exclusively due to a significant increase of the small ones (Fig. 5C), while the number of larger dots (Fig. 5C, arrowheads) remained virtually unchanged. These results confirmed that FGF7 acts at very early steps of the autophagic process inducing the formation of new autophagosomes.

To unequivocally demonstrate that the FGF7-dependent induction of autophagy is directly mediated by FGFR2 expression and signaling, we analyzed the effect of receptor overexpression or depletion on the amount of LC3-II protein. To this aim, cells were transiently transfected with human FGFR2wt (HaCaT FGFR2wt), with the FGFR2^{Y656F} kinase-dead mutant (HaCaT FGFR2kin⁻) or with the empty vector (HaCaT pCI-neo). After transfection, cells were pretreated with TG and serum-starved in the presence or absence of FGF7 as above, and the levels of LC3-II and FGFR2 proteins were estimated by western blot analysis. Comparing HaCaT FGFR2wt and HaCaT pCI-neo cells, the forced overexpression of FGFR2wt further enhanced the FGF7-dependent increase of the amount of LC3-II (Fig. 6A); in contrast, the overexpression of FGFR2kin⁻ did not affect LC3-II levels independently of FGF7 stimulation (Fig. 6A), suggesting that FGFR2 expression and signaling is required for the ligand-mediated induction of autophagy. In contrast, SQSTM1 levels were increased by TG as above, but the amount remained unchanged in cells overexpressing different forms of FGFR2 (Fig. 6A). This finding may indicate that, although SQSTM1 degradation was inhibited as a consequence of the TG-mediated block of the autophagic flux toward lysosomes, FGFR2 expression and signaling did not induce any further

increase in SQSTM1 expression. Then, focusing on the effects of FGFR2 depletion, cells were transiently transfected with a small interfering RNA for *FGFR2* (*FGFR2* siRNA) or with an unrelated siRNA (control siRNA) as a control. Western blot analysis showed that, in *FGFR2* siRNA-transfected cells, the evident downregulation of FGFR2 was accompanied by a significant decrease of LC3-II levels in response to FGF7 (Fig. 6B), demonstrating that an efficient depletion of FGFR2 protein inhibits FGF7-mediated LC3-II expression and

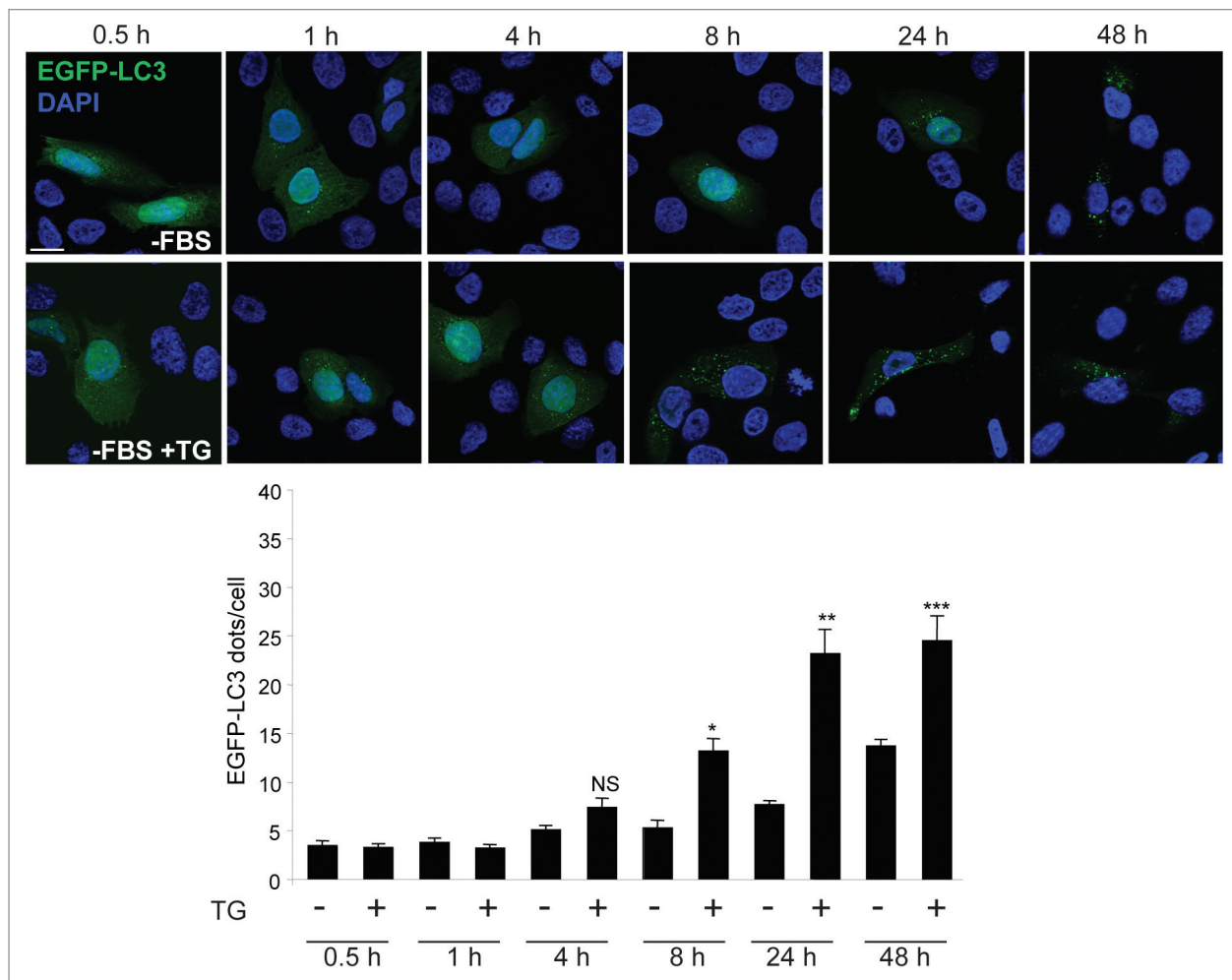


Figure 4. Block of the autophagic flux by thapsigargin. HaCaT EGFP-LC3 cells were pretreated with or without thapsigargin (TG) for 1 h at 37 °C and then serum-starved for different times (0.5, 1, 2, 4, 8, 24, and 48 h). Quantitative fluorescence analysis showed that TG significantly increased the number of the LC3-positive dots already after 8 h and more evidently after 24 h and 48 h. The quantitative analysis was assessed as reported in Materials and Methods and results are expressed as mean values \pm standard errors (SE). The Student *t* test was performed and significance level has been defined as *P* < 0.05; *, **, ****P* < 0.001 vs. the corresponding serum-starved cells; NS vs. the corresponding serum-starved cells. Scale bar: 10 μ m.

consequently autophagosome formation. Again, in the presence of TG, the amount of SQSTM1 protein was not modified upon FGFR2 modulation (Fig. 6B). Thus, the inducing role of FGF7 at very early stages of the autophagic process is directly mediated by FGFR2 activation and signaling.

FGF7 accelerates the fusion between autophagosomes and lysosomes

It has been recently reported that the survival growth factor IGF1 is able to counteract the massive accumulation of autophagosomes by increasing their fusion with lysosomes.⁹ Since it is well known that FGF7 plays a role in epithelial cell protection and survival,¹⁸⁻²⁰ we wondered if this effect could be dependent on the ability of FGF7 to accelerate autophagosome turnover. Indeed this possibility has been already suggested by the finding reported above that FGF7 is able to increase the degradation of SQSTM1 (Fig. 5A). To further address this point, we verified whether FGF7 could counteract the accumulation and clustering of autophagosomes, analyzing the appearance of LC3-positive

dots larger than 0.7 μ m in HaCaT EGFP-LC3 cells subjected to prolonged autophagic stimulus by serum starvation. We found that upon 48 h of serum deprivation, the treatment with FGF7 reduced the number of vacuoles visible in the cytoplasm of the transfected cells (Fig. 7, arrowheads) and the quantitative analysis confirmed that FGF7 stimulation led to a significant shift of the percentage of large vacuoles per cell from 23% to 24% to 5% (Fig. 7). Thus, FGF7 is able to counteract autophagosome clustering. To demonstrate that this effect would be due to the ability of the growth factor to accelerate autophagosome turnover, we investigated by immunofluorescence the kinetics of autophagosome confluence into lysosomes. To this aim HaCaT EGFP-LC3 cells were subjected to a serum starvation time course (0.5, 12, 24, 48, and 54 h) in the presence or absence of FGF7, and the lysosomal compartment was visualized using a monoclonal antibody directed against the specific marker LAMP2. Quantitative analysis showed that, in serum-starved cells, the EGFP-LC3-positive dots colocalized with

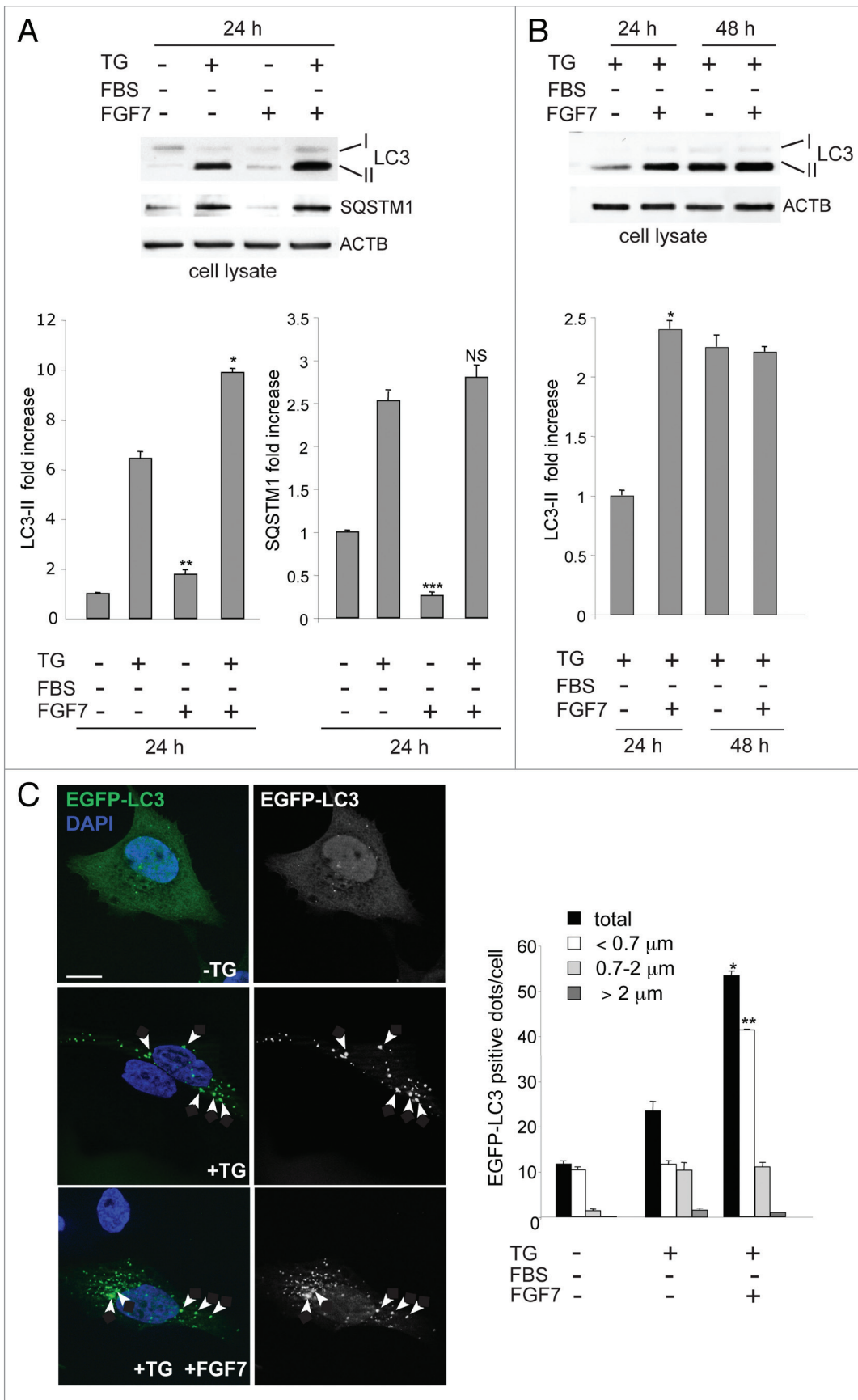


Figure 5. For figure legend, see page 811.

Figure 5 (See opposite page). FGF7 triggers autophagosome formation. **(A and B)** HaCaT cells were pretreated with or without TG and then serum-starved in the presence or absence of FGF7 for 24 h **(B and C)** or 48 h **(C)**. Western blot analysis shows that TG treatment increased the basal levels of LC3-II protein. Upon 24 h of serum deprivation FGF7 significantly increased the LC3-II amount either in TG-treated or TG-untreated cells **(B and C)**; while upon 48 h the very high levels of LC3-II were not affected by FGF7 addition **(C)**. Upon 24 h of serum-starvation TG also strongly increased the SQSTM1 amount independently of FGF7 stimulation **(B)**. Equal loading was assessed with anti-ACTB antibody. The densitometric analysis and Student *t* test were performed and significance levels have been defined as above: *, **, ****P* < 0.05 vs. the corresponding unstimulated cells; NS vs. the corresponding unstimulated cells. **(C)** HaCaT EGFP-LC3 were pretreated with and without TG and serum-starved in the presence or absence of FGF7 for 24 h as above. Fluorescence analysis indicates that serum-starved cells showed very few small LC3-positive dots (< 0.7 μm diameter) and the pretreatment with TG increased the total LC3-positive dots by increasing the number of large vacuoles (> 0.7 μm diameter) (central panels, arrowheads). FGF7 induced a further rise of the total LC3-positive dots exclusively due to a significant increase of the small ones, while the number of larger dots (lower panels, arrowheads) remained unchanged. The quantitative analysis was assessed as reported in Materials and Methods and results are expressed as mean values \pm standard errors (SE). Student *t* test was performed and significance level has been defined as *P* < 0.05: *, ***P* < 0.01 vs. the corresponding unstimulated cells. Scale bar: 10 μm .

LAMP2-positive dots (Fig. 8, arrowheads) only at 24 h and this colocalization reached a peak at 48 h (Fig. 8). In this scenario, the addition of FGF7 resulted in an acceleration of the fusion kinetics, as shown by the significantly higher EGFP-LC3 and LAMP2 colocalization at both 12 h and 24 h (Fig. 8). At 48 h, the colocalization rate in the presence of FGF7 decreased, reaching values comparable to that detected in control cells (Fig. 8). In parallel experiments performed, pretreating cells with TG, no colocalization between EGFP-LC3 and LAMP2 was detected at all time points, confirming the efficiency of this drug in preventing the autophagosome-lysosome fusion (Fig. 8). Thus, similarly to IGF1,⁹ FGF7 is able to accelerate autophagosome turnover preventing their potentially harmful accumulation in the cytosol.

FGF7-mediated autophagy is required for keratinocyte differentiation

We have recently demonstrated that FGFR2 expression and its signaling control early differentiation in keratinocytes.²⁸ Since it has been postulated that autophagy might represent an early crucial event for keratinocyte commitment to differentiation,²⁹ we wondered if the autophagic stimulus of FGF7 could play a role in the triggering of early differentiation. To address this point, we investigated if the ability of FGF7 to increase the early differentiation marker KRT1 (keratin 1) in differentiating HaCaT cells²⁸ would be maintained upon the nonspecific block of autophagy by a general inhibitor of autophagosome formation, 3-MA.^{32,39,40} HaCaT cells were cultured to reach the confluent differentiating condition and then serum starved or treated with FGF7 in the presence or absence of 5 mM 3-MA, a dose that was previously found to efficiently inhibit autophagy in keratinocytes.⁴¹ Western blot analysis clearly showed that 3-MA not only blocked the conversion of LC3-I to LC3-II and the SQSTM1 degradation, as a consequence of reduced autophagosome formation, but it also significantly inhibited the increase of KRT1 expression induced by FGF7 (Fig. 9A). Thus, the induction of autophagy is a process through which FGF7 may control differentiation. To confirm the interplay between FGF7-mediated autophagy and early differentiation, the autophagosome formation and KRT1 expression were visualized by immunofluorescence analysis in HaCaT EGFP-LC3 cells treated as above. Quantitative analysis showed that the presence of 3-MA significantly counteracted the increase induced by FGF7 of either the EGFP-LC3-positive dots per cell or the percentage of KRT1-positive cells (Fig. 9B, upper panels). Similar results

were obtained in primary HKs transiently transfected with EGFP-LC3, cultured in the presence of high Ca^{2+} concentration in the medium to trigger differentiation and treated with 3-MA and FGF7 as above (Fig. 9B, lower panels),

Since 3-MA induces a general inhibition of autophagy, the effect of BECN1/Beclin 1 and ATG5 depletion on FGF7-dependent induction of KRT1 expression was also investigated. In fact, BECN1 and ATG5 are 2 key proteins in the early stages of autophagosome formation,^{1,42} whose depletion specifically blocks autophagy also in keratinocytes.⁴³ HaCaT cells were transiently transfected with a small interfering RNA for *BECN1* (*BECN1* siRNA) or for *ATG5* (*ATG5* siRNA), or with a mixture of both siRNAs. Transfection using an unrelated siRNA was performed as a control. After transfection, cells were serum-starved or stimulated with FGF7 as above. Immunoblot using anti-LC3, anti-SQSTM1 and anti-KRT1 antibodies clearly showed that the efficient depletion of BECN1 or/and ATG5 not only resulted in LC3-II reduction and SQSTM1 increase (Fig. 10), but also in a significant inhibition of the FGF7-mediated increase of KRT1 (Fig. 10). Again, to visualize the effects of BECN1 and/or ATG5 depletion on FGF7-induced autophagy and early differentiation, a coinjection of *EGFP-LC3* cDNA and *BECN1* siRNA or *ATG5* siRNA or both, was performed in HaCaT cells. Microinjection of an unrelated siRNA was performed as control. After injection, cells were serum-starved or treated with FGF7 as above. Quantitative immunofluorescence analysis showed that, in cells microinjected (asterisks) with *BECN1* siRNA, with *ATG5* siRNA or with both, the number of LC3 autophagosomes per cell as well as the percentage of KRT1-positive cells in response to FGF7 appeared significantly reduced compared with the surrounding uninjected cells in the same microscopy fields or to cells injected with control siRNA (Fig. 11). Taken together, these results indicated for the first time that the ability of FGF7 to induce early differentiation in keratinocytes requires autophagy, since it is lost when the onset of the autophagic process is blocked by a general inhibitor or by autophagic key protein depletion.

Discussion

It is well recognized that FGF7/KGF is an important survival factor for epithelial cells.¹⁸⁻²⁰ In this paper we addressed the possible role of FGF7 and its receptor in the regulation of autophagy in keratinocytes. We surprisingly found that, unlike other survival growth factors that generally inhibit the autophagic process,^{6,8,9}

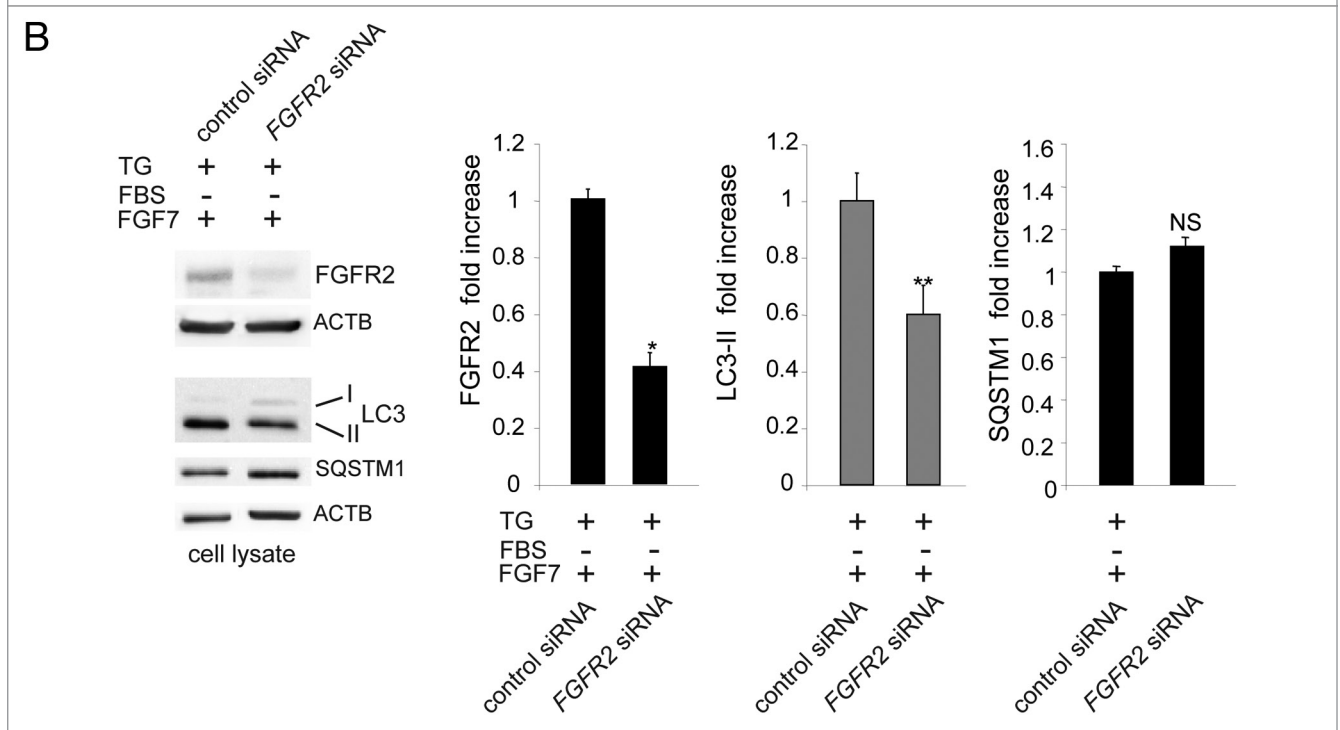
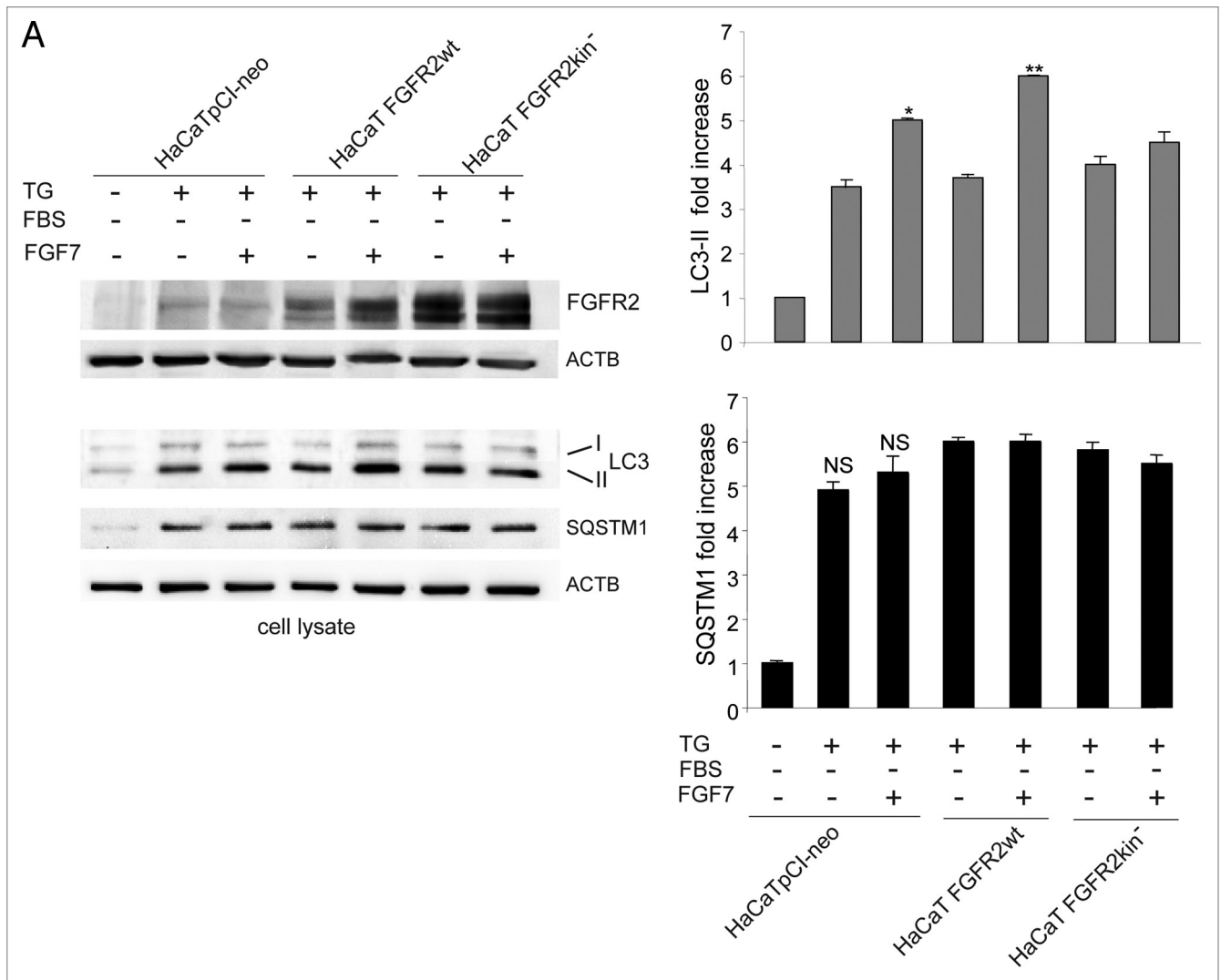


Figure 6 (See opposite page). FGF7-induced autophagy requires FGFR2 expression and signaling. **(A)** HaCaT cells transiently transfected with the empty vector (HaCaT pCI-neo), with FGFR2 (HaCaT FGFR2wt) or whit FGFR2^{Y656F Y657F} kinase-negative mutant (HaCaT FGFR2kin⁻) were pretreated with TG and serum-starved in the presence or absence of FGF7 as above. Western blot analysis performed using anti-SQSTM1 monoclonal antibody, and anti-LC3 and anti-FGFR2 polyclonal antibodies shows that the overexpression of FGFR2wt (indicated by a visible thickening of the band at the molecular weight of 140 kDa corresponding to the receptor) potentiated the increase of LC3-II amount induced by FGF7, while the overexpression of FGFR2kin⁻ did not affect LC3-II levels independently from FGF7 stimulation. SQSTM1 protein amount appeared increased by TG and remained unchanged, independently from FGFR2wt or FGFR2kin⁻ overexpression. Equal loading was assessed with anti-ACTB antibody. The densitometric analysis and Student *t* test were performed and significance levels have been defined as above: **P* < 0.05 vs. the corresponding unstimulated cells; ***P* < 0.01 vs. the corresponding unstimulated cells; NS vs. the corresponding HaCaT FGFR2wt. **(B)** HaCaT cells transfected with an unrelated siRNA (control siRNA) or with a small interfering RNA for *FGFR2* (*FGFR2* siRNA), to obtain receptor silencing, were treated with TG and stimulated with FGF7 as above. Western blot analysis shows that in *FGFR2* siRNA-transfected cells the downregulation of FGFR2 induced a decrease of LC3-II levels in response to FGF7; the level of SQSTM1 protein was unchanged independently from FGFR2 modulation. Equal loading was assessed with anti-ACTB antibody. The densitometric analysis and Student *t* test were performed and significance levels have been defined as above: **P* < 0.001 vs. the control siRNA-transfected cells; ***P* < 0.01 vs. the control siRNA-transfected cells; NS vs. the control siRNA-transfected cells.

Figure 7. FGF7 counteracts autophagosome clustering. HaCaT EGFP-LC3 cells were left in complete medium, serum-starved for 24 h or 48 h in the presence or absence of FGF7 as above. Cells were then fixed, permeabilized, and nuclei were stained with DAPI. Fluorescence analysis indicates that upon 48 h of serum deprivation the presence of FGF7 reduces the number of big dots visible in the cytoplasm of transfected cells (arrowheads). Quantitative analysis performed as reported above confirms that upon 48 h of serum deprivation FGF7 stimulation increases the number as well as the percentage of LC3-positive dots > 0.7 μm diameter per cell. Student *t* test was performed and significance levels have been defined as above: **P* < 0.001 vs. the corresponding cells starved for 24 h; ***P* < 0.001 vs. the corresponding unstimulated cells; ****P* < 0.001 vs. the corresponding unstimulated cells starved for 24 h; *****P* < 0.005 vs. the corresponding unstimulated cells. Scale bar: 10 μm.

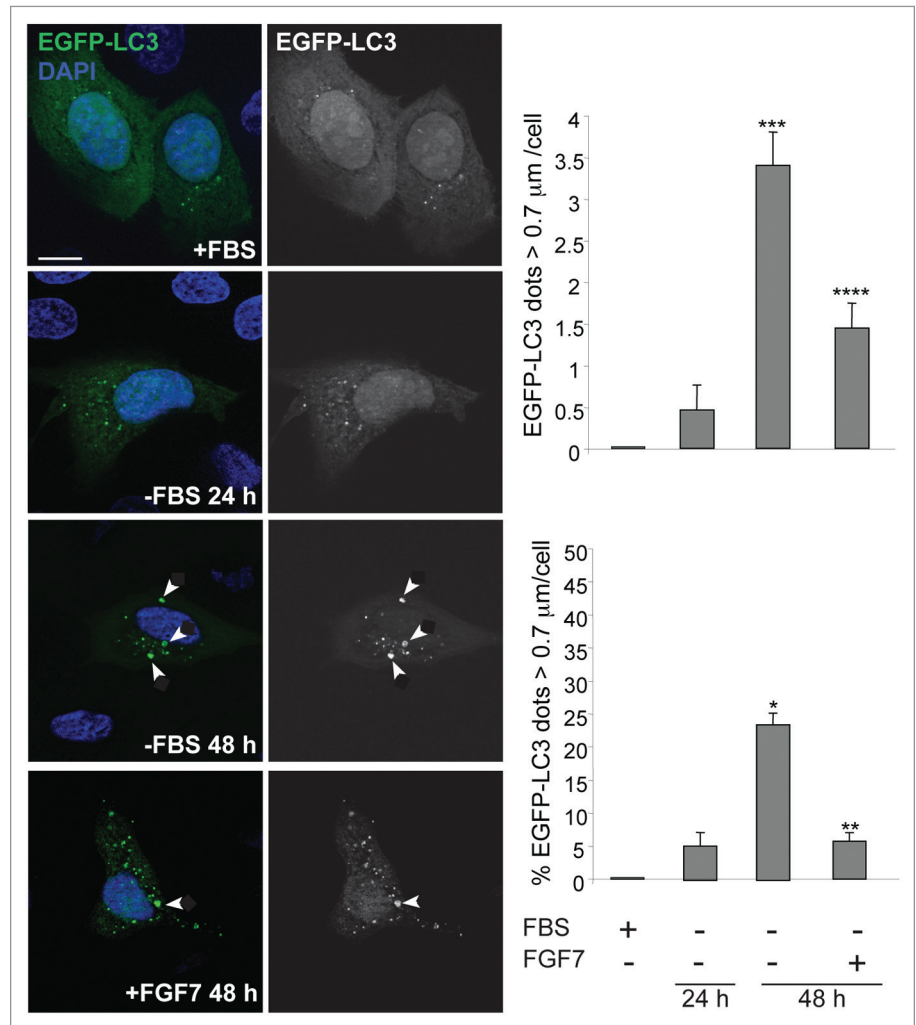


Figure 8 (See next page). FGF7 accelerates autophagosome-to-lysosome fusion. HaCaT EGFP-LC3 cells were subjected to a serum starvation time course (0, 4, 12, 24, 48, and 54) in the presence or absence of FGF7. Parallel experiments were performed pretreating cells with TG. anti-LAMP2 monoclonal antibody was used to visualize the lysosomal compartment, nuclei were stained with DAPI. Quantitative immunofluorescence analysis shows that in serum-starved cells the colocalization between EGFP-LC3-positive dots and LAMP2-positive dots was visible after 24 h and reached a peak at 48 h; the addition of FGF7 accelerated the kinetics of LC3 and LAMP2 colocalization, which at 48 h returned comparable to that detected in control cells. No colocalization was detected in TG-treated cells at all time points. The quantitative analysis of the colocalization of LC3 and LAMP2 signals was assessed as reported in Materials and Methods and results are expressed as mean values ± standard errors (SE). Student *t* test was performed and significance level has been defined as *P* < 0.05; ***P* < 0.001 vs. the corresponding unstimulated cells; ****P* < 0.05 vs. the corresponding unstimulated cells; NS vs. the corresponding unstimulated cells. Scale bars: 10 μm.

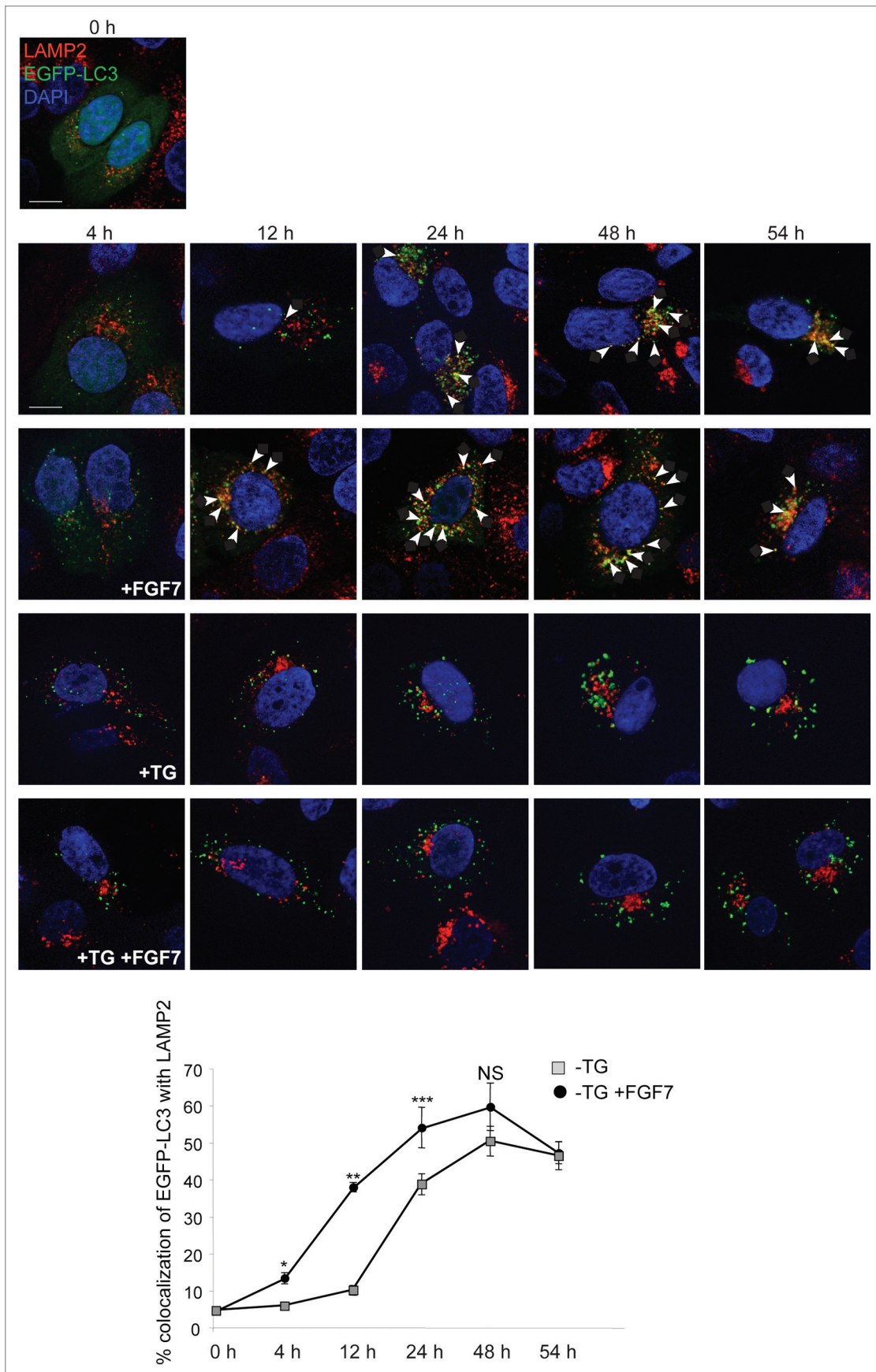


Figure 8. For figure legend, see page 813.

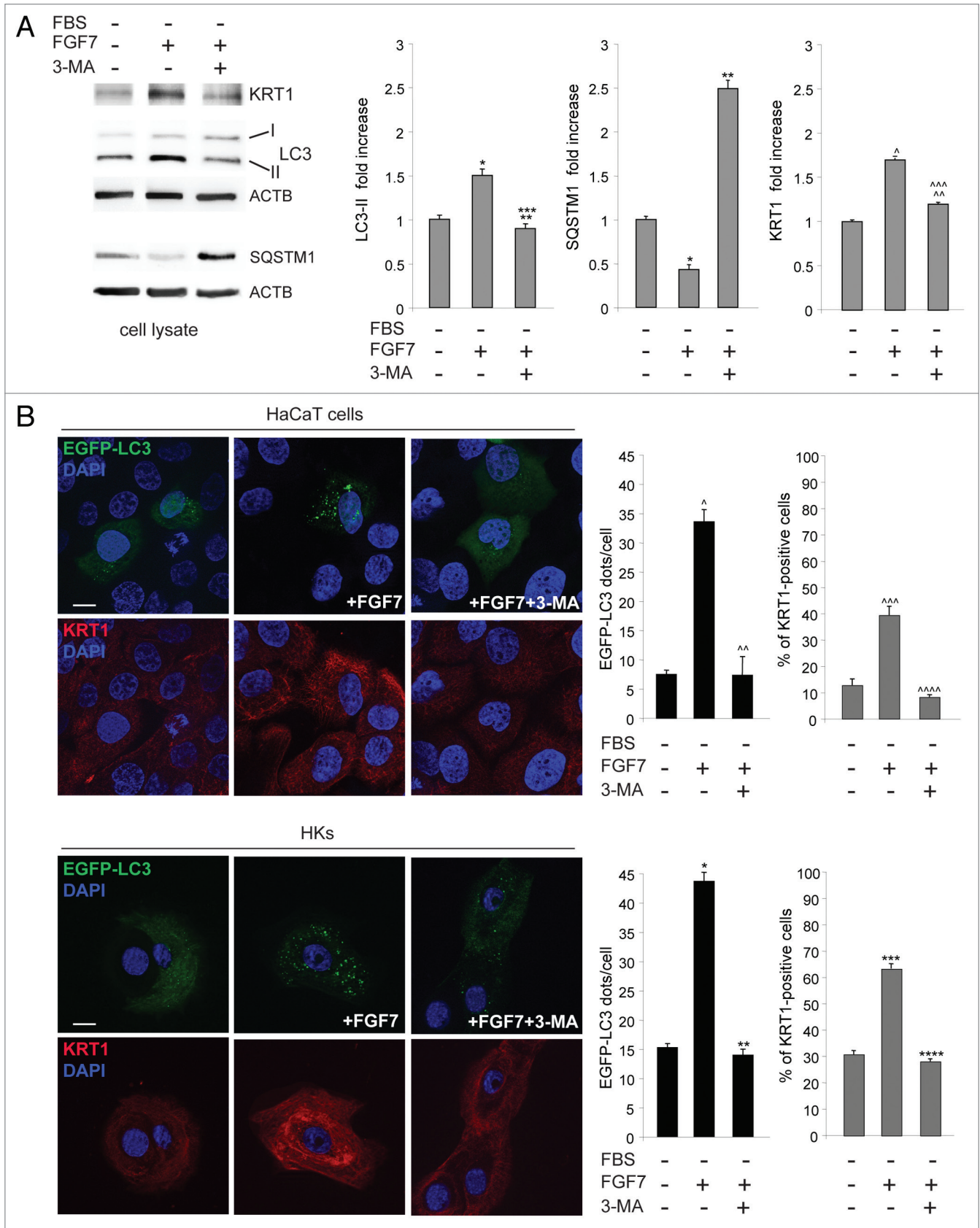


Figure 9. For figure legend, see page 816.

Figure 9 (See previous page). Block of autophagosome formation inhibits FGF7-induced early differentiation. **(A)** HaCaT cells were cultured to reach confluence and then serum-starved or treated with FGF7 for 24 h in presence or absence of 5 mM 3-MA as reported in Materials and Methods. Western blot analysis shows that the presence of 3-MA reduced the FGF7-dependent increase of both LC3-II and KRT1 protein levels and caused a significant rise in the FGF7-dependent decrease of SQSTM1. Equal loading was assessed with anti-ACTB antibody. The densitometric analysis and Student *t* test were performed and significance levels have been defined as above: **P* < 0.05 vs. the corresponding unstimulated cells; ***P* < 0.05 vs. the corresponding FGF7-stimulated cells; ***NS vs. the corresponding unstimulated cells; ^*P* < 0.01 vs. the corresponding unstimulated cells; ^^*P* < 0.05 vs. the corresponding FGF7-stimulated cells; ^^NS vs. the corresponding unstimulated cells. **(B)** Confluent HaCaT EGFP-LC3 cells (upper panels) and HKs EGFP-LC3 grown in high Ca²⁺ (lower panels) were serum-starved and treated with FGF7 and 3-MA as above. Quantitative fluorescence analysis showed that in both cells the presence of 3-MA significantly reduced the increase of either the EGFP-LC3-positive dots per cell and the percentage of KRT1-positive cells observed upon FGF7 stimulation. The quantitative analysis of LC3-positive dots per cell and of the percentage of KRT1-positive cells was assessed as reported in Materials and Methods. Results are expressed as mean values ± standard errors (SE). Student *t* test was performed and significance level has been defined as *P* < 0.05: ^, ^^*P* < 0.001 vs. the corresponding unstimulated cells; ^^, ^^*P* < 0.001 vs. the corresponding cells stimulated with FGF7 in absence of 3-MA; *, ****P* < 0.001 vs. the corresponding unstimulated cells; **, *****P* < 0.001 vs. the corresponding cells stimulated with FGF7 in absence of 3-MA. Scale bars: 10 μm.

FGF7 triggers autophagy in keratinocytes and this effect is directly dependent on FGFR2 expression and signaling. Since autophagy is a dynamic process, an increase in autophagosome number may depend either on increased formation or reduced turnover of these organelles.^{36,37} Alternatively, but more rarely, both these processes can be simultaneously active. Selectively blocking the fusion of autophagosomes with lysosomes by thapsigargin,¹² we demonstrated that FGF7 triggers the very early step of the process stimulating autophagosome formation.

Interestingly we also found that upon a prolonged autophagic stimulus, FGF7 is also able to accelerate the fusion of the autophagosomes with lysosomes, contrasting their accumulation and clustering. This second important function of FGF7 in the later step of the autophagic process is not surprising and is consistent with its well-established survival role in epithelial cells. In fact, similarly to other growth factors, such as IGF1, having an important role in cell protection, FGF7 may accelerate autophagosome turnover protecting cells from the potentially harmful accumulation of vacuoles in the cytosol.

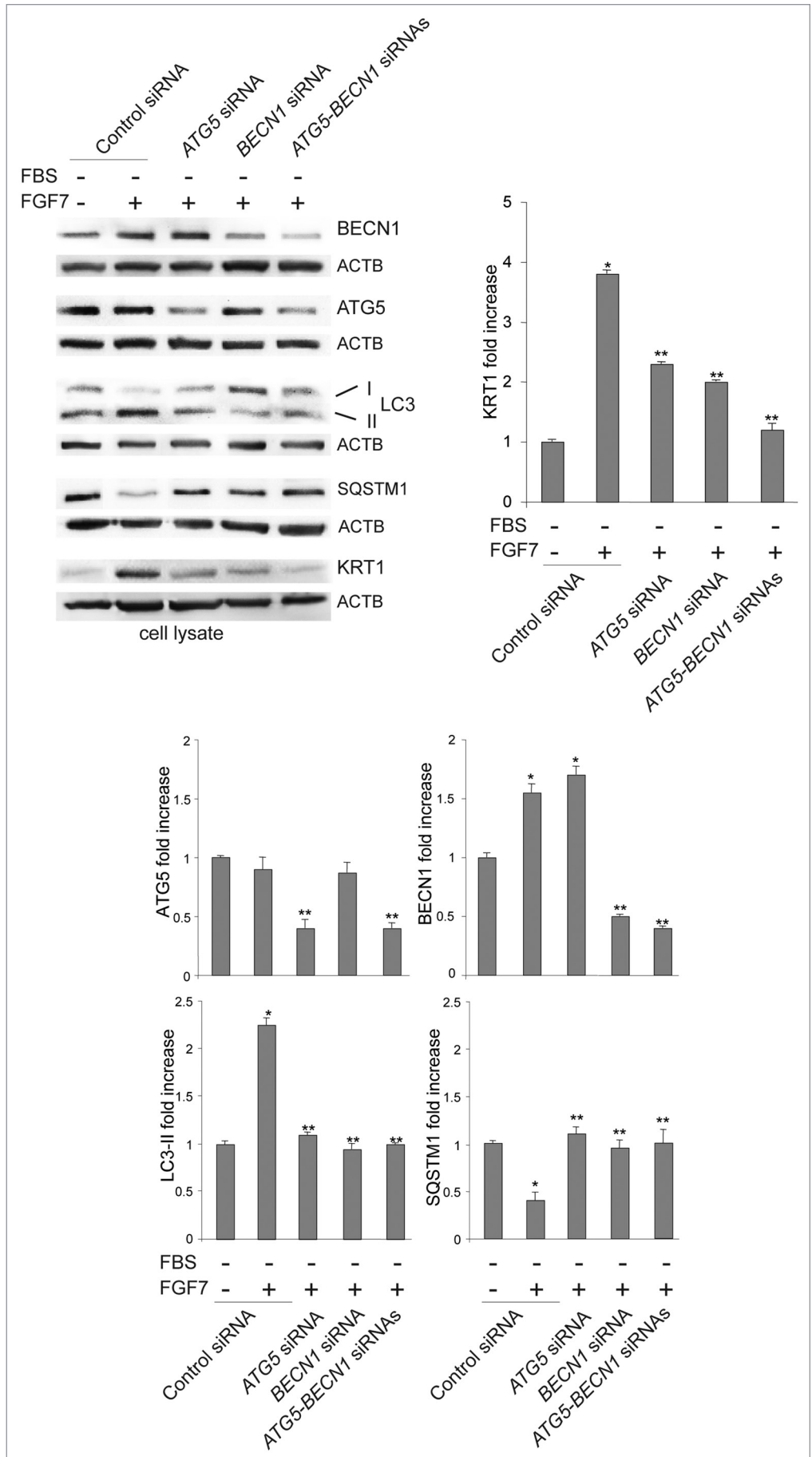
Very recent lines of evidence have highlighted that the protective function of autophagy is also crucial in determining skin color.⁴⁴ In fact, it has been demonstrated that in keratinocytes from light skins, the autophagic activity was higher than in dark keratinocytes, inducing the rapid lysosomal-dependent degradation of melanosomes and contrasting their potentially dangerous accumulation. In agreement with these observations, results from our group have shown that FGFR2 is more expressed in light keratinocytes compared with dark ones⁴⁵ and its FGF7-dependent activation induces melanosome uptake stimulating the phagocytic process.^{30,46} Based on these findings, our present results strongly suggest that the activation of FGF7-FGFR2 axis, through induction of melanosome autophagy, would be likely a crucial event in regulating skin color. In fact, FGF7-dependent activation of FGFR2 not only triggers the melanosome uptake, but it would be also able to induce their efficient removal stimulating both their inclusion in autophagosomes and their rapid lysosome-mediated degradation. Consistent with this possibility, we might also speculate that the UVB-induced persistence of the melanosomes in keratinocytes can be due to a reduced rate of autophagy consequent to the ligand-independent internalization and degradation of FGFR2 triggered by this type of radiation, as we have previously demonstrated.⁴⁷ Therefore, our findings implicate FGF7-FGFR2 signaling as

one of the essential elements underlying the regulation of skin pigmentation.

Several lines of evidence in the past few years have also suggested a possible link between autophagy and cell differentiation. In fact, it has been recently reported that autophagy induces myocardial differentiation⁷ as well as accompanies the early steps of the differentiation program in keratinocytes.²⁹ The involvement of autophagy in cell differentiation might be explained considering the fact that this process represents a rapid self-degrading pathway that may be used by cells to rapidly and efficiently change their protein and organelle patterns and consequently their phenotype.^{3,48} The recent finding that FGF2 prevents the premature differentiation of cardiomyocyte progenitors blocking autophagy⁴⁸ has provided the first direct evidence that a growth factor can affect differentiation directly affecting autophagy. Since FGF7 is a crucial physiological player regulating the early differentiation of keratinocytes during the switch from undifferentiated to differentiating cells, we wondered if this well-established ability²⁸ could be, at least in part, dependent on its capacity to induce autophagy. Using different approaches we found that a general block of autophagosome assembly or a specific inhibition of this process through depletion of key autophagic proteins not only blocks the FGF7-mediated formation of new autophagosomes, but it also significantly inhibits the increase of KRT1 expression induced by FGF7 in keratinocytes. Therefore, our data strengthen the hypothesis of the existence of a direct interplay between growth factor-mediated autophagy and cell differentiation, providing the first evidence that the induction of autophagy by FGF7 is a key event required for the triggering of early differentiation during the switch from basal undifferentiated to suprabasal differentiated keratinocytes.

We also demonstrated that the autophagy induced by FGF7 is not affected by the FGF7-dependent activation of the PI3K-AKT-MTOR signaling pathway. Since we have recently found that this pathway is involved in the induction of keratinocyte differentiation,²⁸ it is plausible that distinct intracellular signaling pathways could be independently responsible for the regulation of autophagy and differentiation. Further investigation will be needed to clarify the molecular mechanisms underlying the FGF7-mediated induction of autophagy, considering that a possible MTOR-independent

Figure 10. BECN1 and/or ATG5 depletion inhibit the KRT1 protein expression induced by FGF7. HaCaT cells were transiently transfected with small interfering RNA for *ATG5* (*ATG5* siRNA) or for *BECN1* (*BECN1* siRNA) or with a mixture of both siRNAs (*ATG5* and *BECN1* siRNAs). Transfection with an unrelated siRNA was performed as a control. After transfection, cells were serum-starved or stimulated with FGF7 for 24 h. Western blot analysis shows that BECN1 and ATG5 are significantly reduced in cells transfected with the specific siRNAs. In HaCaT *BECN1* siRNA, HaCaT *ATG5* siRNA and in HaCaT *BECN1* and *ATG5* siRNAs a strong LC3-II reduction, SQSTM1 accumulation and a significant inhibition of KRT1 increase upon FGF7 stimulation is shown. Equal loading was assessed with anti-ACTB antibody. The densitometric analysis and Student *t* test were performed and significance levels have been defined as above: **P* < 0,05 vs. the corresponding unstimulated cells; ***P* < 0,05 vs. the corresponding control siRNA-transfected cells.



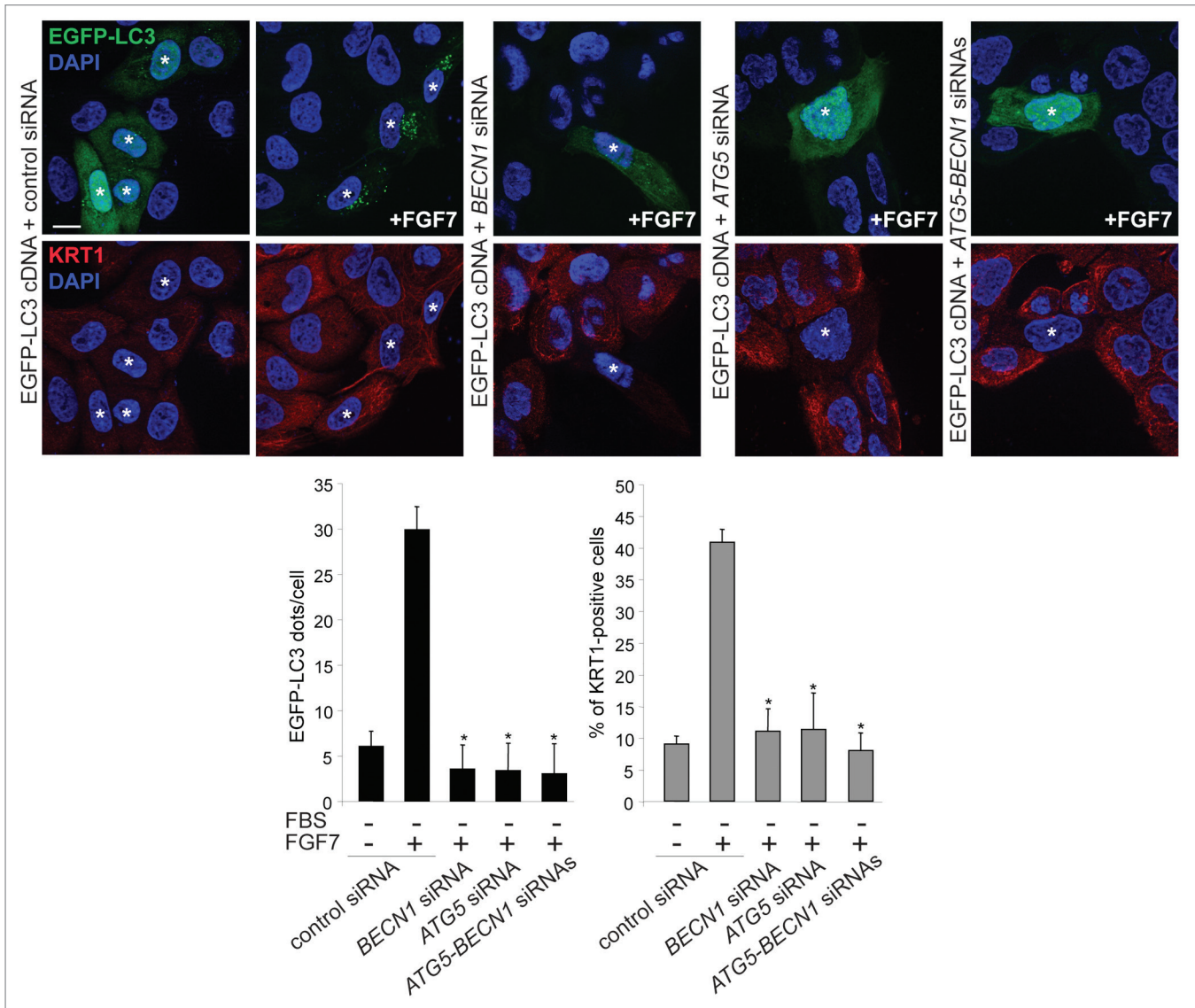


Figure 11. BECN1 and/or ATG5 depletion reduces the percentage of KRT1-positive keratinocytes upon FGF7 treatment. HaCaT cells were coinjected with *EGFP-LC3* cDNA and *ATG5* siRNA or *BECN1* siRNA or both, to obtain specific gene silencing. Microinjection with an unrelated siRNA was performed as control. After injection, cells were serum-starved or treated with FGF7 as above. Quantitative immunofluorescence analysis shows that in cells microinjected (asterisks) with *ATG5* siRNA, with *BECN1* siRNA or with *ATG5* and *BECN1* siRNAs mixture, the increase of the percentage of LC3-positive autophagosomes per cell as well as the expression of KRT1 in response to FGF7 appeared significantly reduced compared with the surrounding uninjected cells in the same microscopic fields or to cells injected with control siRNA. The quantitative analysis was assessed as reported in Materials and Methods, results are expressed as mean values \pm standard errors (SE). Student *t* test was performed and significance level has been defined as $P < 0.05$; * $P < 0,001$ vs. the corresponding control siRNA-injected cells. Scale bar: 10 μ m.

involvement of the RAF-MAP2K-MAPK/ERK signaling pathway cannot be excluded.

In summary, our results demonstrate for the first time that FGF7-mediated FGFR2 signaling exerts an important role in the regulation of the autophagic process, since it is able to either induce autophagosome formation or to accelerate their turnover. Because FGF7 is a crucial factor in the regulation of skin homeostasis, we propose that the dynamic balance between keratinocyte proliferation, survival, differentiation, and pigmentation could be at least partially regulated by FGF7 through a fine modulation of the autophagic process.

Materials and Methods

Cells and treatments

The human keratinocyte cell line HaCaT³¹ was cultured in Dulbecco's modified Eagle's medium, supplemented with 10% fetal bovine serum plus antibiotics. Primary cultures of normal human keratinocytes (HKs) were derived from skin biopsies and maintained in Medium 154-CF (Cascade Biologics, M154CF) supplemented with human keratinocyte growth supplement (HKGS, Cascade Biologics, S-001-5) plus antibiotics and Ca²⁺ (0.03 mM; Cascade Biologics, 50-9702). To induce cell

differentiation, primary human keratinocytes were placed in medium containing Ca^{2+} (1.5 mM).

HaCaT cells were transiently transfected with pCI-neo empty vector or with pCI-neo containing human FGFR2wt (HaCaT FGFR2wt) or a kinase-negative mutant FGFR2^{Y656F} (HaCaT FGFR2Kin⁻). Alternatively, HaCaT cells and HKs were transiently transfected with pEGFP-C2 expression vector containing LC3 (kindly provided by Prof Francesco Cecconi, Tor Vergata University of Rome, Italy). jetPEITM DNA Transfection Reagent (Polyplus-transfection, 10-40) was used for HaCaT cells transfection, while FuGENE 6 Transfection Reagent (Roche, 11815091001) was used for HKs transfection.

For RNA interference and *FGFR2* silencing, HaCaT cells were transfected with *FGFR2/Bek* small interfering RNA (*FGFR2* siRNA) (Santa Cruz Biotechnology, sc-29218), or with unrelated siRNA as a control, using Lipofectamine 2000 Transfection Reagent (Life Technologies, 11668-019) according to the manufacturer's protocol.

For growth factor stimulation, cells were incubated with 100 ng/ml FGF7 (Upstate Biotechnology, 01-118) for different times (4, 8, 12, 24, 48, and 54 h) at 37 °C.

To inhibit AKT or MTOR, cells were respectively incubated with the specific AKT inhibitor 1L-6-hydroxy-methyl-chiro-inositol 2-(R)-2-O-methyl-3-O-octadecylcarbonate (1 μM ; Calbiochem, 124005) or with the specific MTOR inhibitor rapamycin (100 nM; Cell Signaling Technology, 9904) for 1 h at 37 °C before treatment with FGF7 in the presence of each inhibitor.

To generally inhibit autophagosome assembly, cells were treated with the class I/III PI3K/PtdIns3K inhibitor 3-methyladenine (3-MA) (5 mM; Sigma Chemicals, M9281) for 1 h at 37 °C before treatment with FGF7 in the presence of the inhibitor.

To irreversibly block the fusion between autophagosomes and lysosomes, HaCaT cells were incubated with thapsigargin (3 μM) for 1 h at 37 °C. Since TG stock (1 mg/ml) was diluted in the solvent dimethyl sulfoxide (DMSO), control cells were treated with an equal amount of DMSO.

Microinjection

Microinjection was performed with an Eppendorf microinjector and an Axiovert S100 inverted microscope (Zeiss, 451311-0000-000). Injection pressure was set at 80 to 100 hPa and the injection time at 0.5 s. A mixture of 100 nM siRNA for *ATG5* (Santa Cruz Biotechnology, sc-41445) or *BECN1* (Santa Cruz Biotechnology, sc-29797) and 100 ng/ μl EGFP-LC3 cDNA in phosphate buffer saline, PBS (Euroclone, EC B4004L) was microinjected in the cytoplasm to simultaneously induce RNA interference and consequent *ATG5* and/or *BECN1* silencing and EGFP-LC3 expression. Cells were left in complete medium for 8 h at 37 °C, then stimulated with FGF7 for 24 h at 37 °C and processed for immunofluorescence.

Immunofluorescence

Cells, grown on coverslips and incubated with or without FGF7 and TG as above, were fixed with 4% paraformaldehyde (Electron Microscopy Sciences, 157-8) in PBS for 30 min at 25 °C followed by treatment with 0.1 M glycine (Sigma, 50046) for 20 min at 25 °C and with 0.1% Triton X-100 for

an additional 5 min at 25 °C to allow permeabilization. Cells were then incubated for 1 h at 25 °C with the following primary antibodies: rabbit polyclonal anti-KRT1 (1:50 in PBS; Covance, PRB-149P-100) and mouse monoclonal anti-LAMP2 (CD170b, BD Biosciences, 55803). The primary antibodies were visualized, after appropriate washing with PBS, using goat anti-rabbit IgG-Texas Red (1:200 in PBS; Jackson ImmunoResearch Laboratories, 111-075-144) and goat anti-mouse IgG-Texas Red (Jackson ImmunoResearch Laboratories, 115-075-146) for 30 min at 25 °C. Nuclei were stained with DAPI (1:1000 in PBS; Sigma, D9542). Coverslips were finally mounted with mowiol (Sigma, 81381) for observation.

Fluorescence signals were analyzed and cells were scanned in a series of 0.5- μm sequential sections with an ApoTome System (Zeiss, 000000-1189-776) connected to an Axiovert 200 inverted microscope (Zeiss, 491906-9850-000); image analysis was then performed by the Axiovision software (Zeiss, 410130-9850-000) and 3-dimensional reconstruction of a selection of 3 central out of the total number of the serial optical sections was shown in each figure. Quantitative analysis of EGFP-LC3-positive dots per cell and the percentage of KRT1-positive cells was performed analyzing 100 cells for each sample in 5 different microscopy fields from 3 different experiments. The KRT1 signal intensity was measured using the software KS300 3.0 Image Processing System (Zeiss, 000000-1020-771) as previously reported.^{28,49} Briefly, a cut-off value to discriminate between KRT1-positive and negative cells was determined on subconfluent untreated HaCaT cells as the mean fluorescence intensity + standard deviation (SD). Quantitative analysis of the extent of colocalization between EGFP-LC3 and LAMP2 signals and of the nascent autophagosomes and of autophagosomes clustering in vacuoles was performed using the software KS300 3.0 Image Processing System (Zeiss). The diameters of all EGFP-LC3-positive dots per cell were measured and their distribution in 2 size categories (< 0.7 μm and > 0.7 μm) was evaluated. Cells expressing very high level of EGFP-LC3 were excluded by the analysis, since in these cells the fluorescent dots could be due to protein aggregates independent of autophagosome formation.³⁷

Results have been expressed as mean values \pm standard errors (SE). *P* values were calculated using the Student *t* test and significance level has been defined as *P* < 0.05.

Western blot analysis

HaCaT, HaCaT FGFR2wt, and HaCaT FGFR2Kin⁻ cells were lysed in a buffer containing 50 mM HEPES (Eurobio, GAUHEP 00-62), pH 7.5, 150 mM NaCl (Sigma, S7653), 1% glycerol (Sigma, G6279-500ML), 1% Triton X-100 (Sigma, T8787), 1.5 mM MgCl_2 (Sigma, M-1028), 5 mM EGTA (Sigma, E 3889), supplemented with protease inhibitors (10 $\mu\text{g}/\text{ml}$ aprotinin [Sigma, A1153], 1 mM PMSF [Sigma, 93482], 10 $\mu\text{g}/\text{ml}$ leupeptin [Sigma, L-2884]), and phosphatase inhibitors (1 mM sodium orthovanadate [Sigma, S-6508], 20 mM sodium pyrophosphate [Sigma, P-8135], 0.5 M NaF [Sigma, S7920]). A range between 50 and 20 μg of total protein were resolved under reducing conditions by 8 or 12% SDS-PAGE and transferred to reinforced nitrocellulose (Schleider and Schuell,

BA-S 83). The membranes were blocked with 5% nonfat dry milk (Bio-Rad, 170-6404) in PBS 0.1% Tween 20 (Bio-Rad, 170-6531) or with 3% BSA (Sigma, A7030) in PBS 0.1% Tween 20, and incubated with anti-LC3 polyclonal antibodies (MBL, PD014), anti-FGFR2/Bek (C-17, Santa Cruz Biotechnology, sc-122) polyclonal antibodies, anti-KRT1 (Covance, PRB-149P-100) polyclonal antibodies, anti-phospho-AKT (Ser 473, Cell Signaling, 9271) polyclonal antibodies, anti-ATG5 (Novus Biologicals, NB110-53818) polyclonal antibodies, anti-BECN1 (H-300, Santa Cruz Biotechnology, sc-11427) polyclonal antibodies and anti-SQSTM1 (BD Transduction Lab, 610832) monoclonal antibody followed by enhanced chemiluminescence detection (SuperSignal West pico Chemiluminescent Substrate, Thermo Scientific, 34080). The membranes were rehydrated by being washed in PBS-Tween 20, stripped with 100 mM mercaptoethanol and 2% SDS for 30 min at 55 °C, and probed again with anti-AKT1/2 (H-136, Santa Cruz Biotechnology,

sc-8312) polyclonal antibodies or anti-ACTB (Sigma, A5441) monoclonal antibody, to estimate the protein equal loading. Densitometric analysis was performed using Quantity One Program (Bio-Rad Laboratories). Briefly, the signal intensity for each band was calculated and the background subtracted from experimental values. The resulting values from 3 different experiments were then normalized respect to the corresponding ACTB band intensity and expressed as fold increase respect to the control value.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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