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Research Techniques Made Simple: Analysis of Autophagy in the Skin

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Abbreviations: autophagy-and-beclin-1 regulator 1, AMBRA1; cannabidiol, CBD; class III PI3K complex, PI3KC3; green fluorescent protein, GFP; Light Chain 3, LC3; monomeric red fluorescent protein, mRFP; phosphatidylethanolamine, PE; phosphatidylinositol-3-phosphate, PI3P; sequestosome-1, SQSTM1/p62; Δ9-tetrahydrocannabinol, THC; unc-51-like kinase 1, ULK1.

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Abstract

Autophagy is required for normal skin homeostasis and its disordered regulation is implicated in a range of cutaneous diseases. Several well characterised biomarkers of autophagy are used experimentally to quantify autophagic activity, or clinically to correlate autophagy with disease progression. This article discusses the advantages and limitations of different approaches for measuring autophagy, as well as techniques for modulating autophagy. These include analysis of endogenous LC3, a central autophagy regulatory protein, and measurement of LC3 flux using a dual-fluorescent reporter, which provides a quantitative readout of autophagy in cell culture systems *in vitro* and animal models *in vivo*. Degradation of SQSTM1/p62 during autophagy is proposed as an alternative biomarker allowing analysis of autophagy both experimentally and clinically. However, the complex regulation of individual autophagy proteins and their involvement in multiple pathways means that several proteins must be analysed together, preferably over a time course to accurately interpret changes in autophagic activity. Genetic modification of autophagy proteins can be used to better understand basic autophagic mechanisms contributing to health and disease, while small molecule inhibitors of autophagy regulatory proteins, lysosomal inhibitors, or activators of cytotoxic autophagy have been explored as potential treatments for skin disorders where autophagy is defective.

Autophagy is a key cellular mechanism for the degradation and recycling of proteins and organelles, and is defined according to three main classifications. *Macroautophagy* is the canonical form of autophagy involving sequestration of cellular components within a double-membrane autophagosome, which fuses with a lysosome to form a degradative autolysosome (Figure 1). *Chaperone-mediated autophagy* is a selective process where individual proteins/aggregates are directly sequestered into the lysosome, while *microautophagy* is the direct engulfment of cellular contents by lysosomes or endosomes without involvement of a secondary phagosome.

In eukaryotes, autophagy regulatory proteins control homeostasis by interacting with a multitude of other signalling pathways, which allows adaptation to stress, regulation of differentiation or activation of apoptosis. In the skin, autophagy is required for a range of normal physiological activities such as barrier formation, pigmentation and antigen presentation (Sil et al., 2018), while deregulation of autophagy is implicated in a variety of skin pathologies including infection, cancer and ageing. As such a number of research techniques have been developed to study autophagy in cell culture systems, animal models and patient samples and these are increasingly being applied to skin and skin disease. These models primarily focus on detecting and quantifying several important autophagy regulatory proteins, which serve as biomarkers of autophagy.

Biomarkers of autophagy

The initiation of macroautophagy (hereafter referred to as autophagy) is mediated via the unc-51-like kinase 1 (ULK1) complex in response to several signals

such as nutrient deprivation and stress (resulting in the accumulation of damaged proteins). The ULK1 complex phosphorylates components of the class III PI3K (PI3KC3) complex, which triggers nucleation of a double membrane structure to create a phagophore (Figure 1). Several proteins including Beclin-1 and vacuolar protein sorting 34 (VPS34), are contained within the PI3KC3 complex and have become important biomarkers for identifying, measuring and modulating autophagy in an experimental setting. The early stages of autophagy initiation are regulated at multiple points by the autophagy-and-beclin-1 regulator 1 (AMBRA1) protein, which plays a multifunctional role in determining when and where autophagosome formation occurs. AMBRA1 is therefore a critical control protein for regulation of autophagic activity and is also emerging as a useful biomarker of autophagy. The PI3KC3 complex stimulates production of phosphatidylinositol-3-phosphate (PI3P) within the phagophore membrane, which recruits WIPI proteins and the ATG12 complex (composed of ATG12, ATG5 and ATG16L1). The WIPIs and the ATG12 complex promote phagophore elongation via lipid conjugation of ATG8 proteins, such as light-chain 3 (LC3), which become anchored to the developing autophagosome membranes. During selective autophagy, cellular components (e.g. damaged proteins or mitochondria) are labelled with an 'eat-me' signal (often ubiquitin), which interacts with selective autophagy receptors such as sequestosome-1 (SQSTM1/p62, hereafter referred to as p62) to target cargo for sequestration into the autophagosome by binding with LC3. When the autophagosome subsequently fuses with the lysosome, p62 is degraded along with its cargo making it an attractive candidate to be a qualitative and potentially quantitative biomarker of autophagy.

Measuring autophagic activity

Research techniques for measuring autophagy or correlating autophagy with disease status often rely on capturing a snapshot of autophagy regulatory protein expression. However, as most autophagy regulatory proteins are not specific for autophagy multiple biomarkers need to be considered together, and preferably analysed over a time course, to accurately measure autophagic activity. In this section we describe some of the techniques used for measuring autophagy in the skin, and explain the advantages and limitations of each approach.

Given the pivotal role of LC3 in the autophagic process this central autophagy regulatory protein is often used as a biomarker of autophagy, with studies showing the endogenous expression of the LC3B isoform correlates with progression of melanoma skin cancer as well as poor patient outcome (Lazova et al., 2012). Immunofluorescent staining of 446 melanoma tumours for LC3B demonstrated that LC3B levels were significantly higher in nodal, visceral and cutaneous metastases. The same study also showed that LC3B expression strongly correlated with Ki-67 staining. Similarly, a variable LC3A expression pattern was observed in cutaneous squamous cell carcinoma, with increased numbers of densely stained LC3+ve structures correlating with tumour thickness (Sivridis et al., 2011). These data collectively indicate that LC3 expression increases with tumour progression and is associated with increased proliferation. Here, LC3 is a biomarker of disease severity but additional information is required to accurately determine the status of autophagic activity. To interpret such data, it is important to understand the mechanisms associated with LC3 expression and post-translational processing. Nascent pro-LC3 is proteolytically processed within the cytosol to LC3-I and subsequently conjugated with

phosphatidylethanolamine (PE) to form LC3-II (Figure 1). This lipid subunit allows LC3-II to be incorporated into autophagosomal membranes where it is later degraded or deconjugated back to LC3-I following fusion with the lysosome. LC3-II is therefore a marker for autophagosomes, and can be measured by western blotting for LC3-II or immunofluorescence to detect LC3-positive puncta. An important caveat is that changes in LC3 levels and puncta formation may arise as a consequence of reduced autophagosome degradation, or independently to autophagic activity (Klionsky et al., 2016). Therefore the amount of LC3-II at a single time point does not reflect autophagic flux (the complete process of autophagy); flux can be measured by following LC3 turnover using inhibitors of lysosome-mediated degradation of LC3-II, such as chloroquine, which prevents autophagosome-lysosome fusion therefore inhibits degradation of LC3-II. Increased accumulation of LC3-II in cells treated with an autophagy inducer in the presence of a lysosomal inhibitor, compared to either treatment alone, can indicate autophagic flux. Care must be taken when measuring endogenous LC3 as antibodies may have a different affinity for LC3-I and LC3-II, or different specificities to LC3 isoforms, which can create artefacts in analysis (Klionsky et al., 2016). Alternatively, tandem fluorescent labelled-LC3 reporter probes (where both a monomeric red fluorescent protein (mRFP; or mCherry) and green fluorescent protein (GFP) are fused with LC3) are available for monitoring LC3 turnover in various experimental models, including live cells or *in vivo*, to provide a more accurate measure of autophagic activity (Kimura et al., 2007). This assay is based on the sensitivity of the GFP signal to the acidic conditions of the autolysosome, whereas the mRFP/mCherry signal is stable. In fluorescence microscopy, autophagosomes appear as a yellow signal (merged green and red fluorescence) while autolysosomes appear

red (Figure 2). While these reporters are not applicable to clinical analysis of autophagy in patient tissue they are commonly used for pre-clinical analysis of autophagic activity.

An alternative biomarker for quantifying autophagy in clinical as well as pre-clinical samples is the autophagy receptor p62 as it is degraded alongside sequestered substrate when autophagosomes are broken down. Therefore, reduced p62 protein levels would appear to be indicative of high autophagic activity, while conversely an increase in p62 expression likely indicates autophagy inhibition causing p62-containing protein aggregates to accumulate. Immunohistochemical staining of 121 primary cutaneous melanoma tumours for p62 demonstrated that tumours with low levels of p62 (< 20% cells were positive for p62) were associated with significantly reduced disease-free survival (Ellis et al., 2014), suggesting aggressive tumours have a high level of autophagic activity. Nevertheless, p62 analysis must be interpreted with caution as p62 changes can be independent of autophagy (Klionsky et al., 2016). Conversely, in early stage melanoma, autophagy regulatory proteins such as ATG5 are downregulated, which is associated with decreased autophagy (Liu et al., 2013). This approach of using immunostaining to measure levels of autophagy regulatory proteins adds to the body of evidence of temporal regulation of autophagic activity during cancer progression (Galluzzi et al., 2015). Collectively, these data suggest that autophagy is lost during melanoma initiation to avoid induction of endogenous tumour-suppressor mechanisms, but as the disease progresses autophagy is reactivated in advanced stages of melanoma to support the high metabolic demands of cancer cells, often associated with a poor patient outcome (Lazova et al., 2012).

In addition to analysis of autophagy regulatory proteins, transmission electron microscopy is also often used in a research setting, where sample preparation can be optimised, to demonstrate autophagosome formation. The presence and number of double membrane autophagic vesicles (autophagosomes) within a cell relative to other organelles is an accurate measure of autophagic activity. However, this technique is labour-intensive and requires extensive training to operate, analyse and interpret correctly so its suitability for determining autophagic activity in patient tissue or in high-throughput studies is limited.

Modulation of autophagy regulatory proteins

In recent years several molecular tools have been developed to modulate autophagy regulatory proteins with the aim of understanding the autophagic mechanisms that contribute to normal cutaneous biology, and develop more effective therapeutic interventions for disorders caused by defective autophagy. However, research has revealed a dual role for autophagy in the development and progression of skin cancer, creating problems for the development of treatments aimed at modulating autophagy. Autophagy is a critical tumour suppressor mechanism that signals with multiple other mechanisms to prevent cancer development. Therefore, the influence of the tumour-suppressor function of autophagy is thought to be silenced in the early stages of cancer development. However because tumour progression is an energy intensive process, the growth-promoting function of autophagy is a survival advantage and is often reactivated to drive growth of advanced tumours (Galluzzi et al., 2015). Therapy aimed at modulating autophagy to kill cancer cells faces a double-edge sword because while inhibiting autophagy may slow the

growth of advanced cancers by cutting off a vital energy supply, it could also promote the progression of early stage tumours by removing autophagy-induced tumour suppression. Alternatively, some drugs may activate a cytotoxic form of autophagy, potentially of early *and* late stage tumours, but could also drive tumour growth.

Experimentally, it is possible to block autophagy using RNA interference-mediated knockdown of autophagy regulatory genes by transfecting cells in culture with a silencing RNA (siRNA). Using this technique, it was shown that inhibition of autophagosome formation by knockdown of ATG5 in mouse embryonic fibroblasts prevented the elimination of skin-resident group A *Streptococcus* bacteria, revealing a critical role for autophagy in our innate immune response to cutaneous infections (Nakagawa et al., 2004). Alternatively, autophagy can be blocked by chemical inhibition using drugs such as chloroquine and hydroxychloroquine. In a meta-analysis of 7 clinical trials these drugs have been shown to improve the response of cancer when administered in combination with chemotherapy or radiotherapy compared to therapy without autophagy inhibition (Xu et al., 2018). Similarly, small molecule inhibitors of the autophagy regulatory proteins can be used *in vitro* and *in vivo* to investigate the function of autophagy as well as to model potential clinical utility. For example, use of 3-methyladenine (a PI3KC3 inhibitor) in a mouse model of wound healing demonstrated the potential of autophagy inhibition as a strategy to improve wound healing in diabetic patients (Guo et al., 2016). Furthermore, targeted inhibition of VPS34 results in a block in autophagy, evidenced by the accumulation of LC3 and p62, sensitises metastatic melanoma cells to inhibition of MAPK signalling and reduces invasion of melanoma cells *in vivo* (Verykiou et al., 2019) (Figure 3). Therefore, autophagy inhibitors may have a role in the clinical management of patients with

advanced stage skin cancer. However, an alternative approach may be to activate autophagy-induced cell death. In a mouse xenograft model of human metastatic melanoma the cannabinoids ∆9-tetrahydrocannabinol (THC) and cannabidiol (CBD) increased immunofluorescent punctate staining of LC3B in treated tumours, which correlated with reduced Ki67 and increased TUNEL staining indicating these drugs induce cytotoxic autophagy within tumour cells (Armstrong et al., 2015). This response also appears to be specific for cancer cells as corresponding non-transformed cell types (e.g. melanocytes in the case of melanoma) did not undergo apoptosis upon exposure to the same cannabinoid concentrations *in vitro* suggesting that cannabinoids, which are currently undergoing clinical trials in other cancer types, may be a safe and effective approach for the treatment of some patient subgroups. Nevertheless, further research is required to elucidate the mechanisms mediating the anti-tumour effects of cannabinoids. There is also potential for the use of cannabinoids, or other drugs that activate autophagy such as dithranol (unpublished data and Figure 2), for the treatment of other skin diseases including psoriasis where autophagy is also defective (Akinduro et al., 2016; Mahil et al., 2016).

In summary, autophagy is central to skin homeostasis and dysfunctional autophagy is now recognised as a key feature of a range of cutaneous pathologies. Multiple techniques must be utilized in order to accurately evaluate autophagic activity, its role in the pathogenesis of skin disease, and the potential to exploit this process for therapeutic intervention.

Conflict of Interest: The authors have no potential conflicts of interest to disclose.

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Figures

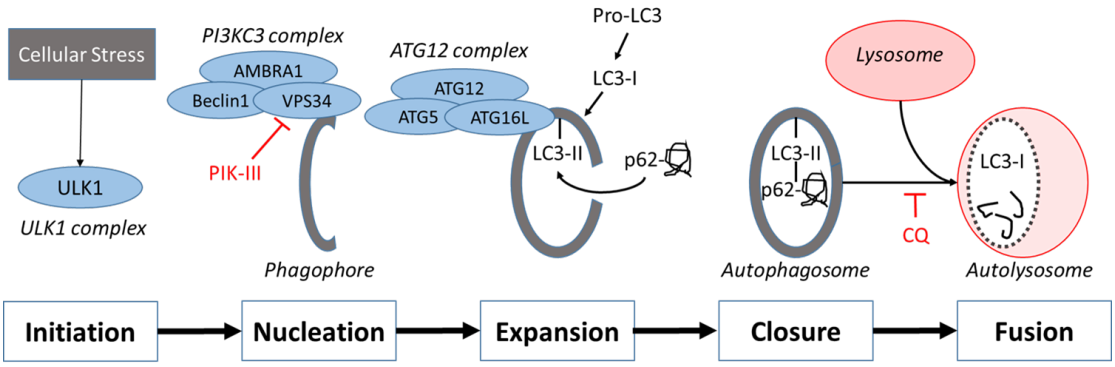

Figure 1: Simplified schematic of autophagy. Autophagy is induced in response to cellular stress, such as nutrient deprivation or infection, and acts to provide nutrients for cellular processes, as well as to maintain cellular health by removing harmful components. The process of autophagy progresses in five distinct phases (bold), each of which refer to the stages of autophagosome maturation. Analysis of LC3 and p62 is a common approach to assessment of autophagy, while autophagy can be inhibited by targeting autophagosome formation or lysosome fusion.

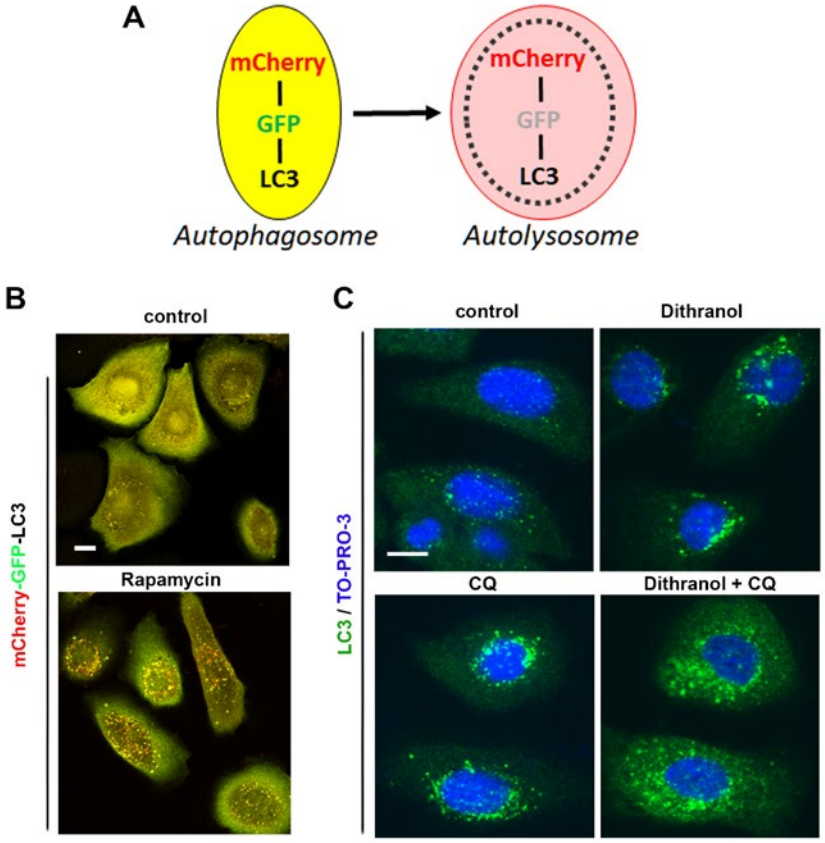

Figure 2: Analysis of autophagy using LC3. A) Following introduction into cells, tandem fluorescent-labelled mRFP/mCherry-GFP-LC3 is incorporated into the autophagosome membrane during autophagy. Colocalisation of green and red fluorescent signals (resulting in yellow fluorescence) indicate an autophagosome, whereas a red signal alone corresponds to an autolysosome. B,C) Normal human epidermal keratinocytes expressing mCherry-EGFP-LC3B were treated with vehicle (DMSO) or Rapamycin (10 nM) (B), or CCD-1106 keratinocytes were treated with vehicle (DMSO) or Dithranol (1 μM) in the absence or presence of chloroquine (10 μM) for 24 hours, followed by immunostaining for LC3B. Representative fluorescent micrographs are shown (bar, 10 μm).

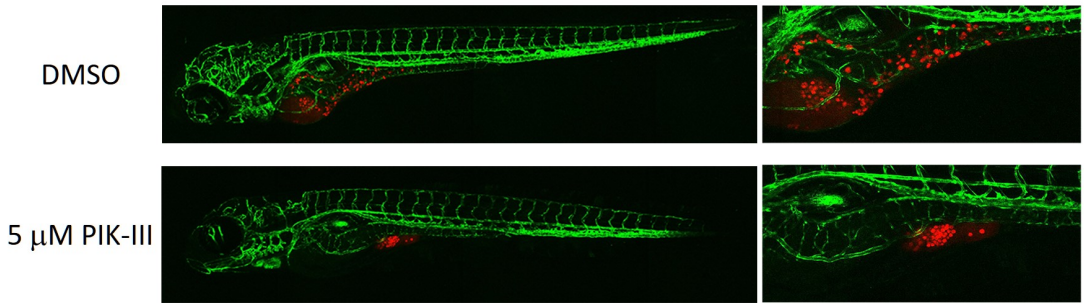

Figure 3: Effect of autophagy inhibition on invasion of metastatic melanoma cells. Fluorescence microscopy images of 5 day old zebrafish embryos with GFP-expressing endothelial blood vessels (Green), which were injected with DiI-labelled A375 metastatic melanoma cells (Red) at 2 days post-fertilisation and treated for 72 hours with either DMSO or 5 μM PIK-III (a small molecule VPS34 inhibitor; Verykiou et al., 2019).

Summary points

What information the techniques provide

- Autophagy is a dynamic process essential for skin homeostasis.
- Autophagy can be measured at a single time point by observation of autophagy proteins or autophagy structures, using techniques such as western blotting, immunofluorescence, immunohistochemistry or electron microscopy.
- Multiple methods should be used to enable accurate interpretation of autophagy in vitro
- Autophagy can be measured dynamically by introduction of fluorescent tagged markers into cultured cells *in vitro*.
- Autophagy can be modulated in cultured cells or model systems using small molecule inhibitors of autophagy proteins or inhibitors of lysosomal function.

Limitations

- Accumulation of LC3-II may reflect activation of autophagy or decreased turnover.
- Changes in SQSTM1/p62 protein levels can be independent of autophagic activity
- It is not currently feasible to monitor autophagic flux in clinical samples.

Multiple Choice Questions

1) Autophagy disruption is implicated in which of the following skin pathologies?

A. Infection
B. Cancer
C. Ageing
D. All of the above

Explanation: Autophagy is central to skin homeostasis, and plays vital roles in pigmentation, differentiation, barrier formation, antigen presentation, pathogen clearance, resolution of inflammation and wound healing. Dysfunctional autophagy has now been implicated in a number of pathological conditions, including infection, skin cancer, psoriasis and skin ageing.

2) Autolysosome formation proceeds through the stages of initiation, nucleation, expansion, closure, fusion.

A. True

B. False

Explanation: Autophagy is a catabolic pathway in which cellular components are delivered to lysosomes for degradation and recycling. Central to this process is the formation of an autophagosome, in which cellular material destined for degradation are transported to lysosomes. Various cellular signals can activate autophagy (initiation), which leads to formation of a cup-shaped membrane structure (termed the phagophore; nucleation) generated by the action of ATG proteins, which are also required for elongation (expansion) and sealing (closure) of the membrane to form a double-membraned vesicle, the autophagosome. In the final stage, the autophagosome matures and fuses with a lysosome (fusion) forming the autolysosome.

3) Using immunolabelling of LC3 and fluorescence microscopy, increased levels of LC3-positive puncta could be caused by

A. An accumulation of autophagosomes.
B. A reduction in autophagosome degradation.
C. Mechanisms independent of autophagy.
D. All of the above.

Explanation: In cells undergoing autophagy, LC3 is conjugated to a lipid (forming LC3-II) and localised to the autophagosome membrane, creating LC3-positive puncta which can be detected by immunolabelling and fluorescence microscopy. However, while LC3 is a marker for autophagic structures, accumulation of LC3-puncta could also be due to increased synthesis of LC3, a reduction in autophagsome degradation, formation of LC3 aggregates independently of autophagy, or association of LC3 with non-autophagic structures. Analysis of LC3 should therefore not be used alone to measure autophagy, and additional assays of autophagic flux (LC3-II turnover in the presence and absence of inhibitors of lysosomal degradation, or using tandem-fluorescent labelled LC3) used to measure the dynamic activity of autophagy.

4) What colour is the tandem fluorescent-labelled LC3 reporter protein when it is localised to the autophagosome?

A. Green
B. Yellow
C. Red
D. Blue

Explanation: LC3 is incorporated into the autophagosome membrane during autophagy. Colocalisation of green and red fluorescent signals (resulting in yellow)

indicate an autophagosome, whereas a red signal alone corresponds to an autolysosome.

5) Which two drugs are able to inhibit autophagy in vivo?

A. Rapamycin
B. ∆9-tetrahydrocannabinol
C. VPS34 inhibitor
D. Chloroquine

Explanation: Rapamycin and ∆9-tetrahydrocannabinol are able to activate autophagy, whereas both the VPS34 inhibitor PIK-III and chloroquine are autophagy inhibitors. PIK-III inhibits VSP34 with 100-fold greater selectivity over related kinases, and reduces autophagic activity by inhibition of *de novo* lipidation of LC3. Chloroquine increases lysosomal pH and blocks fusion between the autophagosome and the lysosome, thereby preventing lysosomal degradation and autophagic activity.