UNIVERSITY OF EASTERN PIEDMONT

SCHOOL OF MEDICINE

Department of Health Sciences

Master's Degree in Medical Biotechnologies

Master's Thesis

Differential Modulation of Beclin-1 Splicing in 2D *versus* 3D Breast Cancer Cell Cultures: An In-Vitro Modelling.

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Academic Year 2023 - 2024

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ABSTRACT

Rationale: Traditional 2D monolayer cell cultures have been used in cancer research. However, these models fail to replicate the microenvironment and heterogeneity of real solid tumors. Researchers are adopting 3D cell culture models, which more closely mimic *in vivo* tumor characteristics. 3D tumor models accurately reproduce gradients found in solid tumors, such as oxygen and nutrients decreasing towards the center, a condition known to activate autophagy and to maintain cancer cell dormancy and stemness. Autophagy maintains cellular homeostasis by degrading unnecessary or damaged proteins and organelles. The study focuses on BECN1, a key regulator of autophagy, that was shown to undergo splicing and whose expression is frequently found decreased in breast cancers due to monoallelic deletion. The aim of this study was to examine how three alternative splicing isoforms of BECN1 (wt, α, β and γ) are modulated during the transition from 2D to 3D and back to 2D cell culture conditions.

Study Plan: In this study, we investigated the differential splicing of BECN1 in breast cells grown in suspension versus adherent conditions. We compared the growth kinetics of MDA-MB231 and MCF-10A cells and expression of mRNA transcripts and protein levels of the BECN1 isoforms in 2D and 3D models. After 96 hours, we transferred the spheroids back to adherent culture conditions and monitored them for reattachment, secondary colony formation, BECN1 isoform transcript and protein expression. We treated spheroids with Spautin-1 (10μM), an inhibitor of BECLIN-1, and with Madrasin (30μM), an inhibitor of the spliceosome, to study the regulation of alternative splicing and the functioning of the isoforms.

Results: Our findings show that MDA-MB231 cells (a triple-negative representative breast cancer) grown in monolayer conditions proliferated faster and formed larger spheres than MCF-10A cells (a benign representative counterpart) over four days. We observed changes in splicing in 3D cell culture conditions characterized by downregulation of BECN1 isoform mRNA transcripts in both cell lines. Notably, on switching from 3D to 2D conditions, MDA-MB231 cells exhibited enhanced secondary colony formation and a reversion in alternative splicing, while MCF-10A cells showed no phenotypic change. Treatment with Spautin-1 caused fluctuations in BECN1-wt mRNA expression over the four days, while Madrasin dysregulated BECN1 isoform transcription after only 24 hours with a detrimental impact on spheroid development of MDA-MB231 cells.

Conclusion: This study presents substantial evidence that the BECN1 isoforms are differentially spliced in adherent and non-adherent cell culture conditions.

INTRODUCTION

1. INTRODUCTION

1.1. BREAST CANCER

Breast cancer (BC) stands as the second most commonly diagnosed cancer worldwide and the most prevalent tumor among women as per the latest global statistics from 2022 (Ferlay *et al., 2024)*. Having the fourth highest mortality, BC poses a grave threat to women's health on a global scale attributed to increasing incidence due to various behavioural factors, including alcohol consumption and low physical activity. Additionally, the management of the disease with improved screening, diagnostic, and therapeutic advancements has increased the life expectancy in developed countries. Despite these advancements, the mortality rates have risen significantly in developing or underdeveloped regions like Sub-Saharan Africa, primarily due to inadequate and late-stage detection and limited access to healthcare services (Arnold *et al.*, 2022).

The Cancer and Steroid Hormone Study conducted by the Centre for Disease Control and Prevention showed compelling evidence for a genetic component to breast cancer. The analyses conclusively demonstrated that individuals with breast cancer were significantly more likely to have a family history of the disease than controls (Claus, Risch and Thompson, 1990). The prevalence of germline mutations in *BRCA1* and *BRCA2* portray a high risk of breast cancer, characterized as Hereditary Breast and Ovarian Cancer (HBOC) (Huber-Keener, 2022).

Breast cancer arises from DNA damage and genetic mutations that can be influenced by estrogen exposure and that affect the repair of DNA damage, cell cycle regulation, maintenance of genome stability, and other important physiological processes. In some cases, the inheritance of DNA defects in tumor suppressor genes like *BRCA1* and *BRCA2*, occurs. Consequently, a family history of ovarian or breast cancer indicates a heightened risk for the development of BC (Yoshida, 2021). *BECN1*, our gene of interest, is located on chromosome 17q21 in close to *BRCA1* and displays monoallelic loss in a considerable percentage of breast cancer cases. However, unlike the *BRCA1* mutation which is considered to be a driver mutation,the *BECN1* mutation has been considered to be a passenger mutation due to it's proximity with *BRCA1* (Laddha *et al.*, 2014). This concept has been challenged by the study of Tang et al. in 2015 who demonstrated that decreased mRNA expression of the autophagy gene BECN1 may contribute to the pathogenesis and progression of HER2-enriched, basal-like, and TP53 mutant breast cancers independently of *BRCA1* deletion (Tang *et al*, 2015). Rajendran and Deng

identified genes (*TP53*, *PIK3CA*, *MLL3*, *TTN*, *FLG*, *DNAH14*, *GATA3*, *ERBB2*, *RYR2*, *HRNR*, *NBPF12*, *RUNX1*, *NOTCH2*, and *OBSN* as driver genes in breast tumorigenesis (Rajendran and Deng, 2017).

Due to the groundbreaking work of Perou, Sørlie, and colleagues, breast cancer has been categorized as four clinically relevant molecular subtypes that include: luminal A, luminal B, HER2-enriched, and basal-like. Histological evidence has contributed to further division of these subtypes by steroid hormone receptor (estrogen receptor (ER), progesterone receptor (PR), and HER2 status, as well as tumor proliferation measured by Ki67. They are subdivided as follows: luminal A-like subtype (ER+ or PR+, or both, HER2-, low proliferation); luminal B-like subtype (ER+ or PR+, or both, HER2-, high proliferation); HER2 subtype, non-luminal (HER2+ and ER- and PR- negative) or luminal (HER2+ and ER+ or PR+, or both); basal-like subtype (HER2- and ER- and PR-; triple-negative breast cancer (TNBC)) (Perou *et al.*, 2000) (Sørlie *et al.*, 2001). In general, the molecular distinctions also lead to differences in clinical outcomes and treatment responses. As shown in Fig. 1, the basal-like tumors clinically have the worst prognosis among all, whereas the luminal A tumors display a better prognosis (Polyak, 2007).

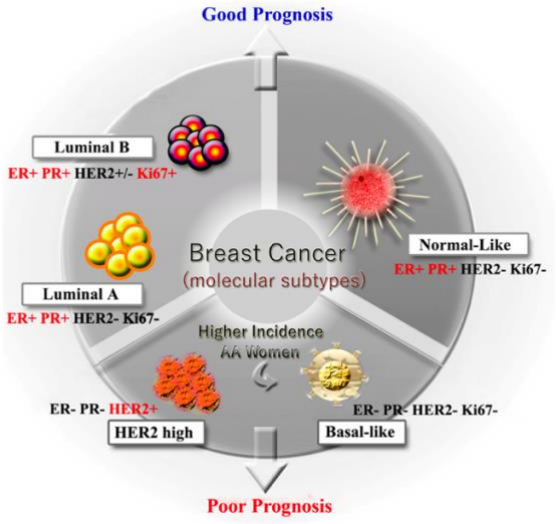

Figure 1: Different molecular subtypes of BC and the clinical prognosis (*Adapted from* (Charan *et al.*, 2020))

Diagnosis of breast cancer patients in early stages is one of the important aspects of breast cancer treatment. Diagnosis is usually done through screening techniques such as mammography. Mammography leads to a 19% overall reduction in breast cancer mortality and

is mostly beneficial for women in their 60s compared to those in their 40s. However, these techniques combined with the other imaging techniques (PET and SPECT) have limitations such as being expensive, lacking sensitivity and specificity. Therefore, biochemical markers including proteins (i.e., Her2, ER, and Ki67), mRNAs (i.e., ERα, ERβ, and ERRγ), enzymes (i.e., CEA and TSGF), and microRNAs (i.e., miR-21, miR-10b, miR-155, and miR-145) could be used as diagnostic biomarkers for early detection and monitoring in the breast cancer patients to improve the overall management of the disease (Jafari *et al.*, 2018).

Due to the heterogeneous nature of BC, morphologically and molecularly, numerous approaches such as MammaPrint, Oncotype DX, and PAM50 have been developed to classify breast tumors to inform prognosis and guide treatment. The Oncotype DX has been widely used clinically to predict the recurrence risk of patients with estrogen-receptor-positive (ER+) breast cancer. It employs 16 marker genes and 5 control genes to calculate a recurrence score, which is used to stratify patients into three prognostic groups which are high-, intermediate, and low-risk(Gianni *et al.*, 2005). The MammaPrint genomic test employs a panel of 70 genes to predict the recurrence risk of patients with breast cancer irrespective of estrogen receptor status(Cardoso *et al.*, 2016).

1.2. ALTERNATIVE SPLICING IN BREAST CANCER

During the process of gene expression, as we move from gene to protein, numerous modifications occur to the gene itself as well as the resulting protein. Among these modifications, alternative splicing stands out as a significant event. Alternative splicing is a vital process in post-transcriptional mRNA processing and gives rise to numerous mature mRNAs with various structures and functions. In this process, exons are taken together in different combinations and introns are removed (Piovesan *et al.*, 2016). Alternative splicing enhances protein diversity by expanding the limited number of gene sequences to a much larger proteome based on different combinations of the sequences in the mRNA of one gene. More than 90% of multi-exon genes undergo alternative splicing (Pan *et al.*, 2008). A proteome-wide mass spectrometry analysis estimated that approximately 35%-40% of the genes undergo alternative splicing to generate multiple protein isoforms (Kim *et al.*, 2014).

Dysregulated alternative splicing represents a significant contributing factor to many human diseases including cancer. When splicing errors arise within genes involved in critical cellular processes such as cell adhesion, proliferation, and cell cycle regulation, there’s a profound

impact on cancer progression (Martínez-Montiel, Rosas-Murrieta and Martínez-Contreras, 2015). For instance, Bcl-x, a regulator of apoptosis, could be alternatively spliced to produce two isoforms with opposed roles in the modulation of apoptosis: the short isoform Bcl-xS promotes whereas the long isoform Bcl-xL suppresses apoptosis. Furthermore, the overexpression of anti-apoptotic Bcl-xL has been linked to an increased risk of metastasis in breast cancer (Mercatante *et al.*, 2001). Similarly, the cell surface molecule CD44 possesses nine variable exons between its constitutive exons, which account for the generation of more than 20 splice variants. The inclusion of one or more of the variable exons generates CD44 variant isoforms (CD44v) while skipping all of the variable exons results in CD44 standard isoforms (CD44s). CD44v is expressed predominantly in epithelial cells, while CD44s is found largely in mesenchymal cells. Therefore, the splicing switch from CD44v to CD44s represents a critical event in the epithelial-to-mesenchymal transition (EMT) and breast cancer metastasis (Brown *et al.*, 2011).

BRCA1, a well-established tumor suppressor gene involved in DNA damage repair, is associated with hereditary breast cancer. *BRCA1* gene, as a result of alternative splicing, has three major isoforms that depend on the regulation of exon 11, including BRCA1 full-length (inclusion of all coding exons), BRCA1-Δ11 (skipping of exon 11), and BRCA1-Δ11q (partial skipping of exon 11). The BRCA1-Δ11q isoform is formed from the usage of an alternative donor splice site in exon 11, leading to the exclusion of most exon 11 sequences. It has also been reported that breast cancer patients having exon 11 mutations exhibit worse overall survival rates in comparison to non-exon 11 mutation carriers. In addition, BRCA1-Δ11q is associated with tumorigenesis and drug resistance(Nielsen, Van Overeem Hansen and Sørensen, 2016)(Wang *et al.*, 2016).

The oncogene HER2, which encodes a tyrosine kinase receptor, plays a pivotal role in the pathogensis of a distinct breast cancer subtype characterised by HER2 overexpression or amplification. The HER2-positive breast cancer subtype is characterised by a high mitotic index and elevated metastatic potential. Δ16HER2, a splice variant of HER2, lacks exon 20 which encodes a small extracellular region. Emerging evidence suggests that the co-expression of the wild-type HER2 oncoprotein with Δ16HER2 significantly increases the heterogeneity of HER2-positive disease, affecting its biology, clinical course, and treatment response (Weigelt and Reis-Filho, 2013). It is also reported that Δ16HER2 initiates a key oncogenic

signal that has a significant impact on HER2-driven breast cancer stemness, tumorigenesis and drug resistance as compared to its full-length counterpart (Jackson *et al.*, 2013).

Another gene of interest is KLF6, which encodes multiple protein isoforms involved in tumorigenesis and tumor progression. The isoforms include KLF6-SV1, KLF6-SV2, KLF-6-SV3. KLF6-SV1 is a key driver of breast cancer metastasis, thus providing great therapeutic potential for invasive breast cancer(DiFeo, Martignetti and Narla, 2009).

The above examples support the view that understanding the underlying mechanisms in the dysregulation of alternative splicing might highlight potential targets for therapeutic intervention.

1.3. AUTOPHAGY

Autophagy is a ubiquitous, evolutionarily conserved process in eukaryotic cells that involves the intracellular engulfment and bulk degradation of cellular structures via lysosomes. Depending on the lysosomal pathway, autophagy can be divided into three categories: a) Macroautophagy, which involves the formation of double-membrane structures called autophagosomes which enclose cytoplasmic proteins and organelles, and fuse with lysosomes leading to degradation of the contents. b) Microautophagy, which involves the direct invagination of cytoplasmic contents by lysosomes. c) Chaperone Mediated Autophagy (CMA), which involves cytoplasmic proteins binding to chaperones and then subsequently translocated to the lysosome for degradation (Yu *et al.*, 2024).

Among the described types of autophagy, macroautophagy is the most extensively researched. Macroautophagy (henceforth referred to as Autophagy) is induced in stressful conditions such as those of low nutrient or energy availability, by breaking down cellular contents into substances that can be utilised for biosynthesis or energy generation, enabling the cell to sustain its survival. In normal conditions, autophagy is induced for cellular homeostasis by targeting damaged or redundant organelles. This indicates that autophagy is principally cytoprotective, however stimulation of this process is detrimental. Autophagic dysfunction is implicated in a variety of disorders such as lung, liver and heart disease, neurodegenerative diseases, cancer, and metabolic diseases (Parzych and Klionsky, 2014).

Autophagy has been shown to have a dual role in cancer, having both tumor-suppressive and tumor-progressive roles. In its tumor suppressive role, autophagy aids in the elimination of misfolded proteins and damaged organelles which would otherwise lead to perturbations in cellular functions, accumulation of reactive oxygen species (ROS), or inflammation, which make the cell vulnerable to malignant transformation. The tumor suppressive role is derived from studies of BECN1, an important regulator of autophagy, when found deleted in breast cancer cell lines or mice hemizygous for BECN1, were susceptible to spontaneous tumorigenesis. Paradoxically, autophagy has been shown to support tumor growth and survival, in numerous ways such as metabolic adaptation of tumor cells, evading immune detection, metastasis, and resistance to cell death (Debnath, Gammoh and Ryan, 2023).

Autophagy is regulated by the metabolic sensory kinase, the mechanistic target of rapamycin (mTOR), which is a serine/threonine kinase that belongs to the PI3K-related kinase (PI3KK) superfamily and is highly conserved throughout evolution. mTOR exists in two distinct complexes known as mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2). Among these complexes, mTORC1 plays a vital role in initiating autophagy by sensing changes in extracellular and intracellular nutrient levels, particularly amino acids, as well as responding to growth factor signalling, energy status, and oxygen levels (Rabanal-Ruiz, Otten and Korolchuk, 2017).

When amino acids are abundant, mTORC1 is active near the lysosome within the cytosol. It phosphorylates unc-51-like kinase 1 (ULK1) and autophagy-related protein 13 (ATG13), two critical proteins involved in autophagy initiation. The phosphorylation at serine 757 (S757) inhibits the catalytic activity of ULK1 and negatively affects the function of ATG13, preventing the ULK1 complex from translocating to the autophagy initiation sites. However, when amino acid levels decrease, mTORC1 becomes inactive and dissociates from the lysosomal surface (Meng *et al.*, 2021). Consequently, both ULK1 and ATG13 are dephosphorylated, leading to the activation of ULK1 kinase. This activation triggers the initiation of autophagy (Zachari and Ganley, 2017).

Autophagy initiation is stimulated in response to the formation of the ULK1 complex which is composed of ULK1, ATG13, Focal adhesion kinase family Interacting Protein of 200kDa (FIP200), and autophagy-related protein 101 (ATG101) (Grasso, Renna and Vaccaro, 2018). ATG13 has also been known to interact with Atg9 to recruit Atg9 vesicles to the pre-autophagosomal structure (Levine and Kroemer, 2019).

In the continuation, the ULK1 complex phosphorylates the Ambra1 protein, facilitating the recruitment of the BECN1-Vps34 complex or BECN1-PI3KC3 complex (BECN1-Vps34-Vps15-ATG14L) (Gómez-Virgilio *et al.*, 2022). BECN1 acts as an overall scaffold for the PI3KC3 complex, enabling the localization of autophagic proteins to the phagophore. BECN1 possesses a BH3 domain which is bound by BCL-2 and BCL-XL and can lead to the disruption of BECN1-Vps34 complex formation. Vps34 phosphorylation decreases its interaction with BECN1. AMBRA1, Vps15, and ATG14 all work together to enhance Vps34 and BECN1 interaction (Levy, Towers and Thorburn, 2017). Vps34 plays a crucial role in generating phosphatidylinositol 3-phosphate (PI3P), which then recruits WD-repeat protein interacting with phosphoinositides (WIPI) and double-FYVE containing protein 1(DFCP1) effector proteins to the initiation site (Liu *et al.*, 2023). Accumulation of the PI3P-binding domain proteins at the membrane facilitates the binding of extra ATGs, which are essential for elongation and closure of the autophagosome membrane (Kocaturk *et al.*, 2019).

The expansion of the autophagosome involves two ubiquitin-like conjugation systems: ATG12-ATG5-ATG16L and ATG8 (microtubule-associated protein 1 light chain 3 (MAP1LC3 or LC3)) – phosphatidylethanolamine. In the first system, ATG12 is conjugated to ATG5 with the assistance of ATG7 (an E1-like ubiquitin-activating enzyme) and ATG10 (an E2-like ubiquitin-conjugating enzyme). The ATG12-ATG5 conjugate then binds to the ATG16L complex, which then participates in the conjugation of LC3-phosphatidylethanolamine. LC3 is derived from Pro-LC3 by ATG4 protease and is conjugated to phosphatidylethanolamine with the help of ATG7, ATG3(another E2-like enzyme), and the ATG12-ATG5-ATG16L complex (acting as an E3-like enzyme complex). The lipid-conjugated LC3 (LC3-phosphatidylethanolamine) also known as LC3-II, localizes to the autophagosomal membranes and contributes to the formation and elongation of the autophagosome (Kim and Lee, 2014) (Li *et al.*, 2017).

Besides its role in the generation and maturation of autophagosomes, the ATG8/LC3/GABARAP protein family also acts as a connector between the ubiquitous cargo and the central autophagic machinery, ensuring effective recognition and capture of the cargo within the autophagosomes (Li *et al.*, 2021). LC3-II facilitates the attachment of specific cargos and adaptor proteins to the phagophore membrane. Examples of such adaptors include p62, which can identify the ubiquitin-decorated cargo and direct them for degradation (Ascenzi *et al.*, 2021).

Following the formation of the autophagosome, it undergoes a maturation process that involves the packaging of cargo materials. This involves the participation of SNARE proteins, members of the Rab GTPase family, and Tethering factors. Autophagosome and lysosome fusion are facilitated by two specific sets of SNARE complexes: STX17-SNAP29-VAMP8 and YKT6-SNAP29-STX7. Tethering factors, including HOPS, play a role in capturing vesicles and bringing the SNARE complexes closer to the target membrane during their transport within cells. The components of HOPS contribute to the fusion of autophagosomes and lysosomes by interacting with STX17 (Chen *et al.*, 2023).

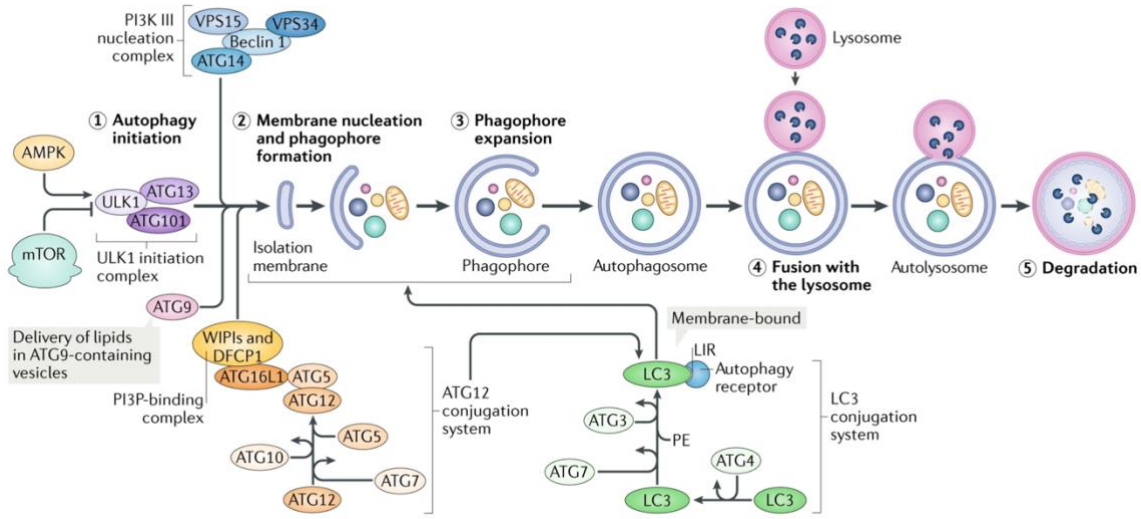

Figure 2: An illustration showing the process and key regulatory machinery of autophagy. (adapted from (Hansen, Rubinsztein and Walker, 2018)

1.4. THE GENE: BECLIN-1

Beclin-1 (BECN1), known as BCL2-interacting coiled-coil protein was the first mammalian protein indicated to be involved in autophagy. In yeast, it is known as ATG6 or VPS30 (He *et al.*, 2013). The ground-breaking research paper published by the Levine lab described BECN1 as a BCL2 binding protein connecting the two fundamental pathways of autophagy and apoptosis (Liang *et al.*, 1998).

BECN1, located on chromosome 17q21 is frequently mono-allelically deleted in various types of cancer, including breast tumors, ovarian tumors as well as prostate tumors. This suggests that the deletion of BECN1 may play a role in the development of multiple cancer types (Liang

et al., 1999). Similar to its involvement in autophagy, BECN1 exhibits a dual nature in its functionality. Initial evidence that autophagy has a role in tumor suppression in mice with allelic loss of the essential autophagy gene *BECN1*. The mice exhibited partial autophagy defects and eventually developed liver tumors (Gong *et al.*, 2012). Furthermore, BECN1 expression has been observed to be upregulated in mammospheres and the knockdown of *BECN1* in several breast cancer cell lines resulted in a decrease in the size of mammospheres, indicating that BECN1 is critical to the maintenance of breast stem cells (Gong *et al.*, 2013). Evidence has also shown that expression of BECN1 is correlated with histological subtypes in ovarian cancer. Researchers found that type I tumors, which are less aggressive than type II, were more frequently expressing high levels of BECN1 and this expression was well correlated with overall survival of patients (Valente *et al.*, 2014).

BECN1 plays a crucial role as an essential regulator of autophagy. It is the key component of the BECN1-PI3KCIII/VPS34 complex, which forms an integral part of the molecular machinery involved in autophagy. This complex consists of VPS15, VPS30/BECN1, PI3KCIII/VPS34 and ATG14/ATG14L collectively known as PI3KCIII-complex I. Its primary function is to facilitate the process of autophagosome biogenesis, contributing to the formation of autophagosomes. Additionally, there exists another complex, called PI3KCIII- complex II, which replaces ATG14 with UVRAG. This complex is essential for various cellular processes, including endocytic trafficking, phagocytosis, and autophagy (Nakatogawa, 2020), (Li, He, Zhang, *et al.*, 2012).

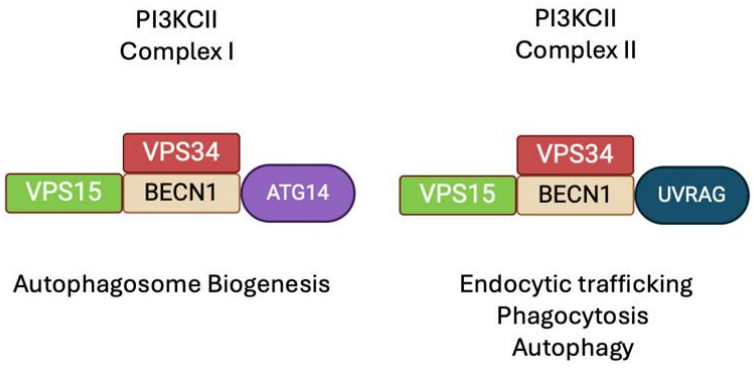

Figure 3: PI3KCIII complexes of BECN1

1.4.1. BECN1 and Its Splicing Variants

DNA sequencing of AML cells carried out by Niu and colleagues revealed the existence of a 3' alternative splice BECN1 variant, in which exon 11 is skipped resulting in a truncated C terminus. (Niu *et al.*, 2014). Another group found an additional BECN1 splice variant that lacks both exon 10 and 11 and designated BECN1 short isoform (BECN1s). It has been reported in multiple cell types. Whereas BECNIs is unable to initiate macroautophagy, its expression supports mitophagy. Further investigation showed that BECN1s selectively associates with the outer mitochondrial membrane and induces mitophagy in a PINK1 (PTEN induced kinase 1)-PRKN/PARKIN (parkin RBR E3 ubiquitin protein ligase) – dependent manner (Cheng *et al.*, 2015). Our group was also able to identify two more BECN1 splice variants (BECN1β and BECN1**γ**). BECN1β lacks exons 5, 6, 10, and 11 while BECN1**γ** lacks exons 5, 6, and 11. They found that overexpression of the BECN1β isoform reduces autophagy with a dominant negative effect over the endogenous *wild-type* BECN1 while overexpression of BECN1-γ has little effect on autophagy (Maheshwari *et al.*, 2022).

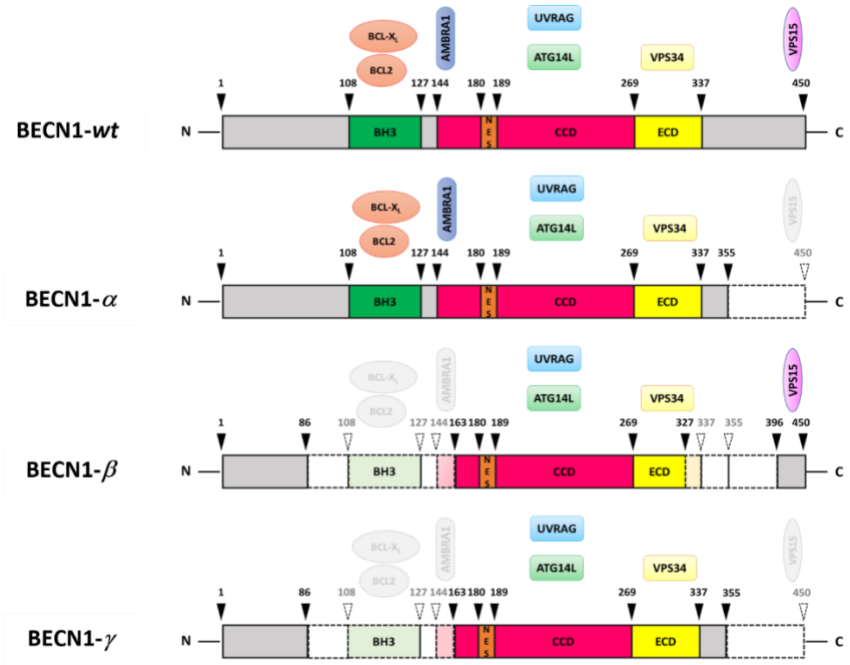

Figure 4: BECN1 (wild-type or wt) and the alternatively spliced isoforms Alpha (α), Beta (ß), and Gamma (ɣ) (adapted from Maheshwari *et al.*, 2022)

1.4.2. BECN1 Structure

The human BECN1 protein is 450 amino acids long and has a molecular weight of 60 kDa. It encompasses several structural domains that contribute to its functionality. These domains include a B-cell lymphoma 2 (BCL-2) homology-3 motif (BH3), a flexible helical domain (F), a coiled-coil domain (CCD), and an evolutionarily conserved domain (ECD) together with a β/α repeated autophagy-related (BARA) domain (Ye *et al.*, 2023). Within the CCD, there is a leucin-rich amino acid sequence responsible for its efficient nuclear export, the Nuclear Export Signal (NES) (Kang *et al.*, 2011).

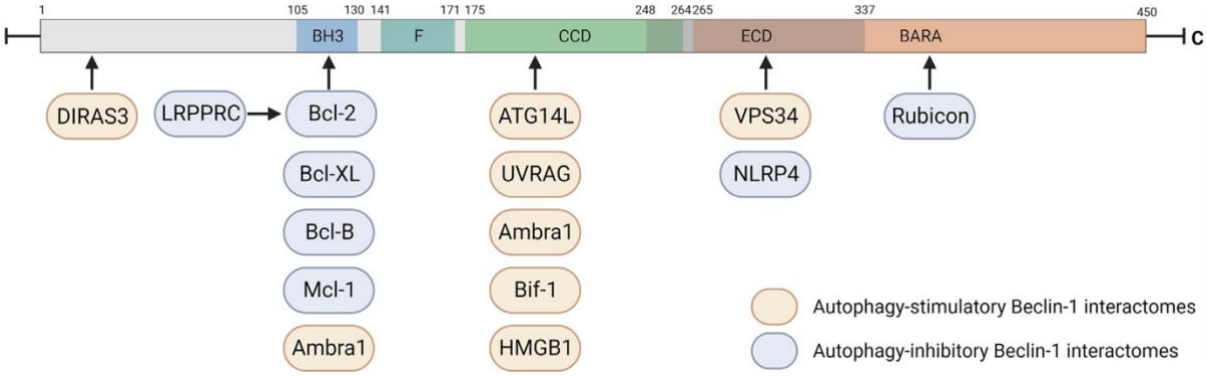

Figure 5: The molecular structure of BECN1 and its interactomes. (Adapted from Ye *et al., 2023*)

The BECN1 interactome serves as a crucial driver of the canonical autophagy pathway. The BH3 domain can bind to the BH3 binding groove present in the antiapoptotic members of the BCL-2 family, including BCL-2, BCL-xL BCL-w, and MCL-1. Research studies have demonstrated that the BCL-2 protein interacts with BECN1 impeding the formation of the BECN1-Vps34 complex and thus inhibiting autophagy(Sinha and Levine, 2008)(Zhou, Yang and Xing, 2011). The BCL-2/BCL-xL-BECN1 interaction can be influenced by some post-translational modifications. For instance, phosphorylation and ubiquitination of BECN1 and BCL-2 can either stabilize or disrupt their interaction, which may lead to inhibition or initiation of autophagy respectively (Fu, Cheng and Liu, 2013).

Defects in either BCL-2 or BECN1 affect both autophagy and apoptosis. For instance, increased Beclin 1 expression may release BAK/BAX from BCL-2 to promote apoptosis, while decreased BCL-2 expression may result in excessive BECN1-dependent autophagy (Su, Mei and Sinha, 2013). The interaction between BECN1 and BCL-2 is also regulated by c-Jun N-terminal protein kinase 1-(JNK1-) which phosphorylates BCL-2 in response to stress and

starvation. The multisite (T69, S70, and S87) phosphorylation of a BCL-2 structured loop disrupts binding of BH3-domain-containing proteins such as BECN1 and BAX (Wei *et al.*, 2008).

The activating molecule in BECN1-regulated autophagy or Ambra1 is known to interact with BECN1 and positively regulates PI3KCIII. This interaction occurs via the BECN1 BH3 domain suggesting that Ambra-1 and BCL-2 compete for BECN1. Findings suggest that Ambra1 may be important in inhibiting the tethering of BECN1 to BCL-2 consequently stimulating autophagy (Strappazzon *et al.*, 2011).

The Coiled-Coil Domain (CCD) of BECN1 is responsible for its self-association in-vivo and for acting as a central interaction platform to engage with factors including ATG14, UVRAG, Bif-1, and Rubicon to regulate VPS34 activity (Li, He, Che, *et al.*, 2012). Within the CCD, there is a leucin-rich amino acid sequence responsible for its efficient nuclear export, the Nuclear Export Signal (NES). Mutations of the BECN1 NES have been shown to interfere with its abilities to promote starvation-induced autophagy and suppress tumorigenesis (Liang *et al.*, 2001). Beyond the CCD is the evolutionarily conserved region (ECD) which is ～70 residues long. The ECD is essential for the interaction of BECN1 and VPS34 forming a complex that mediates the recruitment of other autophagy proteins including Atg5 and Atg7 to the pre-autophagosomal site(Furuya *et al.*, 2005). At the C-terminal region of BECN1 exists a BARA domain which consists of three β- sheets and α-helices (Noda *et al.*, 2012).

1.4.3. BECN1 and the Post-Translational Modifications

Post-traslatnional modifications (PTMs) encompass the modification of amino acid side chains in proteins after biosynthesis. With over 400 distinct types of PTMs identified, they exert influence over various facets of protein functionality. These modifications serve as vital molecular regulatory mechanisms, orchestrating a wide array of cellular processes. Consequently, they exert a profound impact on protein structure and function. Disruption in PTMs can lead to the dysfunction of vital biological processes and hence to various diseases (Ramazi and Zahiri, 2021).

The most common PTM of BECN1 is phosphorylation. The Sr/Thr protein kinase ULK1 which is involved in the initiation of autophagy phosphorylates BECN1 at S15 and this phosphorylation is crucial for VPS34 activation during amino-acid starvation-induced autophagy (Russell *et al.*, 2013). Nutrient deprivation-induced autophagy requires

phosphorylation of BECN1 S90 by MAPK p38 (Wei *et al.*, 2015). BECN1 is phosphorylated at S93/S96 by AMPK to enable interaction with Vps34 and form pro-autophagic PI3KC3 complexes (Kim *et al.*, 2013). The BECN1 BH3D is phosphorylated at T108 enhancing the interaction between BECN1 and Bcl2 which stabilises the BECN1 homodimer and significantly impairs BECN1-ATG14 and BECN1-Vps34 interaction (Maejima *et al.*, 2013). In nutrient deprivation conditions, ROCK1 or DAPK phosphorylate BECN1 at its BH3D anchor region, promoting BECN1-Bcl2 dissociation, while ROCK1 inhibition increases BECN1-Bcl2 interaction, downgrading starvation-induced autophagy. AKT phosphorylates BECN1 CCD at S234 and S295. EGFR tyrosine kinase binds and phosphorylates BECN1 at the CCD Y229, Y233 and BARAD Y352.

Beyond phosphorylation, BECN1 is as well acetylated by p300, a lysine acetyltransferase at K430 and K437 and is deacetylated by SIRT1 (sirtuin 1)(Xu *et al.*, 2009). BECN1 also undergoes ubiquitination, it undergoes K11-, K63- and K48-linked ubiquitination (Shi and Kehrl, 2010).

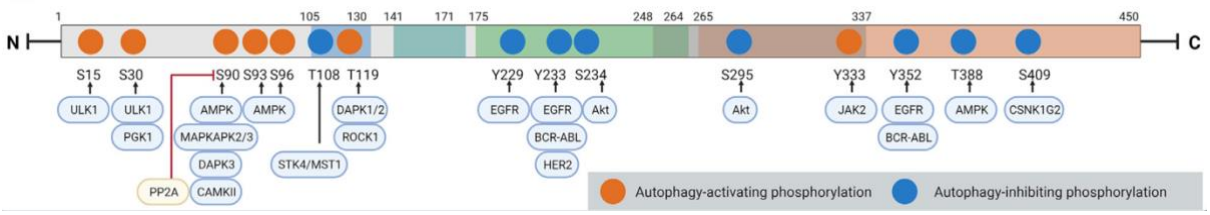

Figure 6: Phosphorylation of BECN1. Phosphorylation-modified residues that lead to the inhibition of autophagy are indicated in orange, and those with autophagy-activating effects are indicated in blue. (Adapted from Ye *et al., 2023*)

AIM OF THE STUDY

2. AIM OF THE STUDY

Traditional 2D in vitro monolayer cell cultures have been used in cancer research for decades, but these fail to capture the microenvironment and heterogeneity of solid tumors. Cells grown in 3D culture, just like in vivo, exhibit decreasing gradients of oxygen and nutrient deprivation-conditions that have been shown to activate autophagy. BECN1, a regulator of autophagy undergoes a monoallelic deletion in numerous cancers and this correlates with poor prognosis underscoring its potential as a reliable biomarker for assessing disease progression and patient prognosis.

This study focuses on analyzing the differential modulation of alternative splicing of BECN1 in two breast tumor cell lines, MCF-10A (with a benign fibrocystic phenotype) and MDA-MB231 (with a malignant triple-negative phenotype) between 2D and 3D cell culture conditions. In detail, my study aims is to analyze the mRNA transcript expression of the BECN1 isoforms in the adherent and the non-adherent cell culture conditions as well as the protein level of these isoforms to elucidate a potential role of these isoforms in the 3D culture environment.

MATERIALS AND METHODS

3. MATERIALS AND METHODS

3.1. CELL CULTURE AND TREATMENTS

For the study of *BECN1* gene splicing, we employed two breast cell lines – MCF-10A and MDA-MB-231. MDA-MB231 represents Triple Negative Breast Cancer and was derived from a 51-year-old white female by isolating the mammary gland of an adenocarcinoma via metastatic pleural effusion. MCF-10A was used as the human normal breast epithelial cell line and was isolated in 1984 from the mammary gland of a White, 36-year-old female with fibrocystic breasts.

Both the cell lines were obtained from the American Type Culture Collection (ATCC, Rockville, MD). MCF-10A cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 15% Horse Serum, 1% L-Glutamine, 1%Penicillin/Streptomycin, EGF (20ng/mL), Insulin from Bovine Pancreas (10µg/mL), Hydrocortisone (50mg/ml) and Cholera Toxin (100ng/mL). MDA-MB231 were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% FBS, 1% L-Glutamine, and 1%Penicillin/Streptomycin. All cells were cultured under standard conditions of 37°C with 5% CO_2. Cells were grown to 80% confluence prior to harvesting for any assay.

The culture medium was changed every 48 hours to maintain an adequate nutritional intake of cells. At the beginning of each assay, cells were washed with the phosphate-buffered saline (PBS) and then trypsinized with a 100X solution of trypsin (Sigma) dissolved in Puck's Buffer: this allows the cells to detach from the substrate by the cleavage of anchoring proteins, therefore allowing monolayer resuspension and subsequent plating.

Spautin 1 (SP-1, cod. SML0440; Sigma-Aldrich) was dissolved in DMSO and used at a final concentration of 10 µM. Madrasin (Mad, cod. ML-1409; Sigma-Aldrich was dissolved in DMSO and used at a final concentration of 30 µM.

3.2. CELL COUNTING

Cells were plated in sterile 12-well plates (20,000 cells/cm^2) and allowed to adhere. Cells were collected and counted in duplicates for each experimental condition. At the beginning of each

assay, cells were washed with the phosphate-buffered saline (PBS) and then trypsinized with a 100X solution of trypsin (Sigma) dissolved in Puck's Buffer. After trypsinisation, we added 400µl of new fresh media and resuspended. We then added 20µl of the cell suspension into a 96-well plate and we then added 20µl of trypan blue stain. We resuspended the mixture and then added 20µl of the mixture into the FastRead chamber and observed under a light microscope at 10X magnification. This assay is based on the principle that live cells possess intact cell membranes that exclude trypan blue, whereas dead cells do not. This enables us to distinguish between viable cells (with a clear cytoplasm) and nonviable cells (with a blue cytoplasm).

3.3. SPHEROID-FORMING ASSAY

In the 3D multicellular spheroid formation,. the cells were cultured in specific 12-well plates coated with 5 mg/mL poly 2-hydroxyethyl methacrylate (poly-HEMA, cod. P3932; Sigma-Aldrich) to prevent cell adhesion. The poly-HEMA stock solution (120 mg/mL) was prepared in 95% ethanol and dissolved under rotation overnight at 50 °C. The day after, the stock solution was diluted in 95% ethanol. The multiwells were coated with the poly-HEMA working solution (5 mg/mL) and left under the biological hood to completely dry. Then, 100,000 cells/cm^2 were seeded and maintained in culture for 4 days after treatment. Fresh medium was gently added every 48 h and supplemented with Spautin-1 (10µM) or Madarasin (30µM).

The spheroids' growth was monitored by taking pictures with a phase contrast microscope (magnification 20×, Zeiss AXIOVERT 40 CFL, Jena, Germany) at each time-point. The area of 3D spheroids was calculated using the ImageJ software (v.1.52) and indicated as an arbitrary unit (A.U.).

In a 3D-to-2D experiment, MCF-10A cells and MDA-MB231 cells were initially plated in a poly-HEMA-coated 12-well plate and allowed to grow as mamospheres until the fourth day; then, the 3D cell aggregates were collected, centrifuged and reseeded in adherent in 12 multiwell plates. The adhesion and growth capacity of the transferred mamospheres were monitored for up to 48 hours by imaging. Finally, after 48h of culture in the adherent condition, the cells were harvested in Tris-HCL buffer and processed for Western blot analysis. Total mRNA was purified with TRIzol reagent.

3.4. REVERSE TRANSCRIPTION AND PCR

Cells plated in monolayers at a density of 20,000 cells/cm^2 or in spheroids at 100,000 cells/cm^2 were treated in different conditions. After culturing, these cells were washed with cold phosphate-buffered saline (PBS), and total mRNA was purified with TRIzol reagent (T9424, Sigma-Aldrich). The mRNA samples obtained were then measured at an absorbance of A260 and A280 by the NanoDrop™ 2000 Spectrophotometer. 1 µg of mRNA was reversely transcribed into cDNA using RevertAid First Strand cDNA Synthesis Kit (K1622, Thermo Scientific, Waltham, MA, USA).

After reverse transcription, BECN1 cDNA was then amplified by PCR using Taq DNA polymerase recombinant (10342-020, Invitrogen, Waltham, MA, USA) and primer designed to identify the BECN1 isoforms, forward primer F-908: 5'-AGC TGA AGA AAA CCA ACG-3' and reverse primer R-1674: 5'-GGC AAC TAT AGA TGG CAT G-3'. The PCR product was analyzed by agarose gel electrophoresis and visualized by the ChemiDoc MP imaging System (BioRad). Densitometric analysis was performed to analyze band intensity using Image Lab Software (Bio-Rad).

3.5. BRADFORD ASSAY

The Bradford assay was conducted to quantify the amount of protein present in cultured cells. Cells, after reaching a required confluence, are lysed with the 300µL Tris-HCL buffer for each P35 Petri dish, which contains a cell membrane denaturing agent NP40, a Protease Inhibitor Cocktail (1000x) and phosphate inhibitors (Na_3VO_4, and NaF). Next, cells were maintained on ice for 30 minutes with the lysis buffer and, further, homogenates were subjected to sonication. The homogenization and sonication process enables the liberation of enzymes or proteins from the cells and, so, the quantification using Bradford reagent, the standard curve is prepared using a dilution of the bovine serum albumin (BSA), whereas samples are prepared with 5µL of homogenates diluted in 45µL of water, as indicated in the following table. In the end, 950µL Bradford's solution is added to each sample and incubated for 30 minutes at 37°C. The samples are measured at absorbance of 595mm by the NanoDrop™ 2000 Spectrophotometer using cuvettes.

Standard Curve	BSA 0.2mg/ml	H_2O	Lysis Buffer	Sample	Bradford Solution
1µg A	5µL	40µL	5µL	/	950µL
2µg B	10µL	35µL	5µL	/	950µL
3µg C	15µL	30µL	5µL	/	950µL
4µg D	20µL	25µL	5µL	/	950µL
5µg E	25µL	20µL	5µL	/	950µL
6µg F	30µL	15µL	5µL	/	950µL
BLANK	/	45µL	5µL	/	950µL
SAMPLES	/	45µL	/	5µL	950µL

Table 1) Protein Dosage with Bradford Assay.

3.6. WESTERN BLOT ASSAY

Cell monolayers or spheres were cultured on sterile 12 multi-well plates coated with or without poly-HEMA. At the end of the treatment, cells were washed with cold phosphate-buffered saline (PBS) and lysed with Tris-HCL buffer (50mM Tris HCL, 1% NP-40, 150mM NaCl and, 5mM EDTA) containing Protease Inhibitor Cocktail and phosphate inhibitors (Na_3VO_4 and NaF). After sonication, protein quantification was performed by Bradford assay: 25µg of total protein was added to 5x-Loading Buffer, followed by heating at 95 °C for 10 minutes. Cell homogenates were electrophoresed on 12.5% polyacrylamide gels at 80 V for 30 minutes and then 120V, following transfer to a PVDF membrane (Bio-Rad) using a wet transfer system at 100 V for 2 hours (Bio-Rad Laboratories).

Membranes were then blocked with 5% skim milk for one hour at room temperature and probed overnight at 4 °C with primary antibody. Thereafter, membranes were probed with peroxidase labeled secondary antibodies (anti-rabbit IgG HRP or anti-mouse IgG-HRP-linked whole antibody (Bio-Rad)) for at least one hour. The chemiluminescent signal was detected by luminol solution (PerkinElmer Inc., Waltham, MA, USA) and visualized using the ChemiDoc

XRS Imaging System (Bio-Rad). The Intensity of the bands was estimated by densitometry using the Quantity One Software (Bio-Rad).

3.7. ANTIBODIES

For immunoblotting, we employed a rabbit polyclonal anti-BECN1 antibody (PA5-96649, Invitrogen) recognizing the 1-280 amino acid region, a mouse monoclonal anti-β-Actin antibody (A5441, Sigma-Aldrich) and a mouse monoclonal anti-Histone-H3 antibody (61475, Acive Motif)

3.8. STATISTICAL ANALYSIS

Statistical analysis was carried out using GraphPad Prism v8.4.2 software (Graph Pad Software, San Diego, CA, USA). Bonferroni's multiple comparison test was performed after a two-way ANOVA analysis (unpaired, two-tailed). *p*-values < 0.05 were considered significant. All data are expressed as mean ± S.D.

RESULTS

4. RESULTS

4.1. *Protein isoforms of BECN1 have enhanced expressions in the 3D cultures versus the 2D monolayers.*

We set out to explore the growth patterns of various breast cell lines representing distinct molecular subtypes. Our study employed two well-characterized cell lines - MCF-10A and MDA-MB 231 to investigate the expression pattern of BECN1 isoforms between the cells grown in adherent (2D) and (3D) conditions. When cultured in suspension, the cells form multicellular aggregates with a spherical shape-like shape in which the cellular behaviour is different from that in the 2D system. Cells grown in 2D culture conditions were collected and counted daily from the time of plating (t0) through day 4 to document the rate of growth exhibited by these cells. The dimension of the spheroids was also measured parallelly to assess the rate of growth of both cell lines.

The cell counts and the growth curves shown in **Figure 7A** indicate that the triple-negative breast cancer MDA-MB231 cells not only proliferate faster than the benign breast epithelial MCF-10A cells in the monolayer, but also consistently form bigger spheres in the suspension over 4 days of cultures.

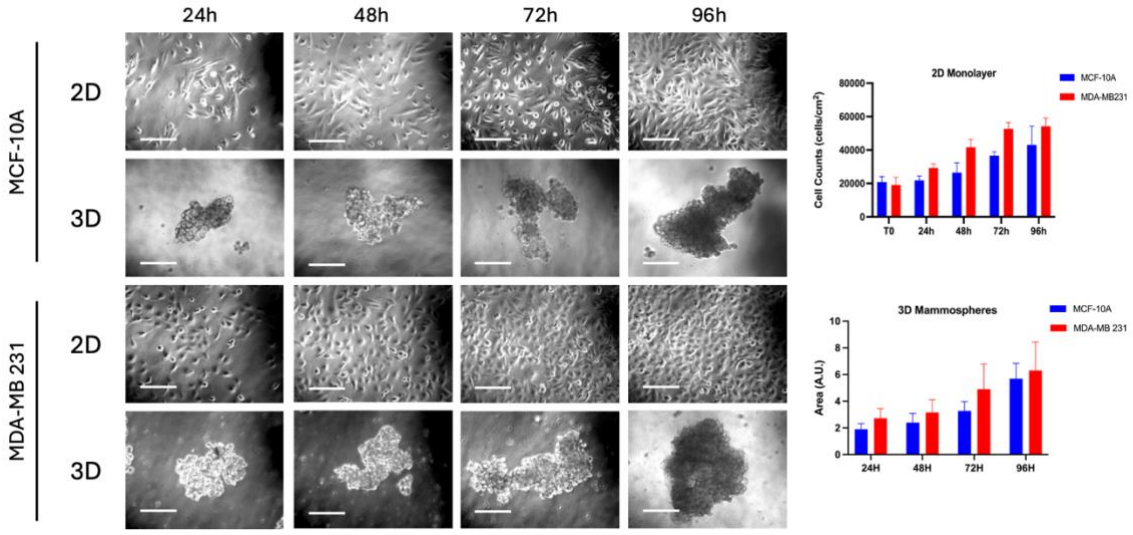

Figure 7A: 2D proliferation and 3D spheroids' growth of MCF-10A and MDA-MB231 monitored by the phase contrast microscope. Images were taken at time points 24h, 48h, 72h and 96h. Scale bar: 200µm, magnification = 20x. Calculated cell counts for each time point are presented in the bar graphs. The area of the spheroids was quantified by ImageJ and indicated as an arbitrary unit (A.U.)

To investigate the expression patterns of BECN1 isoforms under distinct culture conditions, we extracted the total mRNA from the cells grown in both 2D and 3D conditions which was reverse transcribed and amplified with the specific set of primers to detect the BECN1 isoforms, as described in Maheshwari *et al.*, 2022. **Figure 7B** showed the presence of three other isoforms, namely BECN1-α, -β, and -γ, in addition to the BECN1-wild-type (wt). Interestingly, we found that the change from 2D to 3D cultures of MCF-10A led to a steep decline in the expression of the mRNA transcripts for all the identified isoforms of BECN1 (wt, α, β and γ). Simultaneously, the transition of MDA-MB231 cells to 3D did not exert any noticeable impact on the expression of the BECN1-wt, but showed a persistent reduction of the isoforms Alpha, Beta, and Gamma.

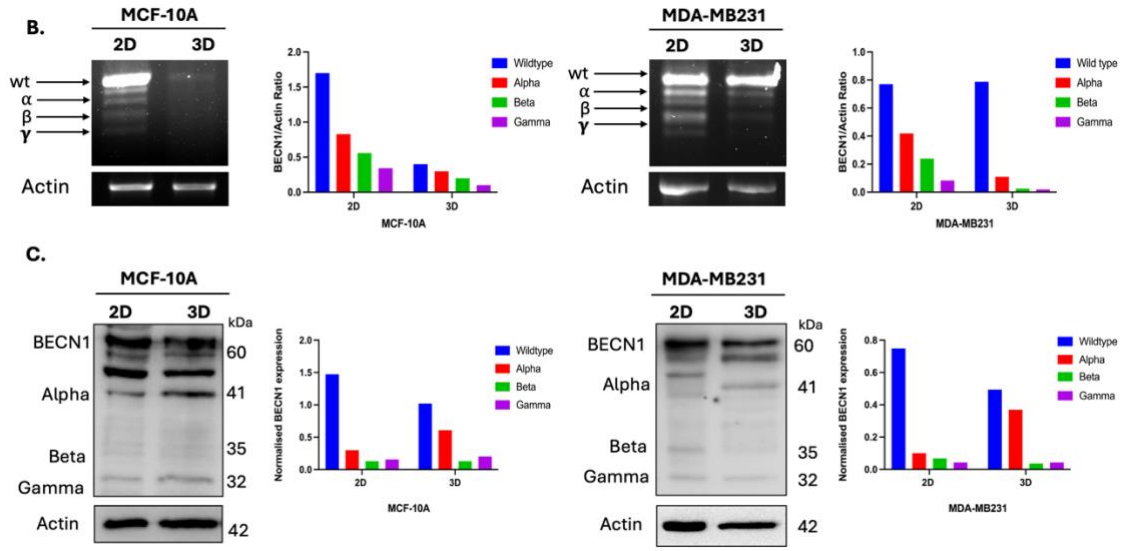

Figure 7 B and C: *BECN1* gene expression in 2D and 3D cultures of MCF-10A and MDA-MB231, respectively. **B)** Agarose gel electrophoresis indicating the PCR amplification of the BECN1 isoforms – wild-type (wt, 765bp), Alpha (α, 680bp), Beta (β, 600bp), Gamma (γ, 550bp). Densitometric analysis of the BECN1/Actin ratio is shown as bar graphs. **C)** Western blotting analysis indicated the endogenous expressions of BECN1 isoforms - wt (60kDa), Alpha (41kDa), Beta (35kDa) and Gamma (32kDa). The bands were normalized to Actin as a loading control. Densitometric analysis is presented.

By immunoblotting, we also analyzed the protein expression of the BECN1 isoforms. As shown in **Figure 7C**, in MFC-10A cells, we observed a moderate reduction in the expression of BECN1-wt isoform which appears to be compensated by the increase in BECN1-α expression in response to the shift from 2D to 3D cell culture conditions. While the expression of BECNI-β was found low in both cell culture conditions. However, there was a slight increase

in BECN1-γ from 2D to 3D cell culture conditions. In MDA-MB231 cells, we also observed a slight decrease in BECN1-wt expression, however, the increase in BECN1-α expression was only slight in comparison to MCF-10A. We also observed a low expression of BECN1-β in both cell culture conditions and a considerable increase in BECN1-γ expression in the transition from 2D to 3D cell culture conditions.

From these data, we conclude that alternative splicing of BECN1 is differently modulated in MCF-10A and MDA-MB231 cells cultured in anchorage-dependent and anchorage-independent conditions. Our findings also suggest that these BECN1 isoforms may play distinct roles in cellular adaptation to different culture conditions notably the BECN1-α isoform which is consistently increasing from 2D to 3D cell culture conditions.

4.2. Reversal of growth conditions reverts the splicing of BECN1 in MDA-MB231 cells.

Invivo, small clusters of circulating tumor cells can reach distant organs where they must attach and grow to form secondary metastasis. To mimic such situations in-vitro, the suspended spheroids were re-placed in multi-wells supporting their re-attachment and were allowed to grow for further 48h. This corresponded to a switch from 3D to the 2D culture condition.

Hence, after 96h of development of the spheroids, we transferred the spheroids back to the 2D culture condition. In **Figure 8A**, we observed the phenotypic shift in the MDA-MB231 spheroids to form ancillary colonies within 24h which further exceeded at 48h showing the dissolution of spheroids back to the monolayer. In contrast, MCF-10A cells were less prone to attach and rescue the growth as adherent colonies. These findings can be attributed to the inherent benign phenotype of MCF-10A, which represents a non-metastatic cell model, and an appropriate metastatic ability of MDA-MB231.

Since MCF-10A did not exhibit the expected phenotypic changes after the transition, we therefore ommitted them from the remaining analyses. The focus of the subsequent experiments and findings was the MDA-MB231 cell line.

Following the transfer of spheroids to 2D cell culture conditions, we further examined the mRNA as well as the protein expressions from all three culture conditions of the breast cancer

cells. MDA-MB231 cells which rescued the downregulated transcripts of the isoforms from 3D to 2D shift with a slight increase, as shown in **Figure 8B**.

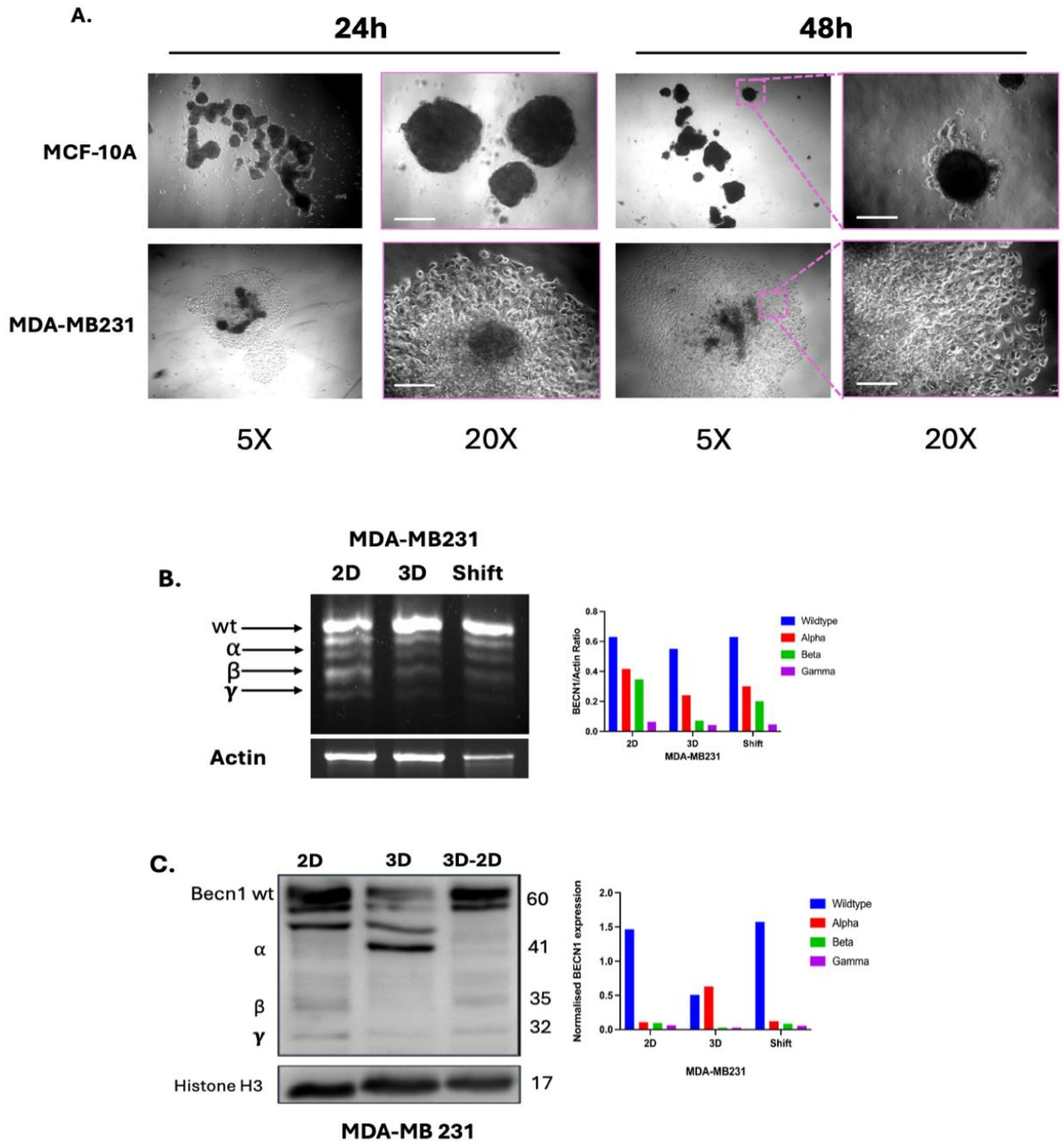

Figure 8: **A.** Images acquired at the phase contrast microscope for 24h, and 48h to monitor cell attachment and growth. Scale bar: 200µm, magnification = 20x.**B.** PCR analyses of BECN1 isoforms normalized to Actin, show the mRNA expressions of BECN1 isoforms in MDA-MB231 cells grown in 2D, 3D and 3D-to-2D shifted (referred to as Shift) cultures. **C.** Western blot analyses to show the protein expressions of the isoforms normalized to Histone-H3 as mentioned.

Furthermore, to complement the findings from the mRNA analysis, the immunoblotting assay shown in **Figure 8C**, demonstrates a notable reversion in protein expression levels of the BECN1 wild-type in addition to the isoforms, particularly of isoform Alpha. These observations suggest that the transition from 2D to 3D to 2D cell culture conditions prompted a dynamic regulation of the alternative splicing of the gene *BECN1* in the malignant MDA-MB231.

4.3. Splicing regulation during the development of the mammospheres

The above data indicates that the BECN1 isoforms are differentially expressed when transferred from 2D to 3D cell culture conditions. We decided to investigate further the splicing pattern of BECN1 isoforms during spheroid development. It is known that the protein product of an mRNA can inhibit its mRNA as a transcriptional feedback inhibition to achieve homeostasis. For instance, an excess of ubiquitin could promote the destruction or inactivation of proteins involved in *UBC* gene transcription, thus providing a straightforward negative feedback loop responsive to intracellular ubiquitin levels (Bianchi *et al.*, 2019). This suggests the existence of a feedback mechanism in gene transcription and protein expression. To elaborate on this, we cultured spheroids in the presence or absence of Spautin-1(SP1) which is a potent inhibitor of autophagy that promotes proteasome-mediated degradation of BECN1(Liu *et al.*, 2011).

Moreover, splicing is often closely associated with tumor occurrence, and that dysregulated changes in alternative splicing machinery could affect tumor progression (Biamonti *et al.*, 2014). Therefore, we cultured spheroids in the presence or absence of Madrasin (MAD), an inhibitor of the early spliceosome assembly at complex A, to investigate the modulation of the alternative splicing of BECN1. (Pawellek *et al.*, 2014).

Data shown in **Figure 9A**, in MDA-MB231 cells, compared to the control, spheroids cultured in the presence of Spautin-1 showed a slightly lower growth rate. Madrasin-treated spheres exhibited a constant decrease in the growth indicating a strong sensitivity to Madrasin.

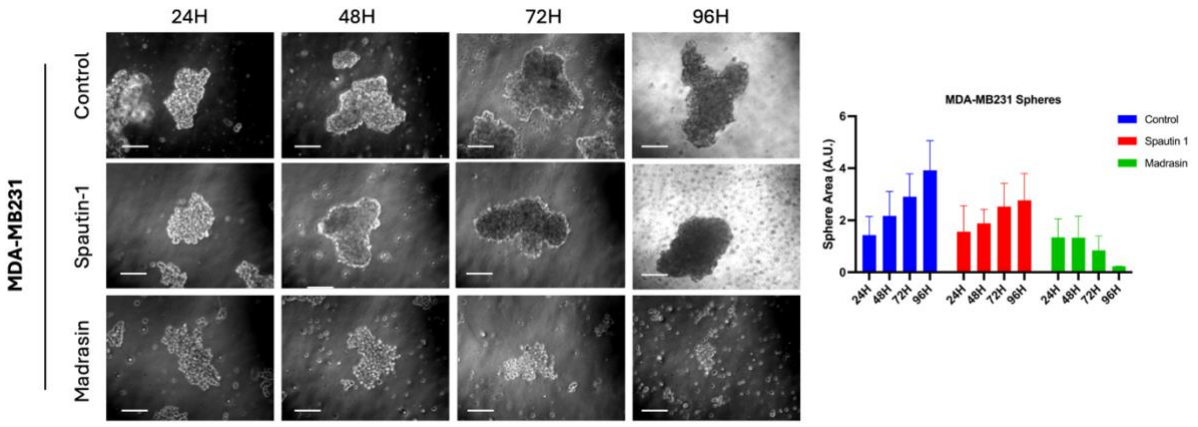

Figure 9A: 3D spheroids' growth of MDA-MB231 monitored by the phase contrast microscope. Images were taken at time points 24h, 48h, 72h and 96h. Scale bar: 200µm, magnification = 20x. Calculated cell counts for each time point are presented in the bar graphs. The area of the spheroids was quantified by ImageJ and indicated as an arbitrary unit (A.U.).

We investigated the effects of the treatment with either Spautin-1 or Madrasin on the spliced mRNAs of BECN1 isoforms. Total mRNA was extracted from these spheroids collected every 24h. Data shown in **figure 9B**, In MDA-MB231 spheroids, in the control conditions, the transcripts of the spliced variants remain relatively stable from 24h to 72h, before declining at 96h. In MDA-MB231 cells treated with SP1, the BECN1-wt isoform reduces at 48h before a consistent increased expression at 96h. Contrarily, the expression of the BECN1-α isoform remains relatively stable throughout the course of 96h, whereas BECN1-β and -γ transcripts decrease from the initial time point till the end of the course. Nevertheless, MDA-MB231 spheroids treated with MAD exhibited a strong reduction in the splicing at 24h which was then rescued at 48h along with a reduction in the wildtype. From this result, we observe that disruption of alternative splicing occurs within 24 hours in MDA-MB231 cells grown in the presence of madrasin while the effects indicate a sensitive splicing machinery in the malignant breast cells.

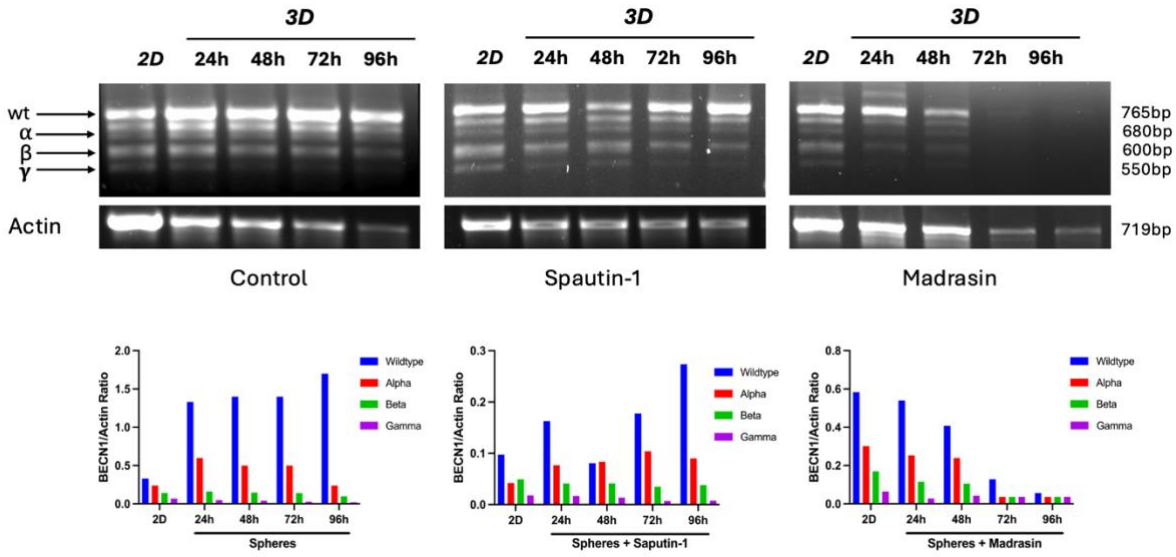

Figure 9B: PCR analyses of BECN1 isoforms normalized to Actin, show the mRNA expressions of BECN1 isoforms in MDA-MB231 cells treated with or without Spautin-1(sp1), and Madrasin (Mad) for 24h, 48h,72h and 96h.

DISCUSSION

5. DISCUSSION

Several studies have shown that dysregulation of alternative splicing is closely associated with numerous diseases including cancer, and particularly with various hallmarks of cancer, including cell proliferation, apoptosis, invasion, migration, and metabolism (Ouyang *et al.*, 2021). In breast cancer, molecular marker genes such as *BRCA1*, *HER2*, *KLF6*, *ERα,* and *Erβ* have been known to undergo splicing to produce isoforms that are involved in breast cancer stemness, tumorigenesis, and drug resistance(Yang *et al.*, 2019). On top of this, our group was able to isolate, characterize, and determine the functional role of the identified BECN1 isoforms (-wt, -α, -β, and -γ) in autophagy (Maheshwari *et al.*, 2022).

Current research has shifted towards three-dimensional (3D) cultures since they can better mimic the more realistic biochemical and biomechanical microenvironments. These models exhibit in-vivo-like gradients of oxygen and nutrient availability leading to central hypoxia and nutrient deprivation conditions that have been shown to activate autophagy. Therefore, the current study delves deeper to explore the modulation of the key autophagy regulator, BECN1 and its splicing in response to the changes in the microenvironment, specifically in 2D and 3D environments utilizing the breast cancer models MDA-MB231 as compared to the benign counterpart MCF-10A and.

The preliminary mRNA and protein analysis indicated that the protein isoforms of BECN1 are expressed more prominently, particularly the isoforms Alpha. However, the mRNA transcripts of the BECN1 isoforms after 96h in 3D cultures showed a steep decline in both malignant and non-malignant breast cells. This data indicates that the protein isoforms have a higher stability (longer half-life) compared to their respective mRNAs, whereas the mRNAs are rapidly degraded. This discrepancy in the stability could be due to various factors including epigenetic regulations through chromatin modifications, micro-RNAs, long-noncoding RNAs, post-translational modifications, etc. (Kouzarides, 2007)(Hunter, 2007)(Rinn and Chang, 2012)(Bartel, 2004).

Next, we investigated whether the transition from 3D spheroid culture to adherent 2D culture conditions also affected the mRNA splicing of the BECN1 isoforms. Notably, in the MDA-MB231 cells, the transition to adherent conditions led to a rescue of the previously downregulated BECN1 isoform transcripts as well as the protein expressions. Our findings suggest that alternative splicing is a dynamic process.

To study the regulation of alternative splicing of these isoforms, we further treated the spheroids with Spautin-1, an autophagy inhibitor that induces the proteasomal degradation of BECN1. MDA-MB231 spheres treated with Spautin-1 displayed a slightly slower rate of spheroid growth in comparison to the control condition. Moreover, we observed a reduction in BECN1-wt after 48 hours followed by a rise again at 72 hours and then a stable expression until 96 hours. However, we observed a stable expression of the spliced isoforms throughout the four days. These results confirm the transcriptional regression of the BECN1 isoform mRNA transcripts. Also, with the help of Madrasin, an inhibitor of the spliceosome, the disruption of BECN1 splicing showed a detrimental impact on the development of the MDA-MB231 spheres.

In conclusion, this study presents substantial evidence that the isoforms are differentially modulated between 2D and 3D cell culture conditions. The increased protein levels of BECN1-α in the spheroids may suggest that it plays a prominent role in maintaining homeostasis in the 3D environment, or it has a longer half-life in comparison to other isoforms. The changes in alternative splicing of BECN1 may also suggest a modulation in autophagy between 2D and 3D conditions, which we plan to further investigate in future experiments. Looking ahead, we aim to better elucidate the roles of individual splicing isoforms in the 3D culture environment. Gaining a deeper understanding of these three BECN1 variants will give novel insight into the mechanism by which cancer cells acquire key properties such as stem cell behaviour, chemoresistance, and dormancy, which are responsible for the failure of complete eradication of cancer.

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6. REFERENCES

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ACKNOWLEDGEMENTS

ACKNOWLEDGEMENTS

First and foremost, I would like to express my upmost gratitude to my mentor, Professor Ciro Isidoro, for giving me the opportunity to join the Molecular Pathology laboratory. Your wisdom, patience and willingness to challenge me have pushed me to strive for excellence in my work. Your consistent support, constructive feedback, and insightful suggestions have immensely helped me during the completion of this project. I deeply appreciate the trust you placed in me and the opportunities you provided for me to thrive and develop as a researcher in your laboratory.

To my tutor, Dr. Chinmay Maheshwari, I am grateful for your unwavering support and guidance. Your expertise and commitment to my training have been instrumental in shaping the outcome of my thesis. Your invaluable feedback, constructive criticism, and dedication to helping me improve have been paramount in enhancing the quality of my work. I am sincerely thankful for your time and effort, which have contributed greatly to the success of our project.

To my lab mates, Denis, Fekadu, Sara, Aya, Shima, Homa, and Ziba, thank you for making every day in the lab enjoyable. To the PhD students, Dr. Andrea, Dr. Beatrice, Dr. Chiara, and Dr. Amreen, and our Postdoc researchers Dr. Alessandra and Dr. Letizia, your knowledge, expertise, and willingness to share ideas have been instrumental in completing my research project. I will forever be grateful for the special opportunity I received by being a part of the Molecular Pathology Lab family.

Finally, I am forever grateful to my family and friends for the love, support and belief that they have shown me through my academic journey. Your belief in my abilities, even in moments of self-doubt, has fueled my determination to overcome challenges and succeed.

Uthman