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Chapter 20

Autophagy in Development and Remodelling of Mammary Gland

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Additional information is available at the end of the chapter

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1. Introduction

Mammary gland is a unique organ, which undergoes the majority of its development in the postnatal life of mammals, especially during puberty and pregnancy. It is a complex tissue comprised of many cell types. The actual glandular part is formed by two epithelial cell subtypes: outer myoepithelia and inner luminal epithelia, which form a complicated net of ducts and lobules involved in milk synthesis and secretion during lactation. The glandular epithelium is embedded in stroma composed of mesenchymal cells, such as fibroblasts, adipocytes, immune cells, and extracellular matrix (ECM). The general features of mammary gland development is universal for animals and humans, however some differences in growth rate and hormonal control of the process can be distinguished between species.

During embryogenesis emergence of epithelial buds from ectoderm into mammary mesenchyme initiates formation of a rudimentary system of ducts, which continue their moderate elongation after birth, simultaneously with the increases in the body weight. In rodents prepubertal mammary gland consist of long, infrequently branching ducts terminated by highly proliferative structures, called terminal end buds (TEBs). TEBs contain two distinct cell types: cap cells organized as a single layer at the leading edge of these structures, staying in direct contact with the thin layer of basal lamina, and body cells, which form the multicellular bulk of the TEB [1] (Figure 1). In ruminants mammary ductal network develops as compact, highly branched structure within loose connective tissue called the terminal ductal units (TDUs), consisting of solid cords of epithelial cells that penetrate into mammary stroma [2]. An accelerated, hormone-dependent expansion of the glandular epithelium occurs at puberty, however the final stages of functional development do not take place until gestation. At the time of pregnancy mammary growth becomes exponential, and driven by pregnancy hor-

mones, giving rise not only to more extensive ductal branching, but also to the development of alveolar structures required for milk production. Mammary alveoli are build by functionally differentiated secretory epithelial cells, showing the ability to synthesize and secrete milk components during lactation. These characteristic structures with hollow cavity are formed in the last stage of mammary gland morphogenesis, termed alveologenesis (Figure 1). Weaning terminates the lactation period causing programmed cell death of a substantial part of the secretory epithelium, which leads to mammary gland involution. The cycle of proliferation, differentiation, and regression can be repeated many times in female's life. That is why mammary gland has become a very good and convenient model for studying processes involved in development and differentiation.

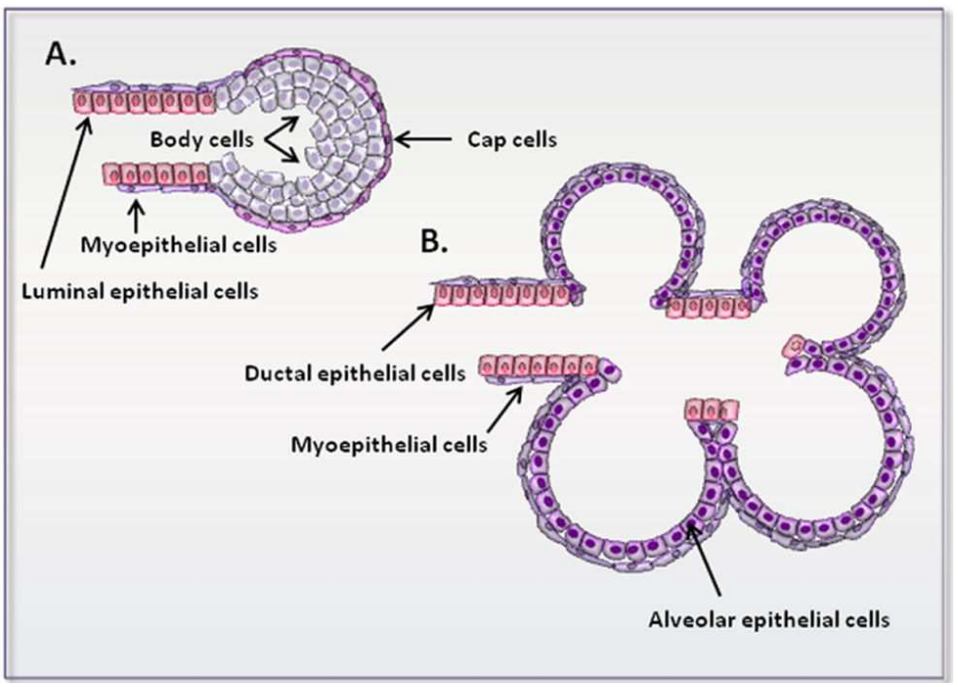

Figure 1. Schematic representation of the structure of mammary epithelium on different stages of differentiation. (A) Pre-pubertal and pubertal mammary gland consists of ducts terminated by highly proliferative terminal end buds (TEBs) that comprise cap cells in direct contact with the basal lamina, and body cells forming multicellular bulk of the TEB. (B) During pregnancy more extensive ductal branching and formation of alveolar structures required for milk production takes place. In functionally active mammary gland alveoli are built by a single layer of milk secreting luminal epithelial cells surrounded by myoepithelial cells, and basement membrane. Myoepithelial cells contractions release the milk to the ducts, and further to the nipple, whereas basement membrane provides cell contact with extracellular environment.

The lactation cycle includes periods of intensive proliferation of mammary epithelial cells (MECs), their functional differentiation during lactogenesis, and tissue involution caused by death of the secretory cells. Nowadays it is well established, that apoptosis plays a crucial role in all stages of mammary gland development. It is involved in lumen formation during ductal and alveolar morphogenesis, in replacement of cells during lactation, when MECs show high secretory activity, and in the involution of mammary gland. However, proper growth, development and remodelling require also well controlled balance between protein synthesis and organelle biogenesis versus protein degradation and organelle turnover in the cells. Since

autophagy is the major cellular pathway for degradation of long-lived proteins and control of the cytoplasmic organelles, this process is particularly important during development and under certain stress conditions.

2. Role of autophagy in mammary gland development

2.1. Role of autophagy in mammogenesis and its relation with apoptosis

During all stages of mammary gland development lumen formation is essential for building functional network of ducts, terminated by milk-producing alveoli at the time of lactation. Lumen formation follows the process of branching morphogenesis, and it is said to be based mainly on the clearance of cells via apoptosis of an inner cell population within newly branched epithelial cords or newly formed acini, creating a cavity [3]. When TEBs invade the mammary stroma they are built by multilayers of epithelial cells, however, the primary ducts behind them posses only a single outer layer of myoepithelial cells and an inner layer of luminal cells surrounding an empty hollow lumen. It was noted that the body cells of TEBs show high rates of apoptosis indicating that this type of programmed cell death contributes to lumen formation [4]. The hypothesis was additionally confirmed with the use of transgenic mice overexpressing an antipoptotic protein Bcl-2 in the mammary gland, because the mammary glands of these rodents showed delayed lumen formation [4].

2.1.1. Autophagy, apoptosis and lumen formation

More information on the mechanisms regulating the lumenization process were obtained in the studies using a three dimensional (3D) cell culture model. This *in vitro* culture system was developed on the basis of the first observations showing, that epithelial cells are able to maintain their tissue structure when grown on ECM components which more closely mimic the *in vivo* microenvironment then the rigid plastic surfaces used in the classic monolayer cultures [5, 6]. It has been shown that mammary epithelial cells (MECs) cultured on laminin-rich reconstituted basement membrane (rBM) are able to recapitulate numerous features of mammary epithelium *in vivo,* including the formation of acini-like spheroids, with a hollow lumen, apicobasal polarization of cells, basal deposition of basement membrane components (collagen IV, laminin I), and the ability to produce milk proteins [5, 7, 8]. Upon seeding within the rBM, normal MECs first undergo a few cycles of proliferation forming small organoids. Next, the structures develop an axis of apicobasal polarity, illustrated among others by basal localization of integrin receptors which are in direct contact with ECM, and lateral (e.g. E-cadherin) or apico-lateral (e.g. ZO-1) localization of junctional complexes [7, 8]. The spherical structures subsequently become unresponsive to proliferative signals, and a bona fide lumen is formed by cavitation, involving the removal of centrally localized cells by death processes [9, 10 , 1] (Figure 2). Lack of cell contact with ECM is regarded to be the direct cause of apoptosis initiation in the MECs placed in the centre of developing spheroid. This type of apoptotic death program is termed anoikis [11, 12]. However, it has been shown that overexpression of antiapoptotic proteins Bcl-2 or Bcl-XL in acini formed by human mammary epithelial cell line

MCF-10A only delayed lumen clearance for a few days, although apoptosis was inhibited, which pointed at a possibility that other processes may contribute to lumen formation [9]. In the same study electron microscopy analysis revealed the presence of numerous autophagic vacuoles in the central cells of developing acinar structures. Since autophagy was observed also in spheroids overexpressing Bcl-2 it was concluded that this process proceeds independently of apoptosis. Thus, it was initially proposed that autophagy may also promote lumen formation by initiating type 2 of programmed cell death [9, 13]. However, this hypothesis had some flaws, as it ignored the fact, that the cells lacking contact with ECM may initially induce autophagy as a cytoprotective mechanism against this stressful condition.

2.1.2. Role of autophagy in cells lacking contact with ECM

Integrin receptors are responsible for sustaining cell-matrix interactions, and mediating signal transduction from ECM into the cells [14]. β1-integrin has been shown critical for the alveolar morphogenesis of a glandular epithelium and for maintenance of its differentiated function [15]. Inhibition of β1-integrin in human MECs using blocking antibodies resulted in induction of autophagy. On the contrary, when laminin-rich basement membrane was added to the cells cultured in suspension autophagy was not induced, which points at a direct relationship between autophagy induction and the loss of cell contact with ECM [16]. Furthermore, it was demonstrated that depletion of some of the major Atg genes responsible for autophagy induction and autophagosome formation, namely: Atg5, Atg6 (beclin1) and Atg7, using siRNA technique resulted in reduced autophagy and enhanced apoptosis in suspended cells. A reduced clonogenic viability upon reattachment of MECs was also observed, indicating the prosurvival function of autophagy in cells lacking the direct contact with ECM during acini formation [16]. Interestingly, these studies have shown that inhibition of autophagy either pharmacologically (using 3-MA) or by knocking down Atg5 or Atg7 genes failed to elicit long-term luminal filling even when combined with inhibition of apoptosis. Similar results were obtained by Karantza-Wadsworth and co-workers [17], who worked on immortalized mouse mammary epithelial cells lacking one allel of beclin1 (beclin1$^{+/-}$). These cells when grown in 3D culture formed acini, which exhibited accelerated lumen formation compared to wild type controls that had both allels of beclin1 gene (beclin1 $^{+/+}$). The authors concluded that defective autophagy may sensitize MECs to metabolic stress, leading to accelerated lumen formation. Central acinar cells of Bcl-2-expressing beclin1$^{+/-}$ spheroids exhibited signs of necrotic cells death, suggesting that necrosis may be the default cell death mechanism upon apoptosis and autophagy inactivation [17]. However, their study also indicated, that defective autophagy compromises the ability of cells to adapt to metabolic stress, which may lead to insufficient ATP generation, accumulation of damaged mitochondria with excessive reactive oxygen species (ROS), and this in turn may cause accumulation of DNA damage, resulting in genome instability and increased risk of cancer progression.

The role of autophagy as the first line survival mechanism of cells centrally localized in the acinar structures was also proven by Sobolewska et al. [18] in the studies on bovine MECs. Bovine BME-UV1 mammary epithelial cells cultured on rBM behave in a similar manner to other described MECs, forming acinar structures composed of an outer-layer of polarized cells

and a hollow lumen in the centre of the spheroids within 16 days of 3D culture. Autophagy was observed on the basis of the punctuated pattern of GFP-LC3 protein in the centre of developing acini by the end of the first week of 3D culture. The induction of autophagy preceded apoptosis, as the expression of apoptosis executor enzyme - cleaved caspase-3 was detected starting from the 9th day of cell culture. Thus, autophagy was observed in the acinar structures when a clear distinction of two populations of cells within the structures could be determined – the outer polarized layer of cells with direct contact with rBM, and the centrally localized cells lacking this contact. Subsequent intensive apoptosis eliminated the inner cells forming hollow lumen of the acini [18]. The importance of autophagy and the time of its activation during formation of spherical structures was further confirmed in the experiments on bovine MECs cultured in 3D system in the presence of 3-MA – the inhibitor of early autophagosome formation. 3MA caused formation of small, underdeveloped organoids, and the cells forming these structures showed signs of apoptosis (cleaved caspase-3 activity) before the process of polarization was completed [18]. However, others have shown that the addition of 3-MA to the 3D culture in the later time points, when minimal luminal apoptosis was already observed, caused only increased luminal cell death, not influencing the shape of the acini [16]. Thus, not only localization of autophagic cells, but also the time of autophagy induction, determines the proper development of mammary alveoli

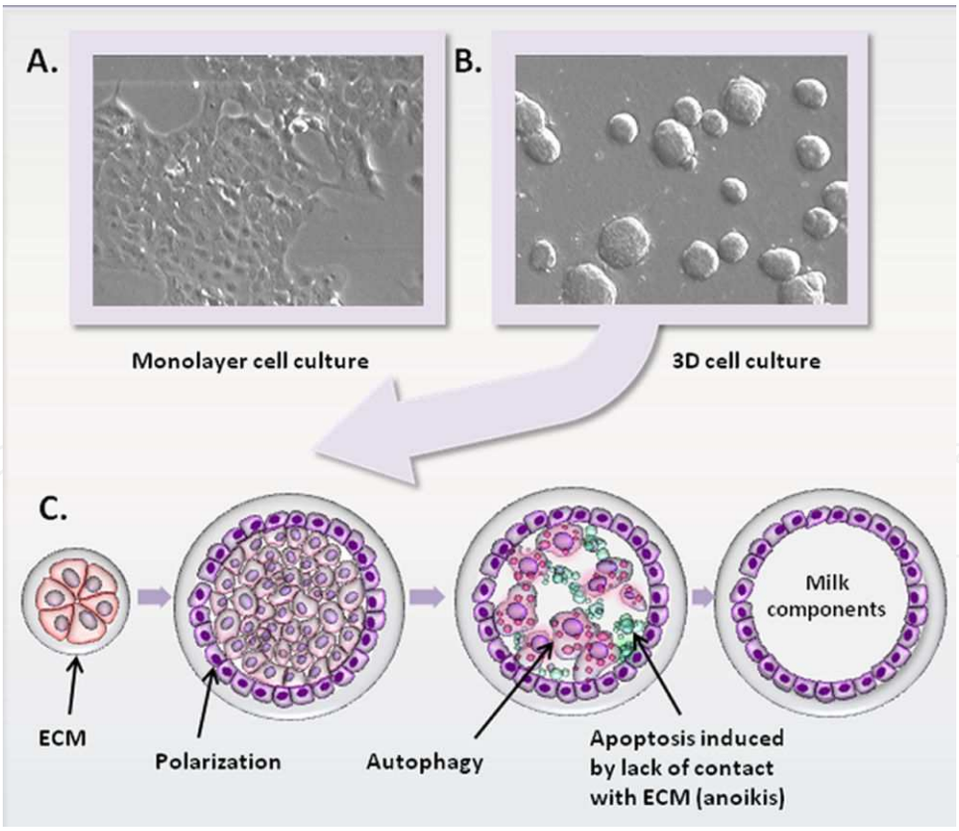

Figure 2. Formation of alveoli-like structures by mammary epithelial cells (MECs). (A) Image of bovine BME-UV1 MECs forming *in vitro* a monolayer on plastic surface. (B) Image of BME-UV1 cells forming 3D spherical structures *in vitro* when cultured on extracellular matrix (ECM) components. (C) Schematic representation of processes that take place

after seeding MECs within ECM: at the beginning of 3D culture cells undergo a few cycles of proliferation, forming small organoids. Next the outer layer of cells in direct contact with ECM develops an axis of apicobasal polarity, while the centrally localized cells lacking the necessary signals from the matrix undergo metabolic changes. At first the inner cells induce autophagy as a survival mechanism, but the sustained stress conditions subsequently lead to lumen formation by apoptotic cell death. The developed alveoli-like structures are able to secrete milk components into the luminal space.

2.1.3. Summary

In the process of mammary alveoli formation the outer layer of epithelial cells, which is in direct contact with ECM undergoes proper apicobasal polarization, and in the later stages develops specific secretory abilities. During alveologenesis autophagy is induced in the cells localized in the centre of the developing alveoli, as a results of the lack of contact of those inner cells with ECM. Autophagy is activated as a survival mechanism under the stress conditions connected with insufficient nutrient and energy supplies, and its main role is cells protection from potential damage of mitochondria and genome instability. The sustained stress conditions in the centre of the alveolar structures lead to apoptosis induction, and elimination of the inner cells by programmed cell death, which results in formation of hollow lumens of the alveoli (Figure 2).

2.2. Extracellular and intracellular factors regulating autophagy in mammary epithelial cells during mammogenesis

2.2.1. Role of endoplasmic reticulum kinase — PERK in the induction of autophagy during alveoli formation

Lack of cell contact with ECM in the centre of developing acinar structures leads to a rapid decrease in glucose intake, which correlates with a drop in ATP levels, and progressive accumulation of reactive oxygen species [19]. In this context the subsequent induction of autophagy supports the hypothesis about the primary adaptive and survival function of this process in the inner population of MECs. Studies on the potential mechanisms taking part in the induction of autophagy during acini formation pointed at the role of endoplasmic reticulum kinase – PERK in this process. PERK kinase is known to attenuate the initiation of translation by phosphorylating eIF2α (eukaryotic initiation factor 2α), when an accumulation of misfolded proteins in endoplasmic reticulum (ER) lumen occurs. It has been shown that upon loss of adhesion MECs activate the canonical PERK-eIF2 α signalling pathway, which serves as an important transcriptional regulator of multiple autophagic genes (ATGs), such as: Atg5, beclin1, Atg8/LC3, involved in autophagosomes formation [20]. PERK not only takes part in the induction of autophagy, but also contributes to the maintenance of ATP production and stimulation of a ROS detoxification response. All together these mechanism protect cells, until the adhesion can be restored, however if the stressful conditions persist the cells finally undergo apoptotic or autophagic death. An evidence for this hypothesis was obtained in the experiment on human MCF10A mammary epithelial cells cultured on rBM. Avivar-Valderas and co-workers [20] observed that enforced PERK activation during the late stages of acinar structures development allowed the centrally localized cells to persistently occupy the luminal space. At the same time an increase in the number of basal cells was noted, suggesting that

some of the surviving MECs reattached to ECM in the outer/basal layer of acinar cells. Complementary observations were made *ex vivo* on murine mammary glands isolated at the lactation period. Immunohistochemical analysis of the expression of activated/phosphorylated PERK (p-PERK) and autophagic marker: LC3 in mammary tissue from lactating mice revealed that PERK was highly activated in the cells found in the luminal space of the mammary alveoli, as well as in the luminal epithelium, whereas LC3 was detected only in the detached cells. On the other hand, the expression of pro-apoptotic protein BimEL was weakly detected in the cells found in the luminal space of the mammary tissue, however and increased staining was observed in the epithelium of female mice with conditional deletion of PERK gene. Simultaneously, autophagy was decreased in the tissue samples from the genetically modified animals, suggesting that activation of PERK promotes autophagy and inhibits induction of apoptosis enabling a sustained survival of mammary epithelial cells during lactation [20].

2.2.2. Regulation of autophagy by signalling pathway mediated by mTOR kinase

Another kinase that may be involved in the induction of autophagy in the inner cells of the developing alveoli is AMP-activated protein kinase (AMPK). This enzyme is activated through the upstream kinase LKB1 when the cellular energy levels are reduced due to intracellular metabolic stress, leading to an increased AMP to ATP ratio. Activation of AMPK in turn leads to phosphorylation of the tuberous sclerosis complex (TSC1/2 complex), causing inhibition of mTOR. mTOR (mammalian target of rapamycin) is a conserved Ser/Thr protein kinase that regulates cell growth, cell cycle progression, protein synthesis and nutrient import [21]. In the nutrient and energy rich conditions it is also considered an inhibitor of autophagy [22]. Thus, the reduction of energy levels leads to mTOR inhibition via the LKB1 and AMPK kinases activation, and stimulation of autophagy in the cells [23]. Since AMPK activity was shown to be significantly increased in the MECs lacking contact with ECM it is highly probable that this pathway is also involved in autophagy regulation during alveolar lumen formation.

The activity status of mTOR constitutes an important switch in cell metabolism and fate. As mentioned above, when the energy and nutrient supply is sufficient the signalling pathway activating mTOR and its downstream targets is involved in translation regulation, mRNA turnover, protein stability, actin cytoskeletal organization, cell cycle progression and inhibition of autophagy [21]. This kinase is a target of a macrolide antibiotic called rapamycin, which specifically inhibits mTOR. Rapamycin was used in the studies on the mechanisms regulating the development of acinar structures formed by bovine mammary epithelial cells cultured on rBM [18]. Addition of the drug from the first day of 3D culture resulted in formation of small, underdeveloped spheroids, because rapamycin blocked cell proliferation. At the same time autophagy was induced in all cells forming the acini, as judged by high expression of the active form of LC3-II protein. The induction of autophagy prevented cells from immediate cell death, since the levels of the apotosis executor enzyme – cleaved caspase 3 were reduced in the rapamycin treated acinar structures. Results of this experiment further confirmed the protective role of autophagy in MECs, but also showed the importance of the proper timing of

autophagy induction, which should not precede the period of intensive growth of the developing acini and the proper polarization of the outer layer of epithelial cells.

2.2.3. Mitogenic function of IGF-I and its effect on the rate of differentiation of mammary alveolar structures

During the time of mammary gland development several growth factors synthesized locally in the stromal or mesenchymal compartment of the gland, such as: IGF-I (insulin-like growth factor-I), EGF (epidermal growth factor), or HGF (hepatocytes growth factor), induce cell proliferation and survival, leading to expansion of the glandular epithelium. IGF-I plays a pivotal role in mammary tissue homeostasis, stimulating cell proliferation and differentiation during mammogenesis and lactogenesis. This growth factor exerts both endocrine and local actions. It is produced in the liver in response to pituitary growth hormone (GH), but is also synthesized and secreted by the cells of the mammary gland. Signals from IGF-I are transmitted into the cell via type I IGF receptor (IGFIR) located on the surface membranes of epithelial cells. When IGF-I binds to its receptor, IGFIR associates with the p85α subunit of phosphatidylinoinositol-3-kinase (PI3K) and activates another downstream target – Akt kinase [24]. Active Akt (phosphorylated at Ser^{473}) initiates other downstream signalling components involved in initiation of proliferation or activation of survival mechanisms. Moreover, the effect of IGF-I on cell growth and metabolism, mediated by Akt involves also activation of mTOR signalling pathway. Therefore, during normal mammary gland development the mitogenic signals from IGF-I must be under control of other locally produced growth factors and systemic hormones in order to maintain the proper homeostasis in the mammary gland. In fact, studies have shown that mammary epithelial cells grown on ECM components in the presence of IGF-I formed large spheroids lacking a hollow lumen in the centre [18]. The MECs showed prolonged proliferative activity, and decreased apoptosis measured on the basis of cleaved caspase-3 expression. At the same time an increased autophagy was observed in the centrally localized cells. The intensive autophagy of these inner cells, however, might have been induced by stressful conditions evoked by the lack of contact with ECM components, and decreased availability of nutrients inside of the large organoids, rather than directly by IGF-I. In fact, another study with the use of human mammary epithelial cell line MCF-10A over-expressing IGFIR, showed that the cells formed large, misshapen acinar structures with filled lumens and disrupted apico-basal polarisation in the presence of IGF-I [25]. The investigators observed that the MECs over-expressing IGFIR showed increased proliferation and decreased apoptosis, which was connected with increased activity of Akt, as well as mTOR. The phenotype of large misshapen spheroids could also be obtained, when MCF10A cells expressed a conditionally active variant of Akt [26]. Sustained Akt activation caused enhanced proliferation, and increased cell size, along with variability in size and shape of the cells forming the large spheroids. However, when rapamycin was added to the 3D culture the morphological disruption was prevented, indicating that mTOR function is required for the biological effect of Akt action during acinar development, and that the activity of mTOR also needs to be tightly regulated [26]. Although the described studies did not examine the role of autophagy in these processes one can expect that the effect of rapamycin addition not only resulted in inhibition

of the proliferative signals induced by conditionally active Akt, but also induced autophagy, which could participate in lumen clearance.

2.2.4. 17β-estradiol and progesterone control mammogenesis by stimulation of mammary epithelial cells proliferation, regulation of gene expression, and induction of autophagy

The proper development of mammary gland is possible thanks to interactions of many cellular signalling pathways induced by intramammary factors, as well as endocrine hormones. Sex steroids belong to the important regulators of normal mammogenesis. Throughout puberty and gestation 17β-estradiol (E2) and progesterone (P4) induce proliferation of mammary epithelium and act as survival factors. Biological responses induced by both hormones are mediated by their receptors, which are located inside the cells, and translocate from the cytoplasm to the nucleus upon activation. There are two types of estrogen receptors (ERα, and ERβ), however ERα is shown to play the major function in the mammary gland. The expression of ERα was found both in the epithelial and stromal compartment of the mammary gland in many species, although in humans and heifers only mammary epithelium express ER. Progesterone can also act through two types of specific receptors: PR-A and PR-B, and the ratio in expression of both isoforms in the mammary gland is critical for the normal response to P4 [27]. PR is expressed only by mammary epithelial cells, and not all MECs show its expression, thus a paracrine interaction occurs between the PR-positive and PR-negative cells, which activates the proliferative and survival signals.

E2 is considered to be responsible for ductal morphogenesis, while P4 is critical for lobulo-alveolar development and transition from ductal to lobulo-alveolar morphology [27]. However, E2 also plays an indirect role in the alveologenesis by stimulating the PR expression in mammary epithelial cells [28]. Additionally, recent studies using the 3D culture system have shown that sex steroids may be involved in autophagy induction during mammogenesis [18]. When bovine MECs were cultured on rBM in the presence of E2 or P4 the cells formed proper acinar structures. During the development of these acini an intensified induction of autophagy was observed in the centre of the structures, as judged by the higher fluorescence intensity of the condensed pattern of GFP-LC3 autophagy marker. Additionally, apoptosis was also elevated in these cells, which led to a faster formation of the hollow lumen inside the spherical structures. Moreover, in case of 17β-estradiol it was shown that this hormone not only accelerated formation of the membrane-bound form of LC3 (LC3-II), but also increased the level of the LC3-I protein [18]. It is well established that both sex steroids exert an influence on target cells through a genomic pathway after binding to their receptors that translocate to the nucleus. Inside the nucleus the activated receptors associate with co-activators, or co-repressors, and finally regulate gene transcription by binding to target genes on the specific sites of the promoter regions, called response elements (ERE – estrogen response element, PRE – progesterone response element) [29, 30]. The observed increase in the total amount of LC3 protein indicates that E2 could enhance the expression of *LC3* gene. More recently steroids, especially estrogen (E2), have been found to exert rapid, non-genomic effects via membrane-bound receptors (mER), causing stimulation of cytoplasmic signalling pathways, such as: MAPK, and PI3K/Akt [31, 30]. So far the non-genomic molecular mechanism of steroids has

not been investigated in regard to their possible influence on autophagy induction. However, John and co-workers [32] reported that beclin1 is able to bind with ERα, and the interactions between these proteins may modulate their action. Thus, sex steroid play a major role in the control of mammary gland development not only by acting as prosurvival factors, and stimulating epithelial cells proliferation, but also by regulating autophagy during alveoli formation. Furthermore, it is possible that E2 and P4 may regulate the action of other intra-mammary factors in the mammary epithelium by interactions of the signalling pathways induced by these endocrine and local factors (Figure 3).

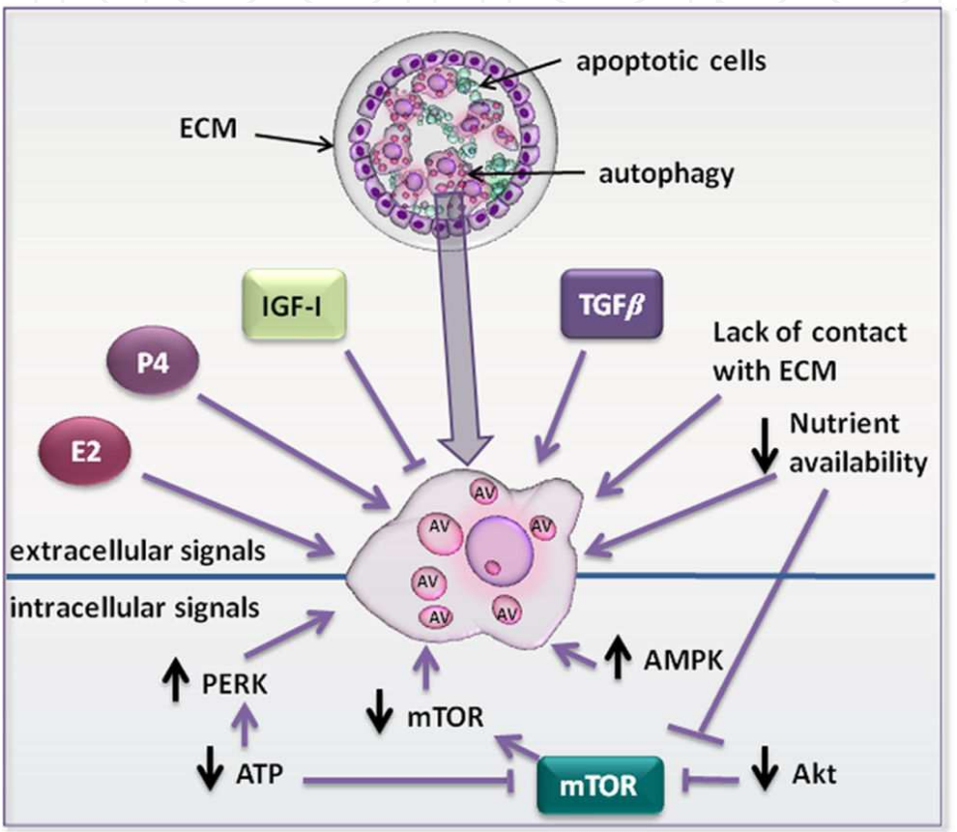

Figure 3. Extracellular and intracellular factors regulating autophagy induction in mammary epithelial cells during the process of lumen formation in mammary alveoli.

2.2.5. Summary

Induction of autophagy in the centrally localized cells of developing acini is regulated by several intracellular pathways and extracellular factors. The stress caused by insufficient nutrient and energy supply in the centre of the alveolar structures activates PERK kinase, which is an important transcriptional regulator, controlling the expression of autophagic (Atg) genes. Increased intracellular metabolic stress inside the developing alveoli also activates AMPK kinase, which inhibits the mTOR mediated signalling pathway leading to autophagy induction. During mammogenesis, the balance between proliferation and cell death processes is also controlled by locally secreted growth factors (i.a. IGF-I), and endocrine hormones (i.a.

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sex steroids). IGF-I is an important survival factor, involved in stimulation of the enhanced growth of mammary epithelium during mammogenesis, whereas E2 and P4 play a major role in the control of mammary gland development by stimulating epithelial cells proliferation, as well as regulating autophagy induction during alveoli formation (Figure 3).

2.3. Role of autophagy in mammary gland involution

Mammary gland shows full functional activity during lactation, when the lobules contain fully developed alveoli formed by differentiated mammary epithelial cells secreting milk components into the luminal space. The surrounding myoepithelial cells contract, releasing the milk further to the ductal network, which delivers the milk to the nipple. The process of milk synthesis is under control of galactopoetic hormones (prolactin – PRL, growth hormone – GH), which stimulate the expression of milk proteins, survival of the glandular epithelium and contractions of the alveoli (oxitocin). After the period of functional activity, when females stop feeding their offspring the mammary gland regresses and returns to the state of development similar to the one prior pregnancy. This stage of remodelling is termed involution. Involution can be gradually initiated in the mammary gland, starting from the peak of lactation, because the young are progressively weaned. In case of dairy animals it starts with the natural, progressive decline in the milk yield. Alternatively, the mammary gland involution can be induced by litter removal (forced weaning), or in diary animals by termination of milking.

The withdrawal of suckling or cessation of milking results in the interruption of the release of galactopoetic hormones, which leads to milk stasis, and a rapid decline in milk synthesis caused by downregulation of genes involved in this process. In rodents, which have been extensively used as a model for studying the progress of mammary gland involution, forced weaning very quickly (within 24h after pup removal) leads to the first signs of apoptotic cell death of the epithelium, as some of the MECs are shed into the lumens of the alveoli. This stage, however, is still reversible, and the renewal of suckling preserves the structure of the secretory tissue. When the involution progresses several other processes take place, leading to the regression of the glandular epithelium.

At the time when the first apoptotic cells can be observed in the luminal space, MECs which remain within the alveoli begin reabsorbing the residual milk. Additionally, it has been shown that these cells undergo a change in their phenotype from secretory to phagocytic, which enables them to actively reabsorb also the apoptotic cells from the lumens by a process resembling efferocytosis [33]. During efferocytosis the cell membrane of phagocytic cell engulfs the apoptotic cell, forming a large fluid-filled vesicle, called efferosome or phagosome, which contain the dead cell. The efferosome subsequently fuses with lysosome, causing degradation of the engulfed material. The change in the phenotype of MECs requires changes in the expression of many genes, and thus, is thought to be transcriptionally-mediated. It has been shown, that more than 20 traditional markers of lysosomal activity are upregulated within 24h of forced weaning, and LC3 was detected among these upregulated proteins [33]. Since it is one of the key proteins involved in autophagosomes formation and fusion of lysosomes with autophagic vacuoles, these results indicate that autophagy is induced during the early stages of involution. Although there is no additional information on the role of autophagy during the

initial phase of involution, there are evidence showing participation of this process when the regression of the mammary gland progresses.

In the second phase of involution proteolysis of the extracellular matrix and further apoptosis of the secretory epithelium takes place, causing the alveoli to collapse. There is an increase in expression of the protease genes, such as plasminogen activators (serine proteases), that induce the formation of active plasmin from plasminogen. Subsequently, plasmin activates matrix metalloproteinases (MMPs), which are responsible for the proteolytic degradation of basement membrane and ECM of the mammary gland. Removal of ECM induces apoptosis of the epithelial cells, that failed to respond to the first phase of apoptotic signals. The large number of apoptotic cells and debris are removed by phagocytosis performed by professional and non-professional phagocytes (macrophages, and epithelial cells, respectively) [34]. Finally, in the last stage of involution the regrowth of stromal adipose tissue is observed, filling the space of the regressed epithelium. The described course of mammary gland regression concerns the situation when lactation is separated from gestation by a dry period, during which the mammary gland remains in a quiescent state. It was extensively studied in rodents, and is often considered to reflect the general changes during the remodelling of the mammary gland in mammalian species. However, it is well documented that the mammary gland involution in ruminants differs in a significant manner. In cows and goats there is a characteristic overlap between the periods of lactation and next pregnancy, which means that these animals are typically pregnant when the involution is induced by termination of milking. Thus, the high levels of pregnancy hormones stimulating the development of new secretory tissue oppose the stimuli for mammary involution initiated by the milk stasis.

2.3.1. Role of autophagy in the regenerative involution of ruminant mammary gland

The nonlactating period before parturition in diary animals is termed the dry period. During this time the morphological changes in the mammary gland of ruminants (especially cows) are less pronounced than those occurring in the involuting glands of mice or rats. They reflect the change in the secretory state of the mammary epithelium, rather than the characteristic features of the tissue regression. The alveolar structure remains mostly intact during bovine mammary gland involution, even after several weeks of the dry period, although about 30 days after milking cessation the luminal area in the mammary tissue decrease and epithelial cells exhibit few secretory vacuoles [35]. Some of the bovine MECs undergo apoptotic cell death during involution, however, this subpopulation is significantly smaller than in rodents. It is considered that the nonlactating period in dairy cows serves to enhance the replacement of the senescent mammary epithelial cells prior to the next lactation, and thus, the processes taking place during that time are described as regenerative involution. Interestingly, studies have shown that bovine mammary tissue during the dry period shows signs of autophagy. It is manifested by increased expression of beclin1 and a high number of cells with typical morphological features of autophagy (autophagosomes and autophagolysosomes) [36]. Furthermore, *in vitro* studies on BME-UV1 bovine mammary epithelial cells revealed that cells partially devoid of nutritional factors and bioactive compounds induce formation of the autophagosome membrane-bound LC3-II form [37]. These experiments aimed at reflecting the

conditions observed in the bovine mammary gland during dry period, when enhanced competition of intensively developing fetus and mother organism for nutritional and bioactive compounds creates a state of temporary malnutrition of MECs. When the concentration of fetal bovine serum (FBS) was significantly reduced in the culture medium of BME-UV1 cells (from standard 10% to 0.5%), the activity of mTOR kinase was significantly decreased, which corresponded with the induction of autophagy. Moreover, autophagy induced by FBS-withdrawal was inhibited by an addition of IGF-I, or EGF. Both growth factors play a prosurvival role in MECs during mammary development, whereas at the dry period their activity is decreased, similarly to the decreased levels of lactogenic hormones. Simultaneously the levels of sex steroids are elevated due to the pregnancy overlapping the period of glandular involution. The *in vitro* studies demonstrated, that in the presence of E2 or P4 bovine MECs cultured in FBS-deficient conditions showed higher levels of autophagy, which suggests, that these hormones additionally stimulate the induction of this process [37]. Thus, autophagy may be induced in bovine mammary epithelial cells as an additional survival process, which participates in preservation of the glandular morphology during involution.

Additionally, it was shown that autophagy can also be stimulated by transforming growth factor – beta 1 (TGF-β1), a cytokine classified as local growth inhibitor and apoptosis inducer in many cell types, including MECs. TGF-β1 expression was shown to be high during puberty and involution, low during gestation, and undetectable at the time of lactation. This growth factor can regulate cellular processes by a specific signalling pathway, which is induced upon binding of TGF-β1 to its membrane receptors (TβRI and TβRII). The receptors then form heterocomplexes and activate downstream components - the Smad proteins [38]. Smads transmit the signal to the nucleus, where they play a role of transcription factors and bind to DNA on the promoter region regulating the transcription of specific genes. TGF-β1 was shown to regulate the expression patterns of cyclins involved in the cell cycle progression, cell adhesion elements, such as integrins, and IGF binding proteins (IGFBP-3,4 and 5), which regulate the activity of IGF within the mammary gland [27, 39]. For example, IGFBP5 prevents binding of IGF-I to its receptor and inhibits the prosurvival signals. TGF-β1 is also able to induce apoptosis in mammary epithelial cells through the mitochondrial pathway involving: activation and translocation of the proapoptotic protein Bax to mitochondrial membranes, release of cytochrome c, and activation of the executor enzyme caspase-3 [40]. The experiments on bovine MECs revealed, that this cytokine also increased the level of LC3 and beclin1 proteins, indicating the direct role of TGF-β1 in autophagy induction. Moreover, it was found that the high expression of TGF-β1 receptors in the involuting bovine mammary tissue correlated with increased levels of beclin1 and downregulation of growth hormone receptor (GH-R) and IGF-I receptor (IGF-IRα) in this tissue [36].

The induction of autophagy by TGF-β1 was also observed during mammary acini formation in the studies with the use of 3D culture system [18]. These results correspond with other findings, showing that TGF-β1 is responsible for regulation of growth and pattering of the mammary ductal tree during mammogenesis, and can partially act by modulation of the effect of IGF-I on the developing tissue [41, 39].

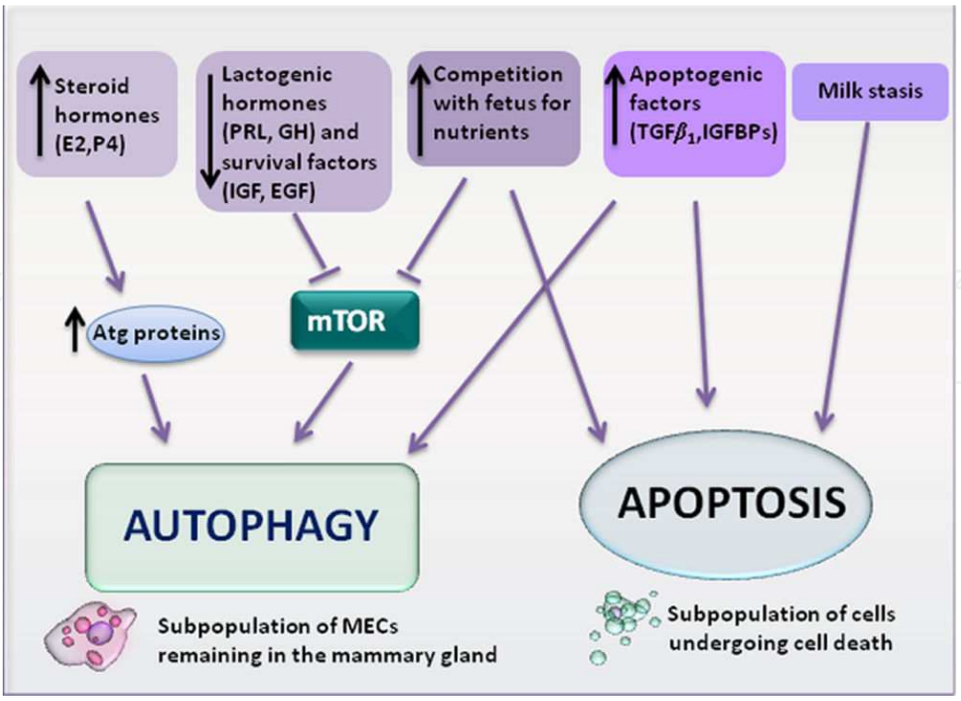

Figure 4. Endocrine hormones, auto/paracrine factors and intramammary conditions inducing autophagy and apoptosis in bovine mammary epithelial cells during regenerative involution.

2.3.2. Summary

In the period of mammary gland involution, during which regression of the secretory epithelium takes place returning the gland to the quiescent state, autophagy seems to be involved in the efferocytosis of the apoptotic epithelial cells. In case of the regenerative involution of bovine mammary gland autophagy is induced as a survival mechanism participating in the preservation of the glandular epithelium prior to next lactation. When milking is terminated a small population of mammary epithelial cells undergo apoptotic cell death, due to milk stasis and increased levels of proapoptotic factors, such as: TGF-β1. The remaining cells down-regulate milk secreting pathways and await parturition by inducing autophagy, as a mechanism which stabilizes intracellular supplies of energy and amino acids at the time of enhanced competition of intensively developing fetus and mother organism for nutritional and bioactive compounds. Additionally, local factors, such as TGF-β1, and pregnancy hormones (17β-estradiol and progesterone) stimulate autophagy during the dry period, suggesting a possible role of these factors in the control of the balance between apoptosis and survival of the epithelial cells in the involuting bovine mammary gland (Figure 4).

3. Autophagy and breast cancer

Most of breast malignancies arise in the terminal duct lobular units (TDLUs). In general, carcinomas are characterized by the loss of epithelial polarity and tissue organization. Cancer

cells, which remain within the basement membrane of the mammary ductal-lobular system are classified as benign in situ carcinomas, whereas when neoplatic cells invade into the adjacent stroma the tumour becomes malignant [42]. Early premalignant breast cancer lesions, such as hyperplastic lesions with atypia and carcinoma *in situ* are characterized by a complete or partially filled lumen [1]. Moreover, *in vitro* experiments with the use of 3D culture system have shown, that human breast tumour cell lines are not able to form acinar structures with the centrally localized hollow lumen, and polarisation of cells surrounding this lumen. Instead they develop into nonpolarized clusters with limited differentiation [43, 44]. Both apoptosis and autophagy have been shown to be involved in the process of lumen clearance, however, autophagy is thought to be induced first as a survival mechanism in the central acinar cells, which are under increased metabolic stress connected with the lack of contact with ECM, hypoxia and decreased nutrient and energy supplies. Karantza-Wadsworth and co-workers [17] have shown that monoallelic deletion of beclin1 (beclin1$^{+/-}$) that leads to defective autophagy, causes increased DNA damage, and genome instability in cells. When defective autophagy is synergized with defects in apoptosis machinery, mammary tumorigenesis can be promoted.

Perturbations in autophagy has been implicated in the pathogenesis of diverse disease states, including cancer. The monoallelic deletion of beclin1 is observed in 50% of breast tumours [45]. Human breast carcinoma cell lines, as well as tumour tissue have decreased beclin1 levels, while mammary tissue from beclin1$^{+/-}$ mice shows hyperproliferative, preneoplastic changes [46]. Moreover, beclin1$^{+/-}$ immortalized mouse mammary epithelial cells, which exhibit compromised autophagy under metabolic stress, cause accelerated tumorigenesis after allogeneic transplantation into nude mice [17]. On the contrary, beclin1 ectopic expression in MCF-7 breast cancer cells, which are tetraploid but have only three beclin1 copies, led to a slower proliferation of these cells *in vitro*, as well as *in vivo* in the xenograft tumours. These findings indicate that beclin1 is a haploinsufficient tumour suppressor [47]. Furthermore, when deficiency in autophagy synergizes with defective apoptosis the response to the environmental stress is impaired and tumorigenecity is increased, promoting tumour growth.

On the other hand, autophagy as a known survival mechanism preserves cell viability during periods of nutrient limitation and hypoxia, which suggests that it can sustain cellular metabolism within the tumour. Metabolic stress is a common feature of solid tumours, resulting from inadequate vascularisation, and causing nutrient, growth factors, and oxygen deprivation [48]. It has been shown that solid tumours formed by cells with defective apoptosis are able to survive the metabolic stress by inducing autophagy [49]. Thus, when tumour cells have intact autophagy it may be induced as an adaptive response to anticancer agents, in which case autophgay may act as a treatment resistance mechanism prolonging tumour cell survival. It is especially important in apoptosis-defective cancers, which rely on autophagy under stressful conditions. In this case inhibition of autophagy should inhibit cancer cells' survival and enhance the efficacy of anticancer treatment [45]. Attempts to use autophagy inhibitors to sensitize cancer cells to treatment have been recently reported. For example, knockdown of autophagic genes in MCF-7 and T-47D breast cancer cells, combined with tamoxifen or 4-hydroxy-tamoxifen treatment, resulted in decreased viability of these cells [50, 51]. Hydroxy-

chloroquine, which is a lysosomotropic agent causing increase in intralysosomal pH, and impairing autophagic protein degradation, has been used in clinical trials to modulate autophagy in metastatic breast cancer [45]. On the contrary, autophagy-deficient cancer cells with intact apoptotic machinery are shown to be particularly sensitive to therapeutically agents inducing metabolic stress (e.g.anti-angiogenic drugs), as these drugs cause apoptosis of the tumour cells. However, in a situation, when tumour cells show defective autophagy and apoptosis the approach to treatment should be different. As mentioned previously, simultaneous deficiency in autophagy and apoptosis makes the tumour cells susceptible to metabolic stress and DNA damage, leading to genome instability. Thus, use of metabolic or replication stress-inducing agents may cause further DNA damage in these cells, resulting in enhanced tumorigenic potential and development of drug resistance [17].

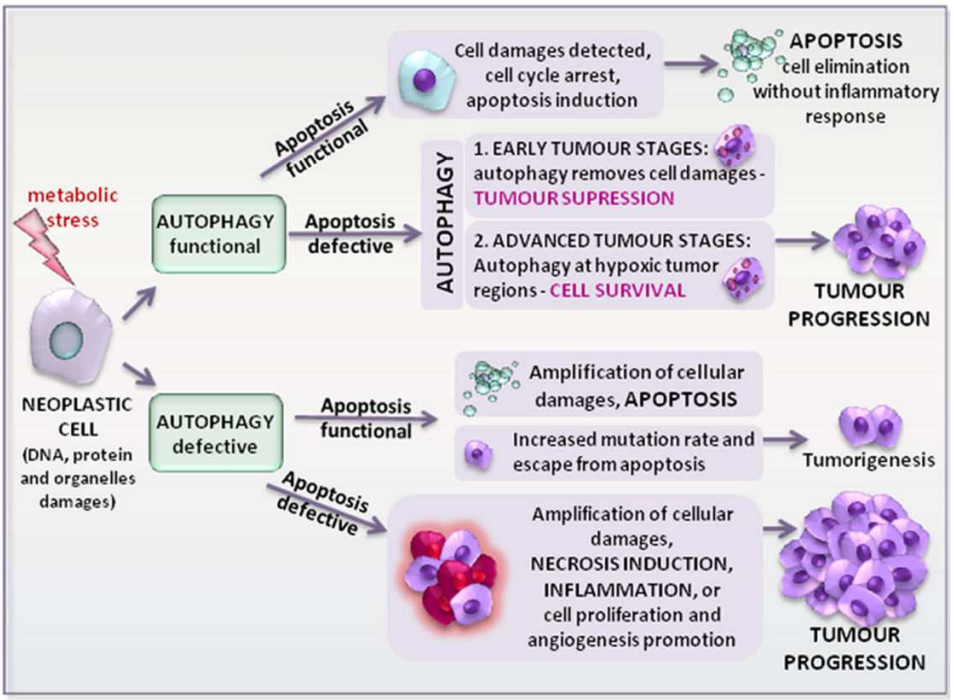

Figure 5. Different scenarios of response to metabolic stress in mammary tumorigenesis, depending on the functional status of autophagy and apoptosis in the tumour cells.

Finally, recent studies have pointed at a possible, yet still unexplored role of autophagy during invasion and metastasis. The studies with the use of 3D culture system revealed, that autophagy is induced in cell lacking the direct contact with ECM. The ability to survive in the absence of normal ECM is critical for metastasis, since cancer cells in the bloodstream or secondary tissue sites are either deprived of matrix or exposed to foreign matrix components [52]. The metastatic, secondary tumours are often resistant to therapy. Disseminated tumour cells, prior to development into the secondary tumours can remain in a dormant state for many years. *In vitro* and *in vivo* studies on mice have shown, that inhibition of β1-integrin, one of the subunits of integrin receptor responsible for cell contact with ECM, prevents proliferation of tumour cells, but not their viability, leading to induction of dormant state [53, 54]. Other studies have

shown that cell detachment from ECM induces autophagy [16, 18], and blocking β1-integrin function in attached human MECs is also sufficient for autophagy induction [16]. Therefore it is possible that detachment-induced autophagy in disseminated tumour cells may promote survival of these cells in the dormant state.

All presented results show the complexity of the role of autophagy in cancer development and progression. The possible role of activated, as well as defective autophagy in tumorigenesis is summarized in Figure 5. Research work continues to determine the molecular pathways regulating autophagy in tumour cells on different stages of tumour development. Results of the future studies may be beneficial for proper modulation of autophagy during cancer treatment and prevention.

Nomenclature

AMPK – AMP-activated protein kinase

E2 - 17β-estradiol

ECM – extracellular matrix

eIF2α – eukaryotic translation initiation factor 2 alpha

ER – endoplasmic reticulum

GFP-LC3 – LC3 (Atg8) protein fused with green fluorescence protein (a marker of autophagy)

GH – growth hormone

IGFBPs - insulin growth factor binding proteins

IGF-I – insulin-like growth factor-I

MECs – mammary epithelial cells

mTOR – mammalian target of rapamycin

P4 – progesterone

PERK – endoplasmic reticulum kinase

rBM – reconstituted basement membrane

TDLU - terminal duct lobular unit

TDU – terminal ductal unit

TEB – terminal end bud

TGF- β1 – transforming growth factor beta1

TSC1/2 – tuberous sclerosis complex (taking part in inhibition of mTOR kinase pathway)

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