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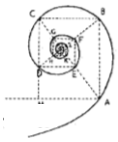

SCUOLA DI DOTTORATO IN MEDICINA MOLECOLARE

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BIO10

THE ROLE OF IFITM3 IN MAMMARY GLAND DEVELOPMENT AND MAMMARY STEM CELLS

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SOMMARIO

Le cellule staminali della ghiandola mammaria sono un modello per lo studio dei processi molecolari che regolano lo sviluppo fisiologico dell'organo e lo sviluppo tumorale. Diversamente da molti organi, la ghiandola mammaria incorre in cicli di rigenerazione e involuzione associati alla gravidanza. Per questo la ghiandola mammaria è uno dei primi organi nel quale si è ipotizzata la residenza di cellule staminali. Dal concetto che le cellule staminali regolano la crescita e la differenziazione della ghiandola con l'avvento della gravidanza è stato ipotizzato che cellule tumorali con proprietà staminali possano contribuire all'eterogeneità del tumore.

Al fine di scoprire come il normale sviluppo dell'architettura della ghiandola mammaria sia originata dalle cellule staminali e il ruolo delle cellule staminali tumorali nella progressione tumorale, in questo lavoro di tesi è stata indagata la funzione di IFITM3. IFITM3 è stato inizialmente identificato dal laboratorio della dott.ssa Zucchi nelle cellule LA7, cellule staminali della ghiandola mammaria di ratto capaci di differenziare in dome, strutture 2D simili agli alveoli che si formano nella ghiandola mammaria durante la gravidanza. È stato dimostrato dal gruppo Zucchi che la funzione di IFITM3 dipende dal cambio di localizzazione della proteina dal citoplasma alla membrana plasmatica, dove risiede nei lipid raft. Questo è stato il punto di partenza di questa tesi di ricerca con lo scopo di identificare se IFITM3 partecipi anche alla formazione di strutture tridimensionali complesse della ghiandola mammaria (strutture tubulo-alveolari) e se svolga una funzione nel mantenimento delle cellule staminali della ghiandola mammaria. Ho utilizzato sistemi transienti e stabili per la modulazione dei livelli di espressione di IFITM3 in cellule LA7 e in cellule MCF7, una linea cellulare umana di ghiandola mammaria. Mentre le cellule LA7 possiedono sia la capacità di generare sfere sia di differenziare morfologicamente e funzionalmente in tutti i tipi cellulari e strutture 3D della ghiandola mammaria, si ritiene che cloni MCF7 utilizzati in questo studio non possiedano proprietà di cellule staminali, non formano sfere e possono differenziare unicamente in un tipo di struttura 3D, le cisti simili agli alveoli della ghiandola mammaria. Abbattendo i livelli di espressione di IFITM3 con siRNA/oligo e tecnologia lenti virale ad RNA a forcina corta (shRNA) in cellule impiegate in saggi funzionali in condizioni di coltura 3D, ho scoperto che IFITM3 è necessario per la formazione di strutture tubulo-alveolari complesse in cellule MCF7 e per il mantenimento delle proprietà di auto-rinnovamento delle cellule staminali LA7. Dato che le mammosfere e le cisti mammarie rappresentano strutture 3D associate rispettivamente a cellule staminali o differenziate, e dato che la sotto-regolazione di IFITM3 inibisce la formazione di entrambe, possiamo ipotizzare che IFITM3 abbia una funzione diversa in base al tipo cellulare. La sotto-regolazione di IFITM3 in cellule staminali LA7 in condizioni di coltura aderenti e non aderenti ha portato ad una graduale perdita delle cellule, suggerendo che IFITM3 svolga una funzione necessaria al mantenimento dell'auto-rinnovamento delle cellule staminali LA7. In cellule MCF7 la sotto-regolazione di IFITM3 ha portato all'incapacità delle cellule di formare alveoli senza effetti sulla proliferazione cellulare. La sovra-regolazione di IFITM3 sia in cellule LA7 sia MCF7 ha portato alla rapida morte cellulare per un meccanismo che è ancora sotto indagine. Complessivamente ho dimostrato che la sotto-regolazione di IFITM3 porta alla perdita delle proprietà di auto-rinnovamento

e della capacità di essere propagate come cellule staminali delle cellule LA7. La sotto-regolazione di IFITM3 in cellule che non hanno proprietà staminali di auto-rinnovamento porta all'incapacità delle cellule di formare strutture 3D.
Lo studio dei profili di espressione genica con tecnologia micorarray ottenuti da cellule LA7 trattate per la sotto-regolazione di IFITM3 supporta il ruolo di IFITM3 nella regolazione del ciclo cellulare, nel trasporto vescicolare e nella modificazione dello stato cromatinico. Geni coinvolti nella proliferazione cellulare sono stati trovati sotto-regolati in seguito alla perdita di IFITM3, insieme a geni del trasporto vescicolare (che coinvolgono proteine che mediano la fusione vescicolare, come le SNARE) che possono essere collegati a IFITM3 in quanto il ruolo di IFITM3 nel prevenire il rilascio delle particelle virali dai compartimenti endosomiali è associato alla formazione di un poro di fusione e alla fusione delle membrane. I geni associati con la regolazione epigenomica e la proliferazione cellulare suggeriscono che IFITM3 possa avere differenti ruoli in cellule staminali e cellule della ghiandola mammaria differenziate, dato che questi geni sono coinvolti nel cancro, nella crescita cellulare o apoptosi e nel differenziamento cellulare.

ABSTRACT

Mammary stem cells (MaSCs) are a model to understand molecular processes that regulate both normal and cancer development. In contrast to many organs, the mammary gland undergoes cycles of re-generation and involution associated with pregnancy. For this reason, the mammary gland was one of the first organs in which stem cells (SCs) were hypothesized to reside. From the concept that SCs regulate normal mammary gland growth and differentiation with onset of pregnancy, arose the hypothesis that cancer cells with SC properties may contribute to tumor heterogeneity. To understand how normal development of the mammary gland architecture arises from SCs and the role of CSCs in tumor progression, the function of the IFITM3 gene was investigated in this thesis research. IFITM3 was initially identified by Zucchi's lab in LA7 cells, rat mammary SCs that differentiate into domes, 2D structures similar to alveoli that form in mammary gland at pregnancy. It was demonstrated by the Zucchi group that the function of IFITM3 was dependent on its shuttling from the cytoplasm to the plasma membrane and being part of lipid rafts. This was the starting point of the thesis research with the aims to identify whether IFITM3 also plays a role in the formation of more complex three dimensional (3D) mammary structures (tubule-alveolar structures) and has a role in MaSCs. I used transient and stable IFITM3 expression modulating systems in the LA7 and the human MCF7 mammary cell line. In contrast to the rat LA7 SCs that have both the capacity to generate spheres and differentiate morphologically and functionally into all the mammary cell types and generate tubule-alveolar structures iin 3D culture conditions, the MCF7 clone used in this study are not considered to have SC properties, do not form spheres and can only form one type of 3D differentiated structure called cysts. Targeting IFITM3 in these cells, with siRNA/oligos and short hairpin RNA lentiviral technology in order to down-regulate the expression of the protein, and by performing functional assays in 3D culture conditions, I found that IFITM3 is necessary for the formation of complex alveolar structures in MCF7 cells and for self-renewal of LA7 SCs. As mammary spheres and mammary cysts represent different 3D structures associated with stem and differentiated cells respectively, and since IFITM3 down-regulation inhibits the formation of both, this suggests that IFITM3 may have different functions depending on the cell type. Down-regulation of IFITM3 in LA7 SCs in both adherent or in non-adherent cultures resulted in a gradual loss in the number of cells, suggesting that IFITM3 function is necessary for self-renewal of LA7 SCs. In contrast down regulation of IFITM3 in MCF7 cells resulted in the inability of the cells to form alveoli with no effect in cell proliferation. IFITM3 up-regulation in both LA7 and MCF7 cells resulted in rapid cell death by a mechanism that is still under investigation. Collectively, I demonstrate, that IFITM3 down-regulation results in loss of self-renewal and loss of the ability of LA7 to be propagated as SCs. While IFITM3 down-regulation in mammary cells that do not

have SC self-renewal capacity results in the inability of the cells to generate 3D structures.

Microarray expression experiments, obtained with down-regulation of IFITM3 in LA7 cells, support the IFITM3 function in cell cycle regulation, vesicular transport and in the modification of the chromatin state. In addition to genes involved in cell proliferation found down-regulated with loss of IFITM3, a link among vesicle-transport genes (involving proteins mediating vesicle fusion, such as SNARE), and IFITM3 was also determined, supporting a role of IFITM3 in preventing the release of viral particles from endosomal compartments, associated with membrane fusion and the formation of a fusion pore. Genes associated with epigenomic regulation and cell proliferation suggests that IFITM3 may have multiple and different roles in SCs and in differentiated mammary cells since these genes are involved in cancer, cell growth or apoptosis and in cell differentiation.

INDEX

1 INTRODUCTION

1.1 MAMMARY GLAND DEVELOPMENT

The mammary gland is a branched tubular organ unique to mammals and provides nourishment, hormones, antibodies and growth factors to new born offspring. The mammary gland architecture is composed of lobes containing secretory lobules and alveoli, hollow cavities that produce milk, and ducts that converge into a single lactiferous duct for each lobe (Fig.1). Ducts are bilayered tubuli with an inner layer of luminal epithelial cells and a basal layer of myoepithelial cells with contractile capacity due to expression of smooth muscle actin. The duct system is embedded in a dense connective tissue composed of fibroblast and adipose tissue called the fat pad.

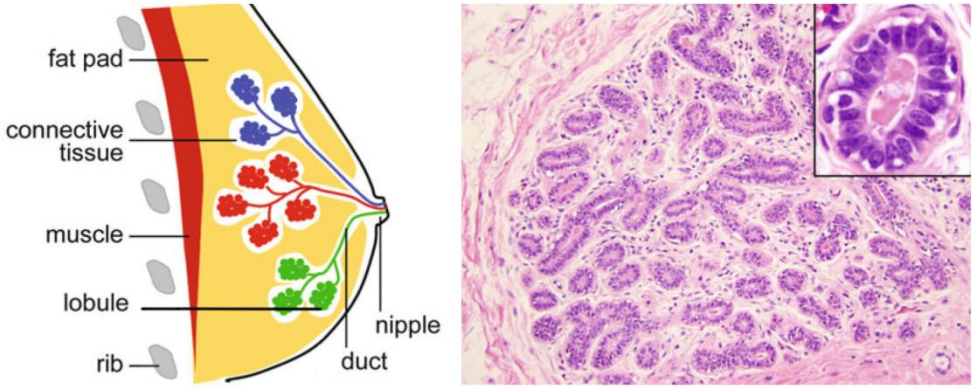


Figure 1. A) Schematic representation of mammary gland anatomy, multiple ducts are in different color, all converging into the nipple. The lobule are embedded into the fat pad. B) Histological section of a lobule of mammary gland, stained with hematoxylin and eosin (nuclei appear in blue). In the corner magnification of an acinus, the functional milk producing structure of the gland. Modified from [1].

The mammary gland has the peculiar feature of undergoing structural and cellular remodeling in multiple stages of development during the lifespan of the female individual (Fig.2).

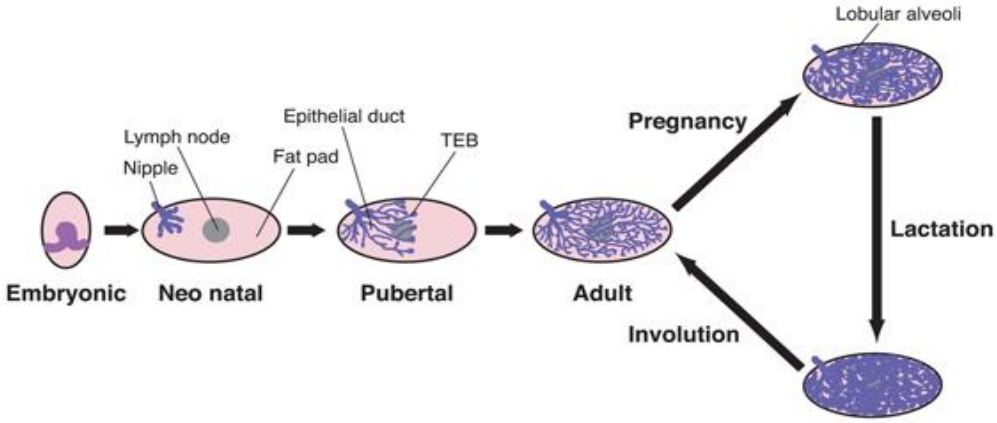


Figure 2. Schematic representation of mammary gland development. The embryo develops the placode and the primitive nipple. With puberty the ducts elongate and the branching morphogenesis take place. In pregnancy the gland reaches it maximum expansion with the formation of alveoli and after weaning the involution phase brings the gland to a state similar to a pre-pregnancy state[2].

1.1.1 EMBRYONIC DEVELOPMENT

The first step takes place during embryonic development with the formation of the mammary lines that are bilateral stripes that extend from the anterior to posterior limbs.

The origin of the mammary line is tightly influenced by the surrounding mesenchyme and continuous crosstalk between the epithelia and fibroblast compartments[3]. The specification of the mammary line depends on the expression of the WNT genes in the ectoderm and flanking mesenchyme[4,5], under the regulation of FGF10[6] signaling from ventral somites. FGF10 along with WNT and BMP4 regulate the expression of the Tbx3 gene in the mesenchyme underlying the mammary line[6–8], which as a result amplifies WNT signalling leading to the formation of placodes.

Five pairs of placodes in mouse and one in human[9] are formed by aggregation of epidermal cells of the mammary line[10] which become columnar and multilayered. In the next days the placodes invaginate into

the mesenchyme forming structures that are shaped like buds that infiltrate into the underlying primitive fat pad. This process is dependent on WNT[4] and the transcription factors MSX1 and MSX2[11,12].

The epidermal bud sprouts through the stroma until it reaches the pre-adipocytes cells and it starts to branch into a rudimental ductal tree in a hormonal independent fashion[13,14]. Branching is followed by cavitation of the primordial ducts, and by nipple formation and sexual dimorphism in mice. In male the ducts are severed by growth of the surrounding mesenchyme under androgen hormone influence (Fig.3).

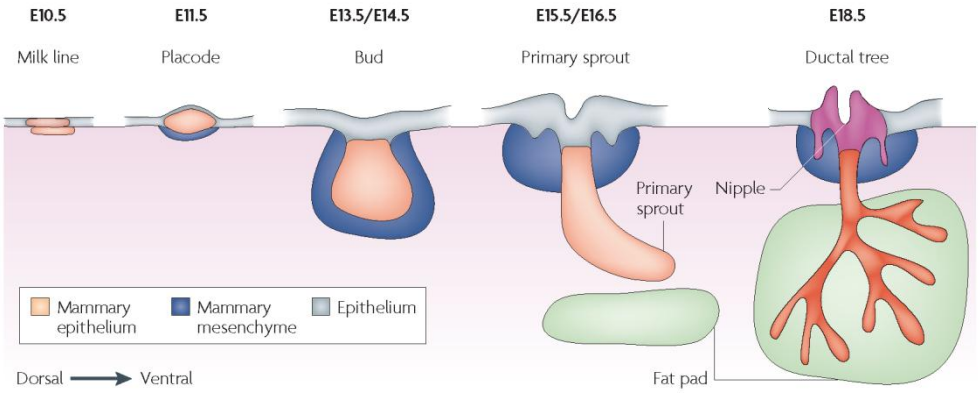


Figure 3. Mammary gland development of mouse. At day 10.5 the mammary line is formed, in following days the epithelial cells will change shape and organize into the placode. Placode invagination forms the epidermal bud, infiltrating thorough the adipocytes of the fat pad. The sprout reaches the fat pad and the primordial ductal tree is formed, with the development of the nipple. Modified from[15].

PTHLH is responsible for this final stage of embryonic development, acting through its mesenchymal receptor PTHR1 modulating BMP4 and WNT signaling[16,17] that will induce the formation of an environment that will support the survival of the epithelial bud, nipple development and growth of the duct system. At this stage the development stops and will proceed in the adult life during puberty.

At the pre-pubertal stage the mammary gland is composed of simple ducts poorly infiltrated into the fat pad, ending in terminal end buds (TEBs) which

are club shaped structures with a multiple layer of epithelial cells (body cells) surrounded by more basal cells forming the cap cell layer[18,19].

1.1.2 PUBERTAL STAGE

At puberty TEBs are stimulated to infiltrate into the fat pat and start the branching morphogenesis process that will remodel the architecture of the gland. Secondary branches grow from the primary elongated ducts and in the further weeks, tertiary branches will follow.

The branching morphogenesis is under the control of growth factors and hormones such as IGF, EGF, FGF, HGF, GH, estrogen and TGFβ, that act directly or indirectly on the epithelial cells, stimulating the fibroblast and surrounding mesenchyme compartments in a paracrine fashion. The pituitary gland releases Growth Hormone, which reaches the stroma of the mammary gland through the blood stream and binds to the Growth Hormone Receptor[20]. Fibroblasts are stimulated to produce IGF1, which acts along with hepatic IGF1 on epithelial cells through the IGF1R, stimulating proliferation and cell survival[21] (Fig.4).

Estrogens have a direct effect on mammary branching[22], as many gene knockout mouse models have shown [23–25]. Estrogens receptors are expressed in fibroblasts of the stroma and epithelial cells, after ligand binding to the fibroblasts produce and release more IGF1 while the epithelial cells produce amphiregulin (AREG). AREG is a member of the EGF family and is cleaved by ADAM17, a metalloproteinase expressed by luminal cells[26]. Once free, AREG binds to EGFR on stromal cells and acts in a paracrine way to stimulate the production and release of FGF. FGF will then bind to FGFR on epithelial cells and promote cell proliferation and branching.

EGFR activates also other matrix metalloproteinases that positively regulate the branching process[27], degrading the extracellular matrix

creating space for the elongation of the TEBs, with cap cells differentiating into myoepithelial cells during elongation[28].

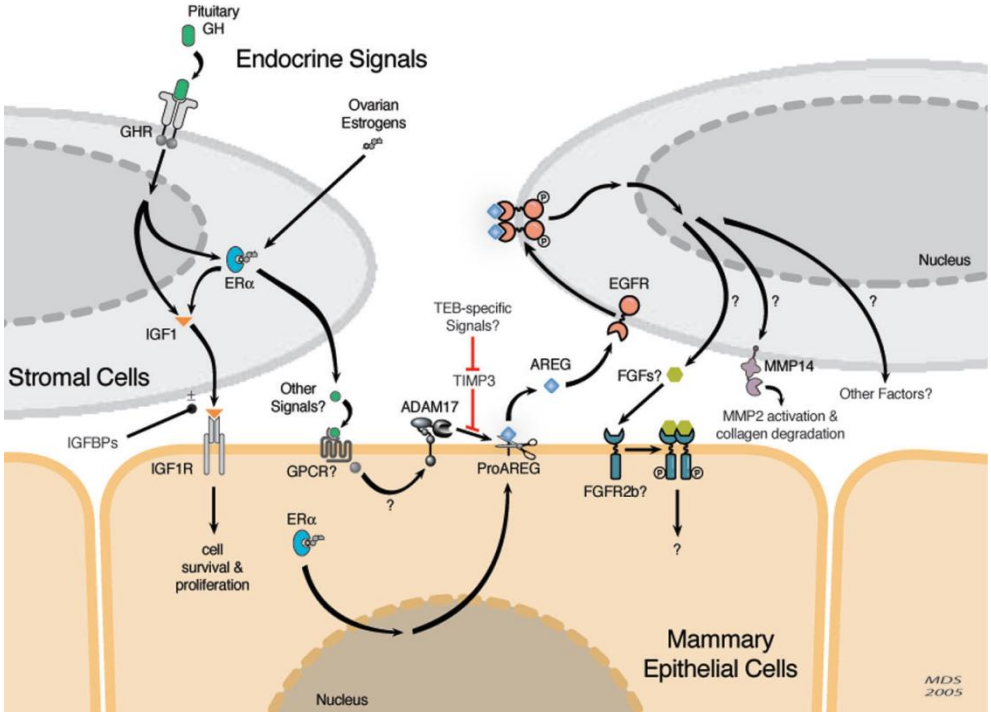


Figure 4. Schematic representation of the growth factors regulating duct elongation. MMPs are involved in the collagen degradation[29].

TEB elongation is dependent of the expression of tissue inhibitors of metalloproteinases (TIMPs), which spatially regulate the activity of MMPs within the stromal/luminal surface and by TGFβ. TGF β acts by inhibiting both duct elongation and branching, limiting the movement of the TEBs and allowing for a properly organized tree architecture[30], and promoting ECM deposition[31], creating distant spaced branched buds. ECM involvement in branching morphogenesis is not only due to its physical spatial effect, but also involves sequestration of growth factors released by proteinase that may also produce bioactive fragments of ECM itself.

1.1.3 LACTATION AND INVOLUTION

The final stage of mammary gland development takes place during pregnancy and following involution after weaning, a cyclic process that occurs at each pregnancy. Lactation is the ultimate function of the mammary gland. During pregnancy the mammary gland will increase its internal complexity completely filling the fat pad, and differentiate creating the specialized structures dedicated to milk production and delivery.

Progesterone (PR) and prolactine (PRL) are the hormones leading the modification of the mammary gland in this last stage. PR is responsible for the massive extension of the branches, and with PRL triggers the differentiation of luminal cells into alveoli[32–34]. PRLR involves the JAK2/STAT5 signalling cascade, that not only promotes the production of milk proteins and enzymes but is also responsible for the differentiation and maintenance of the alveolar cell state[35–37]. PR and PRL both exert their actions through modulating RANKL signalling in a paracrine fashion. RANKL binds to its receptor on epithelial cells starting a cascade that will induce proliferation and block apoptosis[38–40].

The final involution takes place at offspring weaning, when mechanical stimulation of milk production by suckling ceases. Remodelling of the mammary gland then initiates that will bring the gland back to a pre-pregnancy state. Two steps distinguish this last phase (Fig.5), the first one involves accumulation of milk and apoptotic process with accumulation of dead cells in the ducts, without massive remodelling of the tree architecture. This first step can be reversed by re-initiation of suckling stimulation within 48 hours.

The second, takes place in the next days and is irreversible, with a new apoptotic process linked to the ECM degradation[41]. A switch between STAT5 and STAT3 occurs in the first hours of weaning, with consequent suspension of pro-survival input and start of the apoptotic process[42,43], triggered by the Leukemia Inhibitory Factor (LIF) produced by the alveolar

cells in response to suckling withdrawal[44]. Many target of STAT3 are up-regulated in response to LIF, such as C/EBPdelta[45] or the regulatory subunits of the PI3K[46] that reduce the anti apoptotic activity of AKT.

The extrinsic death program is also involved, with ligands binding to the death receptors, activation of the FAS/FASL pathway and promotion of apoptosis[47,48], with IGF1 sequestration by IGFBP5, reducing its pro survival effect[49].

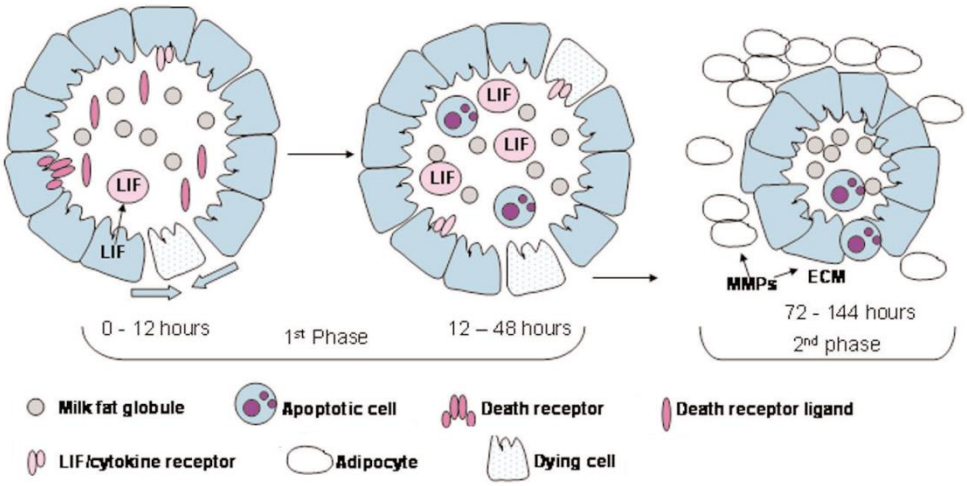


Figure 5. Involution takes place when the milk request to stop. The first reversible phase occurs within the first 48 hours after cessation of suckling. Milk accumulation lead to release of LIF and apoptosis. The second phase is irreversible and involves remodelling of the extracellular matrix. Modified from [50].

The final and conclusive step of involution involve proteinases for a complete degradation of ECM, with the activation of hepatic plasminogen to plasmin, which can directly degrade the ECM[51], and MMPs such as MMP2, 3 and 9 which are no more inhibited by TIMPs [51,52]. Protease influences the multiplication and differentiation of new adipocytes releasing growth factors from the ECM [53,54] for a process that promote cell expansion with recreation of a fat pad similar to the pre-pregnancy state.

1.1.4 MAMMARY GLAND STEM CELLS

As the mammary gland undergoes rounds of remodelling throughout the female lifespan, and cycles of cellular expansion and tissue remodelling are dependent on cells with multi-lineage differentiation and self-renewal potential, these suggest the existence in the mammary gland of cells with stem cell (SC) properties. Stem cell survival relies on a stem cell niche, a microenvironment that provides the signals necessary for the maintenance of self renewal and the undifferentiated state of stem cells.

First observations of the regenerative potential of the mammary gland came from graft experiments of Deome and Faulkin[55]. Transplantation of mouse epithelial cells into mammary fat pad cleared of host's epithelia showed that these cells were able to create a branched tree in the fat pad, and that the transplantation could be repeated with grafted epithelial cells into a cleared fat pad of another mouse.

Cell dilution analysis of mammary epithelial cells showed to contain three different lineage restricted progenitors, which can differentiate into ducts, alveoli or a complete mammary gland[56], raising the question if mammary gland development is due to cooperation of lineage committed progenitors or if a single multipotent stem cell that could be responsible of the complete formation of the gland.

Kordon and Smith used the mouse mammary tumor virus (MMTV) to investigate the clonal origin of the mammary gland. They transplanted infected epithelial cells into a healthy non-MMTV mouse and discovered that primary and secondary outgrowth had the same genomic insertion sites of MMTV. These studies supported that the mammary epithelium originates clonally by a single mammary epithelial cell[57].

Moreover, epithelial cells can be serially transplanted without losing their properties, and are not influenced by the age of the donor, confirming the stem cells feature of epithelial cells[58,59].

In the following years many groups attempted to isolate a cell population with the stem cell features, using marker labelling or dye retention associated with Fluorescence Activated Cell Sorting (FACS). In the late 2000s mouse mammary stem cells were successfully isolated using CD24, CD29 and CD49f as markers[60,61](Fig.6).

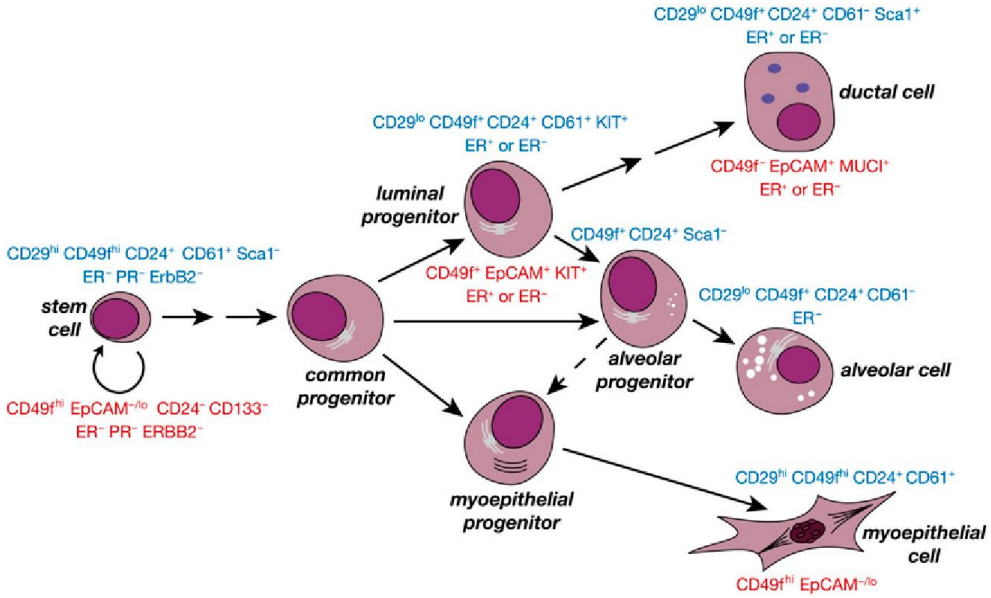


Figure 6. Differentiation hierarchy of mammary gland. In blue and red the surface markers used for isolation of mouse and human cells, respectively. A MaSC originates a common progenitor for luminal and myoepithelial line, which will differentiate to committed progenitors[62].

A similar approach with different markers lead to identification of MaSCs in human [63,64].

1.2 BREAST CANCER

Breast cancer is the second most common cancer worldwide for women. Breast cancer is a very heterogeneous disease which can be classified by molecular profiles that allows for more specific therapy and prediction of

clinical outcome. Human breast cancer can be grouped by the expression of the estrogen receptor, progesterone receptor and human epidermal growth factor receptor 2 (HER2), or by their absence (triple negative cancer), and into subtypes such as luminal A, luminal B, basal like, HER2 like, and claudin low and normal [65–68].

Luminal A tumors are characterized by expression of the ER and/or PR, but are negative for HER2; luminal B, like A, express ER and/or PR and are also positive for HER2. The HER2 like tumors are positive for HER2 and negative for PR and ER, while the basal like are triple negative breast cancer marked by the expression of basal cytokeratins such as CK5/6, CK14, and CK17. Claudin low are triple negative breast cancers which express low levels of the claudin gene and factors involved in the EMT process[69].

Breast cancer progression is divided into different stages depending on the location and size of the tumor, the invasion of lymph nodes and presence of metastasis. The original carcinoma in situ can grow in size and invades adjacent tissue of the skin or the chest wall of the breast. Cancer stages of development are formulated by combination of previous criteria, with the final stage that involves metastasis of the cancerous cells that have spread to other parts of the body of the patient.

1.2.1 CANCER STEM CELL THEORY

Breast cancer originates from a transforming event in a single cell, by accumulation of mutations resulting in the expansion and clonal selection of a tumor single cell. Breast cancer is characterized by heterogeneity both among tumors in different individuals and among population of cells within the same tumor. Inter-tumor heterogeneity origin is difficult to study, as it is nearly impossible to individuate the first transforming cell, as rarely diagnosis take place when the tumor is in its earliest stages of development allowing to follow its molecular evolution. Intra-tumor heterogeneity is due

to the presence of cancer cells with different phenotypes, or epigenetic chromatin states.

To explain tumor heterogeneity two models have been proposed, the clonal evolution and the cancer stem cell model (Fig.7).

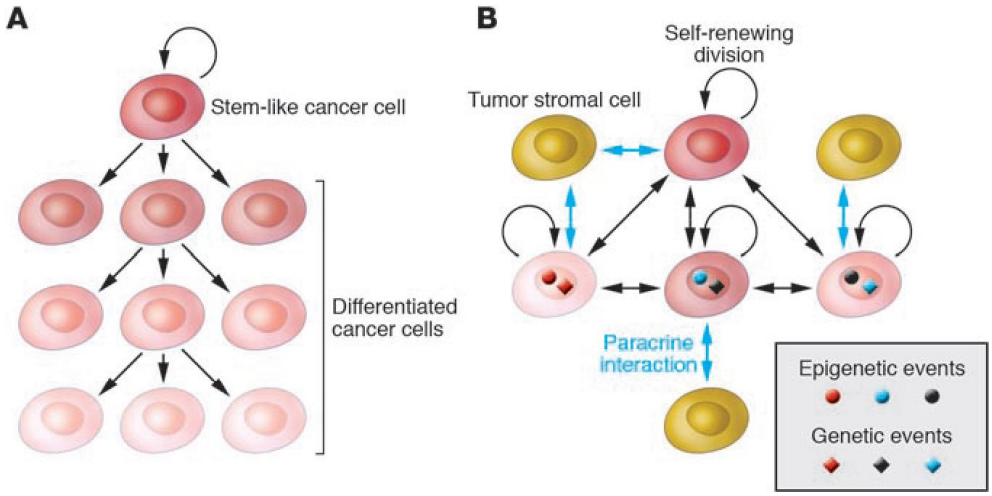


Figure 7. A) Cancer stem cell model, the cancer stem cell is the only one that maintains self renewal properties. Daughter cells form the mass of the tumor but don't contribute to accumulation of new mutations. B) The clonal evolution model. Each cell can acquire the self renewing properties and contribute to tumor progression[70].

In the clonal evolution each cell can be a tumor initiating cell, which starts from initial mutations that provides unlimited proliferation. Individual cancer cells may gain new mutations that can confer advantages against other clones, spreading heterogeneity.

In the cancer stem cell model the tumor starts from mutations in a subset of cells with normal stem cell like properties, or progenitor cells that regain self renewal potential. A cancer stem cells behave as a normal SCs, with asymmetric cell division potential that maintain the stem pool with daughter stem cells and cells which differentiate into all cell types of the body, explaining the heterogeneity.

First observations of cancer stem cells came from the hematopoietic field[71], where the cell capable of initiating an acute myeloid leukemia in mouse possessed proliferative, differentiative and self renewal potential as expected from a stem cell. In 2003 breast cancer stem cells were identified[72], confirming the validity of the cancer stem cell model in breast tumors.

1.3 LA7 CELL MODEL

LA7 cells were first isolated from the RAMA-25 cell line derived from an adenocarcinoma chemically induced in rat by Dulbecco in 1980[73].

The group of Zucchi demonstrated that LA7 cells are a model of cancer stem cells and can be used to study both normal mammary gland and breast cancer development. LA7 cells have the stem cell properties[74,75] of: self-renewal that can be maintained indefinitely in vitro and in vivo, multi lineage differentiation as they have the ability to generate branched ductal-alveolar-like structures in three-dimensional (3D) cultures that morphologically and functionally recapitulate the tubule-alveolar architecture of the mammary tree. In addition, exposure of LA7 cells to lactogenic hormones, lipids, or differentiating agents (e.g. DMSO) results in the formation of dome-shaped structures filled with liquid, that recapitulate the cellular changes that occur in the mammary gland at pregnancy, when alveoli are formed (Fig.8).

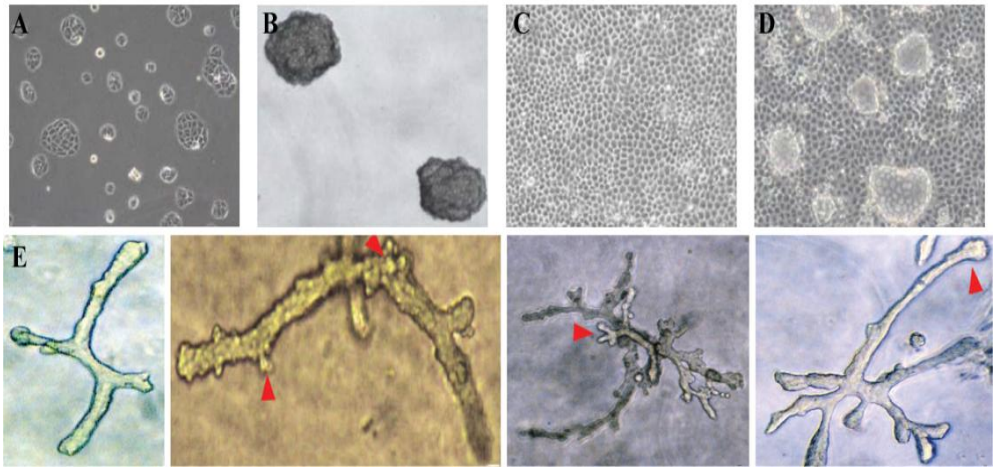


Figure 8. A) LA7 cells cultured in adherent conditions grow forming colonies originated from a single epithelial cell known as holoclone. B) When cultured in non adherent conditions LA7 generate spheres from a single cell with stem cell properties. C) LA7 grown in confluent conditions, spontaneously or treated with differentiating agents, differentiate into D) Domes. E) Tubular structures formed by LA7 cells when cultured in 3D conditions. Red arrows point at TEBs. Modified from[74,76].

LA7 cells have tumor initiation capacity, as one single LA7 is sufficient to develop a tumor in NOD-SCID mouse, and single cells isolated from the dissociated tumor tissues are similar to the injected LA7 in terms of differentiative potential, self renewal and tumor initiation capacity. LA7 also have metastatic potential. LA7 are therefore an ideal model to study breast cancer and breast development in all its facets phases.

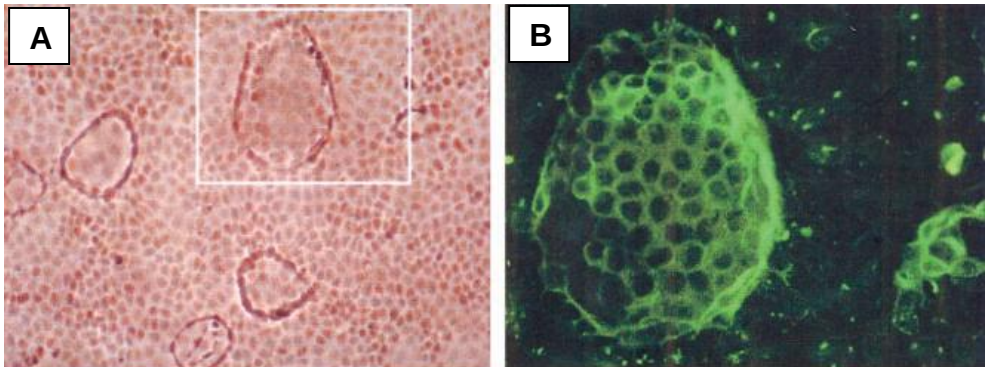


Figure 9. A) LA7 cultured in adherent conditions to differentiate into domes. B) A magnification of A, showing staining of IFITM3 in domes cellular membrane[77]

Zucchi's lab demonstrated that LA7 differentiation into domes is dependent on a gene, rat8, identified during the study of domes differentiation[78] (Fig.9). Rat8 is the rat homologous to human IFITM3 and my thesis research will investigate the role of IFITM3 in other aspects of mammary gland development.

1.4 IFITM3

1.4.1 IFITM3 AS CELLULAR DEFENSE AGAINST VIRUSES

Interferon stimulated Transmembrane protein 3 (IFITM3) is a member of the interferon stimulated genes (ISGs), and basally expressed in many tissues and up-regulated in response to Interferon I and II stimulation[79,80].

IFITM3 is member of the family of the IFITMs, these proteins are highly conserved among species and mostly similar among each other; in human there are five members of the family: IFITM1, IFITM2, IFITM3, IFITM5 and IFITM10 (Fig.10). IFITM5 is specifically expressed in bone cells and is not interferon responsive[81]. Most mammals and other vertebrates have orthologs of these proteins. In mouse there are also the forms IFITM6 and IFITM7.

IFITM1 was first identified in a study in which targeting the protein by an antibody resulted in T lymphocyte aggregation, the antigen was named Leu-13[82] a domain common to all IFITMs, the CD225. IFITIM1 was also the first member of the family to be related to viral resistance. Cells in which IFITM1 is up-regulated are partially protected from the vesicular stomatitis virus (VSV)[83].

IFITM3 was first identified by the Zucchi's lab[78] in subtractive library of differentiating versus undifferentiated LA7.

Identification of viral protection activity of IFITM3 (Brass publication on Cell) was first seen from: Influenza A Virus (IAV), HIV, Dengue virus and West Nile virus[84], demonstrating the efficiency of IFITM proteins in blocking the infection of enveloped viruses. IFITM3 mainly co-localizes with ER markers and was also shown be able to move to the plasma membrane.

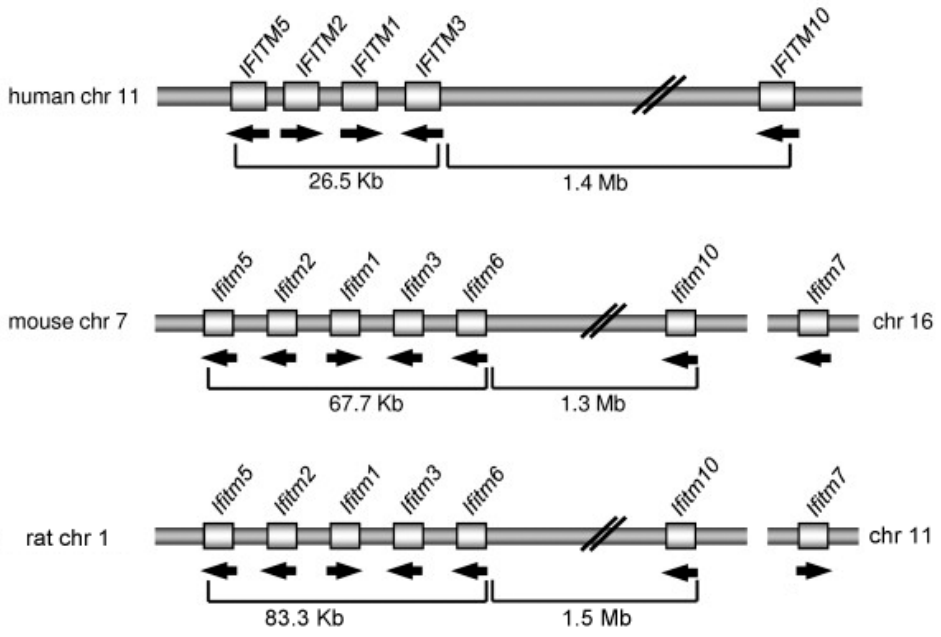


Figure 10. Cluster of IFITM family members in human, mouse and rat. Arrows indicate the direction of transcription. Modified from[85].

Further experiments by other laboratories demonstrated a protective role of IFITMs against other viruses: SARS corona virus, Marburg (MARV) and Ebola (EBOV) filovirus, but MARV and EBOV cell entry was less restricted by IFITM3 with respect to the IAV[86]. Moreover Huang and colleagues hypothesized that IFITM3 mediated restriction could be related to the alteration of the endosomal or lysosomal compartments, as IFITMs do not influence the cathepsin L activity necessary for viral fusion.

Interestingly IFITM3 seems to have a role also in interfering with viral translation[87]. In a STAT1 stable expressing cell line IFITM3 up-regulation reduced the translation efficiency of viral proteins, as confirmed by luciferase assays. This result suggested a putative regulatory role of IFITM3 by its interaction with RNA or DNA by its leucine zipper domain, as suggested by another paper in which IFITM3 expression reduce the level of the osteopontin (OPN) mRNA [88].

These main investigations on IFITM3 are related to the discovery of the mechanisms of action as antiviral protection. The Brass laboratory[89] found that IFITM3 inhibited nuclear translocation of viral pseudo particles and their hemoagglutinin (HA) or VSV's envelope glycoprotein (VSV-G) mediated fusions. Endosomes are membrane bounded compartment implicated in the vescicular transport of the endocytic pathway from plasma membrane to lysosome. IFITM3 is shown to be localized in the late endosomes and exogenous overexpression of the protein or INF treatment resulted in volume expansion and increase of endosome number.
IFITM3 localization into endosomes results in viruses being trapped inside the vesicle compartment, failing to fuse with the membrane and unable to proceed with the following cytosolic entry. The endosomes fuse with lysosomes and viral particles are then degraded.

Another study from Li and colleagues suggest that IFITMs plays a role in preventing viral hemifusion by altering the curvature of endosomal membrane[90]. Hemifusion is promoted by negative curvature and IFITMs may instead confer a positive curvature. Moreover IFITMs may alter the fluidity of the lipidic membrane, reducing the ability of the lipids to undergo movements necessary for achieving hemifusion.

Alteration in cholesterol homeostasis has been linked to IFITM3 antiviral activity, with IFITM3 interaction with Vesicle-membrane-protein-associated protein A (VAPA) and oxysterol-binding protein (OSBP) causing an accumulation of cholesterol in the endosomes, thus stiffening the endosomal membrane and inhibiting viral fusion[91].

Brass and collaborators found that amphotericin B, an antimycotic commonly used in clinical treatment, was responsible for a decreased restriction of viral infection by IFITM3 in treated tissue cultures[92]. This finding is consistent with the proposed way of action of IFITM3 involving membrane stiffness, as amphotericin B is known to bind to lipids and to increase membrane fluidity. They investigated also the role of cholesterol and VAPA on IFITM3 mediated restriction, and found that neither cholesterol nor VAPA does not altered the protective effect of IFITM3. Desai and collaborators agree with these findings, as they directly enriched the cholesterol content of endosomes and evaluated virus-endosome fusion capability, which resulted unaltered compared to untreated cells[93].

Their findings suggest that IFITM3 is not involved in regulating the hemifusion process, but instead contributes to stabilize the cytoplasmatic leaflet preventing the formation of a complete fusion pore. The putative mechanism of action of IFITM3 suggested by their results imply a direct action on the site of arrested hemifusion, as a barrier to stop viral entry, or indirectly modifying the cytoplasmic leaflet protein and or lipid, or disallowing viral back-fusion (a proposed two-step mechanism of fusion

with the viral particle that first fuse with a vesicle inside the maturing endosome, and later with the endosomal membrane).

1.4.2 IFITM3 POST TRANSLATIONAL MODIFICATIONS

IFITM3 can be subjected to many post translational modifications such as phosphorylation, ubiquitination, palmitoylation and methylation. The post translational modifications influence IFITM3 cellular localization, stability and anti-viral activity.

S-palmitoylation at cysteines 72, 73 and 10[94] enhances affinity of IFITM3 to the cell membrane, increasing its antiviral activity, without altering its localization . Ubiquitination at lysines 24, 83, 88 and 104 instead diminishes the IFITM3 mediated viral restriction, since mutations at ubiquitin binding residues increase antiviral activity[95].

The other fundamental modification is phosphorylation at tyrosine 20 by FYN kinase[96]. Phosphorylation regulates IFITM3 localization and thus its antiviral activity since phosphorylated proteins are localized at the plasma membrane level and IFITM3 is required at endosomal level to protect the cell from viral invasion. The majority of IFITM3 proteins are un-phosphorylated and at endosomal level.

Tyrosine 20 is part of the YEML aminoacidic sequence recognized by the μ2 subunit of the AP-2 complex, which is responsible for IFITM3 internalization (Fig.11). Phosphorylation alters the capacity of the complex to bind and promote clathrin mediated endocytosis[97].

It is also known that IFITM3 can influence clathrin dependent endocytosis of v-ATPase[98] suggesting that IFITM3 may play a role organizing proteins at plasma membrane for subsequent internalization.

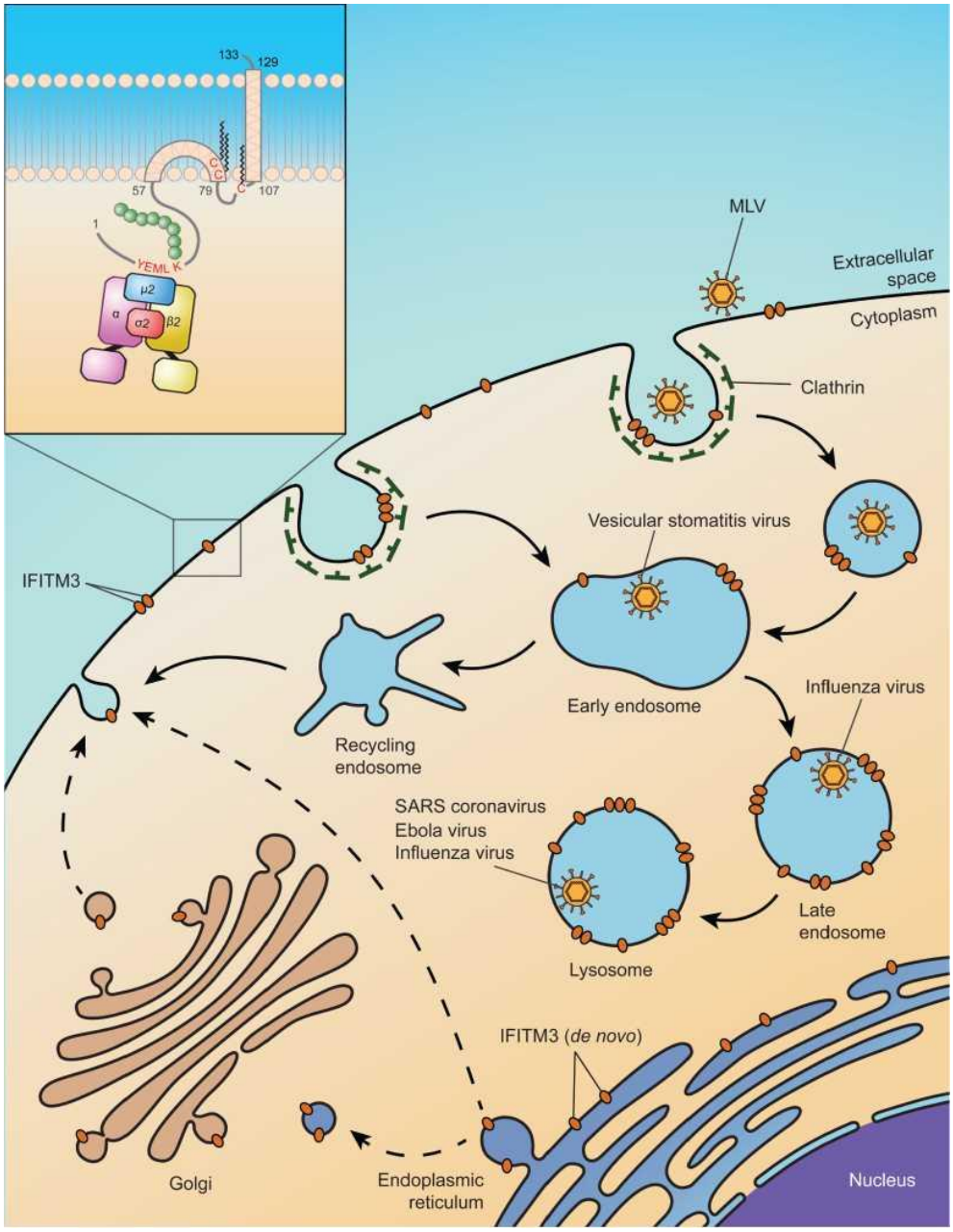


Figure 11. Schematic representation of IFITM3 trafficking and shuttling to plasma membrane. In the corner IFITM3 is shown ubiquitinated at cysteine 24 and palmitoylated. The complex AP-2 bind IFITM3 at the YEML motif with the subunit μ2 and undergoes clathrin dependent endocytosis[97].

1.4.3 IFITM3 STRUCTURE AND TOPOLOGY

IFITM proteins share common domains that are divided into a hydrophobic N-terminal domain, a conserved hydrophobic intramembrane domain, a conserved intracellular loop, a hydrophobic transmembrane domain and a short C-terminal domain. The CD225, shared by all members of the family, includes the intramembrane domain and intracellular loop.

IFITM3 topology is a very discussed argument, as many models have been proposed in the last years. The first one assumes a topology similar to IFITM1, a type III transmembrane protein, with C and N terminals facing the extracellular space, as the N-terminal domain is targeted by the antibody causing aggregation of cells, and both can be detected by flow cytometry and immune-staining, suggesting exposure to the extracelluar side of the membrane[82,90].

Nevertheless, other data suggest a different topology for IFITM3. The protein can be subject to post-translational modifications such as ubiquitylation[95] and phosphorylation[96], those modifications require an N-terminal cytoplasm oriented and accessible to ubiquitin ligase and kinase.

Palmitoylation on conserved and non-conserved residues of murine IFITM1 supported the cytosolic orientation of the C terminus[99], allowing the formulation of a model with both N and C terminus being intracellular. A recent study on murine IFITM3 proposes a new topology, with the N-terminal and conserved internal loop intracellular and the C-terminal extracellular[100] (Fig.12). The authors also observed in some cellular models an alternate topology with the N-Term extracellular, but it is not known if it is a complete inversion of the protein or is involving only the N-terminus. The frequency of the alternate topology is unknown but its existence emphasizes the fact that IFITM3 may change topology under unknown circumstances that are cellular dependent, according also to old literature results.

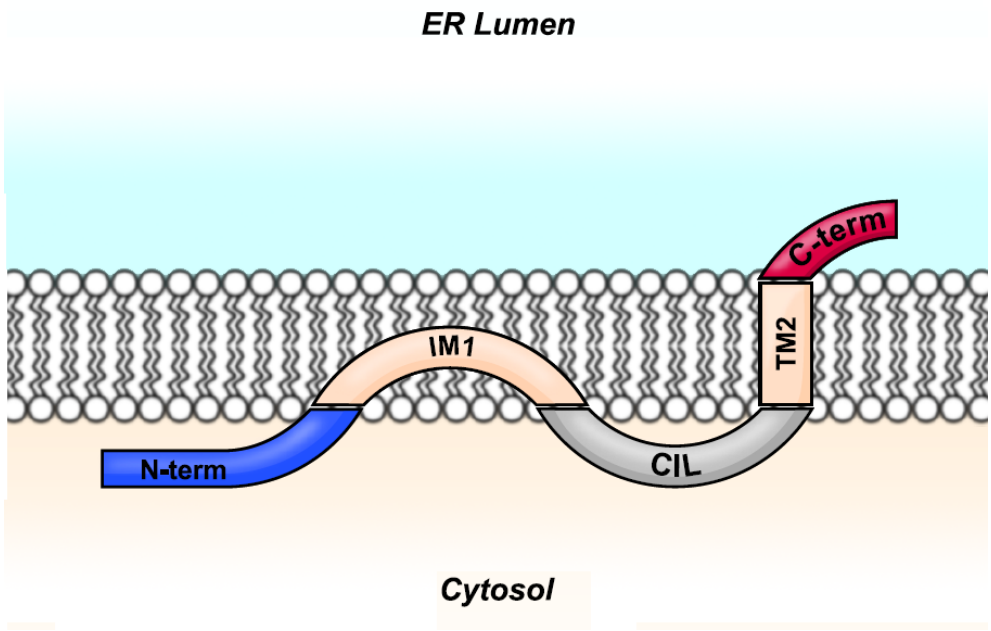


Figure 12. IFITM3 represented as a type II transmembrane protein. N-term and the loop between the two idrophobic domain are intracellular, whereas the C-term region is extracellular or facing the inside of endoplasmic reticulum or endosomal lumen. Modified from [100].

1.4.4 IFITM3 IN DEVELOPMENT

IFITM3 plays a role also in embryonic homing of primordial germ cells (PGCs), where it is expressed and promotes homotypic adhesion of PGC precursors to form a cell cluster in the posterior epiblast[101]. In cooperation with IFITM1, which is expressed by PGC precursors and later in the endoderm and repels the PGCs, IFITM3 is necessary for homing of the cells, as cells ectopically expressing IFITM3 tend to localize to the PGC domain in the posterior endoderm[102].

Surani's group created a mouse model in which IFITM3 or all IFITMs family were deleted, surprisingly no appreciable effect was noticed. Mice were fertile and no defects on development or adult life were observed. Deletion of the entire cluster of the IFITMs highlighted that no redundancy could occur, and single deletion of IFITM3 did not disrupt any putative interaction between family members.

The investigators propose that discrepancy with previous results may be due to different technical approaches used by Surani and Tanaka's laboratories. Tanaka used siRNA technology to silence IFITM1 and 3, whereas Surani used targeted mutation to obtain the loss of function, suggesting that previous results could depend on side effect of siRNA used. Moreover, Tanaka used an experimental model that may suffer delay in development due to the strain or passage of the ESCs used to generate embryos, and since PGCs migration is an event that occurs early in development any slight delay could be significant.
Surani suggest that previous results should be reconsidered in view of their finding.

IFITM3 is a fundamental gene for mammary gland development. IFITM3 was first identified in a screening among cells that can differentiate into domes under treatment with differentiating agents and cells that are unable to form those structures[78]. Down-regulation of IFITM3 by targeted oligos inhibited dome formation capacity of LA7 cells, and oligo treatment on already formed domes lead to collapse of the structures.
IFITM3 in undifferentiated cells is localized to Golgi whereas in domes it stays at the plasma membrane. FYN[77] is required for differentiation of LA7 in domes, as it associates with IFITM3 that shuttle to lipid raft domains of the plasma membrane, and as we do know now[96,103] Fyn phosphorylates IFITM3 and regulates its localization.

1.5 EPIGENETIC AND CANCER

Epigenetics is defined as changes in gene expression due to modifications of DNA or histones in a non permanent way. Epigenetics is a dynamic process that influences cell behavior and can be influenced by changes in

the physiology of the organism. Epigenetic modifications include DNA methylation, histone methylation, acetylation and phosphorylation.

DNA methylation is a post replication modification step that occurs at cytosines in CpG islands, regions rich in cytosines and guanines positioned at the 5' of genes[104], and is catalyzed by DNA methyl transferase (DNMT).

Histone modifications occur at the N-terminal domains of proteins and are catalyzed by the enzymes: histone acetyltransferases (HATs), histone deacetylases (HDACs), histone methyltransferases (HMTs), and histone demethylases (HDMs). The combined modifications involving histones create a complex of information and instructions named "histons code", that can influence affinity of DNA binding proteins and thus their activity[105,106].

Acetylation of histones H3 and H4 are associated with active transcription whereas hypo-acetylated histones are associated with inactive genes.

Phosphorylation of histone H3 is correlated to chromosome condensation during mitosis[107,108] and is necessary for a coordinate segregation of chromosomes without loss of genetic information[109].

HMTs catalyze methylation of histones H3 and H4 at lysines or arginines[110] K9[111], K4[112], 27 and 36. The lysine residues can accept one, two or three methyl groups. Histone methylation can be associated with active transcription or gene silencing depending on the number of methyl groups bound [113,114].

As previously mentioned, breast cancer is a heterogeneous disease which insurgence is related to mutations in oncogene or tumor suppressor genes. Epigenetic alteration of the physiological state of the cells are related to cancer, since tumor suppressor DNA repair genes can be silenced by promoter methylation, resulting also in an increase of genome instability and mutations[115]. In breast cancer there is evidence of hypermethylation of tumor suppressor genes or hypomethylation of oncogenes such as BRCA1, p16 or TMS1[116,117]. For instance, detection of CpG islands methylation of

the promoter of APC, CDH1, and CTNNB1 showed their involvement in cancer onset and progression[118].

In ER negative cancers, histone modifications and DNA methylation is involved in ER regulation. For instance, HDAC1 and DNMT1 are responsible for the transcriptional repression of the receptor and their inhibition by drug treatment can restore ER expression[119,120].

Another example of epigenetic involvement in breast cancer is EZH2, the enzymatic component of the polycomb repressive complex 2 responsible for lysine 17 methylation on histone 3.EZH2 up-regulation correlates with aggressive cancer and poor prognosis. EZH2 dysregulation promotes invasion in vitro and in vivo and could be used as a biomarker for aggressive tumor detection[121].

The epigenetic state of tumor cells and level of enzymes involved in epigenetic modifications can thus be fundamental for early diagnosis and prediction of the evolution of the tumor and also for therapeutic treatment.

2 AIM

Our previous research demonstrated that IFITM3 is necessary for polarization and homotypic adhesion of epithelial cells in the formation of domes.

The aim of this thesis research is to investigate the role of IFITM3 in lumen formation of complex 3D structures associated with mammary gland development and specifically to investigate the role of IFITM3 in the maintenance of the stem cell properties and in the the luminal cell function using rat mammary CSCs and human mammary cancer cells as model systems.

We hypothesize that since IFITM3 has a role in 3D lumen structure formation, loss of or down-regulation IFITM3 protein it may contribute to or tumor formation by promoting loss of cell polarity, loss of cell adhesion and consequently allowing cell invasion.

3 MATERIALS AND METHODS

3.1 CELL CULTURE CONDITIONS

LA7 cells were cultured in DMEM (Invitrogen), 10% fetal bovine serum (FBS, Gibco Laboratories), supplemented with 50 ng/mL insulin and 50 ng/mL hydrocortisone. Cells were harvested with 0,05% trypsin (Invitrogen) containing 0,53 mM EDTA (Gibco Laboratories).

MCF7 cells were cultured in RPMI (Invitrogen), 10% fetal bovine serum, supplemented with 10 µg/mL insulin. Cells were harvested with 0,05% trypsin containing 0,53 mM EDTA.

3.2 CYS FORMATION ASSAY

For cysts formation MCF7 were seeded 500 cells/mL in culture medium and fresh medium was added every two days.

3.3 SPHERE FORMATION ASSAY

For sphere formation LA7 were seeded, at clonogenic density, in culture medium containing 0.5X B27 without vitamin-A (Gibco Laboratories), 10 ng/mL EGF, 10 ng/mL bFGF (Sigma) and 4 µg/mL heparin (Sigma) in low attachment plates (Bibby Sterilin).

3.4 3D STRUCTURE FORMATION ASSAY IN COLLAGEN CULTURES

Monolayer cultures were detached by trypsinization, mixed with collagen derived from rat tails in ratio 1:2 and plated at a concentration of 2500 cells/well in 24 multi-well plates. After collagen had solidified, 500 µL of medium, was added. Medium was refreshed by replacing and half of medium every 2-3 days.

3.5 GENE KNOCK DOWN WITH ANTISENSE OLIGONUCLEOTIDES

The antisense oligomer (AS-IFITM3) sequence was designed as a complementary sequence to the 5'end of the IFITM3 coding region, and the sense oligomer (S-IFITM3) from the same coding sequence but in opposite orientation: AS-IFITIM3 5'-TTTCGTAGTTTGGGGGTTGT-3' and S-IFITM3 5'-ACAACCCCCAAACTACGAAA-3'. The oligos were used at dosage of 40 µg/mL. The cells were pre-treated after plate attachment for 72 hours and then used to generate spheres and tubuli. Fresh oligos were then added to the cells at every change of medium during tubuli and sphere formation.

3.6 shRNA AND LENTIVIRAL TECHNOLOGY

To stably down-regulate IFITM3 expression we prepared a construct containing a short hairpin RNA construct to target IFITM3 transcript (5'-TGCTGTGACAGAAGCCGATCCGTGGGGTTTTGGCCACTGACTGACCC CACGGAGGCTTCTGTCA-3') and cloned it into the pDrive cloning vector. The shRNA was then moved to pCDH-CMV-MCS-EF1-copGFP construct by digesting the pDrive cloning vector with the endonuclease BamHI and NotI (New England Biotechnologies).

A lentiviral approach was used to allow for the genomic integration and constitutive expression of the shRNA to down-regulate IFITM3 expression. Lentiviral components were produced in HEK293 cells by transfecting the pCDH vector together with psPAX2 and pMD2.G using the Lipofectamine 2000 transfection reagent (Life Technologies) according to manufacturer's instruction. The medium of culture containing the viral particles was harvested after 48h and 72h from the time of transfection and precipitated with the PEG-it Virus Precipitation Solution (System Bioscience) according to manufacturer's instruction.

Cells were transduced adding lentiviral preparation to culture medium additionated with 8µg/ml Polybrene (Sigma).

3.7 DESIGNING AND GENERATION OF RAT AND HUMAN IFITM3-eGFP FUSION PROTEIN SUITABLE FOR RAT AND HUMAN PROTEIN LOCALIZATION.

To follow real time the localization of IFITM3, a fusion protein with the enhanced Green Fluorescent Protein (eGFP) was produced. The coding sequence of the protein without the stop codon was inserted upstream the eGFP.

The rat IFITM3-eGFP fusion vector was generated cloning by PCR the CDS of rat IFITM3 without the stop codon from the pDRIVE with the following primer pairs FOR 5'-CGGAATTCATGAACCACACTTCT-3' and REV 5'-TCGTCTCCAGACTGGATCCCG-3'. The PCR product was digested with restriction enzymes EcoRI and BamHI and inserted into the linearized peGFP-N3 (Clontech, discontinued).

The human IFITM3-eGFP fusion vector was generated cloning by PCR the CDS of human IFITM3 from the pCDH with the primer pairs FOR 5'-CGCGGGCCCGGGATCAGTGTGCTGGAATTCG-3' and REV 5'-TGGTGGCGATGGATCCTCCATAGGCCTGGAA-3'. The PCR product was inserted in the peGFP-N3 digested with BamHI using the In-Fusion™ Advantage PCR Cloning Kit (Clontech) and the peGFP-N3 linearized with BamHI.

3.8 FLUORESCENCE ACTIVATED CELL SORTING

Fluorescence Activated Cell Sorting (FACS) was performed with a FACS Canto II cytometer (BD Biosciences) and the software FACS Diva (BD Biosciences). Cells were harvested with 0,05% trypsin (Invitrogen) containing 0,53 mM EDTA (Gibco Laboratories) and washed with PBS prior to perform the analysis.

3.9 RNA EXTRACTION AND QUANTITATIVE PCR

Total RNA was extracted using the TRIzol® (Life Technologies) reagent following manufacturer's instruction. RNA was reverse transcribed using High Capacity RNA Master Mix and mRNA expression was evaluated by Real Time PCR with SYBR Green (Applied Biosystems) with a 7500 Real Time PCR System (Applied Biosystems).

RT-PCR was performed on LA7 cells transfected/transduced with either siRNA oligos or with the lentivirus containing shRNA for the down-regulation of IFITM3. Untreated cells were used as control.

Species	Gene	Gene Bank ID	Forward primer	Reverse primer
Rat	*Ifitm3*	NM_001136124.1	CACGGATCGGCTTCTGTCA	AGCCCAGGCAGCAGAAGTT
Rat	*Hprt1*	NM_013556	TCCATTCCTATGACTGTAGATTTTATCAG	AACTTTTATGTCCCCCGTTGACT
Rat	*Olr1378*	NM_214828.1	CCCATGCTGAACCCTTTTATTT	CCAGTTTTTGTAGAGCCCCTTTC
Rat	*Olr1471*	NM_001000722.1	CCCTGTTCCTGTCCATGTACCT	GAAGGACAAGTTGCTGAGAAAAAAG
Rat	*Olr866*	NM_001000411.1	ATGATTCCAGGCAAGAACCAA	GGTCACTGGAGAAGCCAATCAG
Rat	*Suv39h1*	NM_001106956.1	CGAGGAGCTCACCTTTGATTACA	AGGCCAAAGTTGGAGTCCATT
Rat	*Setdb1*	NM_001271175.1	TGGCTCTGATGGCGATGA	TTTTACTGCCACCTGACGCTTT
Rat	*Prmt3*	NM_053557.1	AGTGGATGGGCTATTTTCTTCTTTTT	CAAGTATTTGCTCTTGGCATAAAGG
Rat	*Kdm1b*	NM_001107343.1	TGGTCCAGCAGGCTTAGCA	GACAGTCACCTTCATCCCAAAGT

Table 1. Primers for RT-PCR. Hprt1 is used as house keeping gene.

3.10 MICROARRAY EXPRESSION AND BIOINFORMATIC DATA ANALYSIS

Total RNA of LA7 cells siRNA transfected or lentivirus transduced for the down-regulation of IFITM3 and untreated cells was extracted using TRIzol® (Life Technologies) following manufacturer's instruction. Expression profile analysis performed using the GeneChip® Rat Gene 1.0 ST Array according to manufacturer's instruction (Affymetrix).

The microarray expression analysis was performed to obtain the "Robust Multichip Average" (RMA)[122] which takes into account subtraction of the background signal, quantile normalization and sum of the value of the probes belonging to the same set. The quality of the normalized data was evaluated generating different charts explorers, such as the density distribution of the signal, principal component analysis, logarithmic expression and the relative dependence of the variance from the mean.

The differential expression of each gene among groups of different samples was calculated using the Student t-test moderate[123]. The p-value associated with the t statistics moderate was used to calculate the values of FDR (false discovery rate) associated with the genes. Analysis were performed using the R language[124] and software packages written in R that contains implementations of the methods described above.
The top 500 genes with the highest absolute fold changes were considered as differentially expressed. In all comparisons such genes had an absolute log2 fold change greater than log2 (1,5).
Pathway analysis was carried out using the hypergeomtric and using NCBI Biosystems database as source of rat gene-pathway associations. Pathways with FDR adjusted p-value < 0.05 were considered as significant.
A second analysis was performed on gene, in order to cluster genes with the same Gene Ontology, or belonging to the same pathway or by known

interaction using the bioinformatics tools DAVID[126,127], Panther[128] and KEGG [129,130].

4 RESULTS

4.1 TUBULE FORMATION ASSAY

It was previously demonstrated that LA7 cells have stem cell properties and are therefore represent an *in vitro* and *ex situ* model for studying the differentiation programs of the mammary gland and the properties of self renewal.

LA7 cells when cultured in 3D culture conditions, form tubular structures similar to mammary gland ducts. Additionally, LA7 cells generate in adherent culture conditions, blister like 2D structures called domes that morphologically and functionally recapitulate alveoli that form in mammary gland at late pregnancy. We hypothesized that the role of IFITM3 in the formation of 2D-like structures was also required for the formation of 3D structures such as tubuli and acini. Therefore to investigate the role of IFITM3 in the formation of more complex 3D structures, tubule formation and cyst assays were developed in our lab using rat tail collagen.

Different approaches were undertaken to evaluate the effect of the inhibition of IFITM3 in the 3D structure formation capacity of cells. In one assay LA7 cells were cultured in adherent conditions and pre-treated with antisense oligo targeting *Ifitm3* in order to down-regulate the level of mRNA and protein, then cells were then embedded in collagen (Fig.13). In other assays LA7 cells were seeded in collagen in the presence of the oligos and treated after collagen solidification (Fig.14). In agreement with IFITM3 being essential for 2D structure formation, as shown for dome formation, experimental results show that inhibition of IFITM3 protein synthesis resulted in no capacity of LA7 cells to generate complex three dimensional structures such as tubuli (Fig.13, 14).

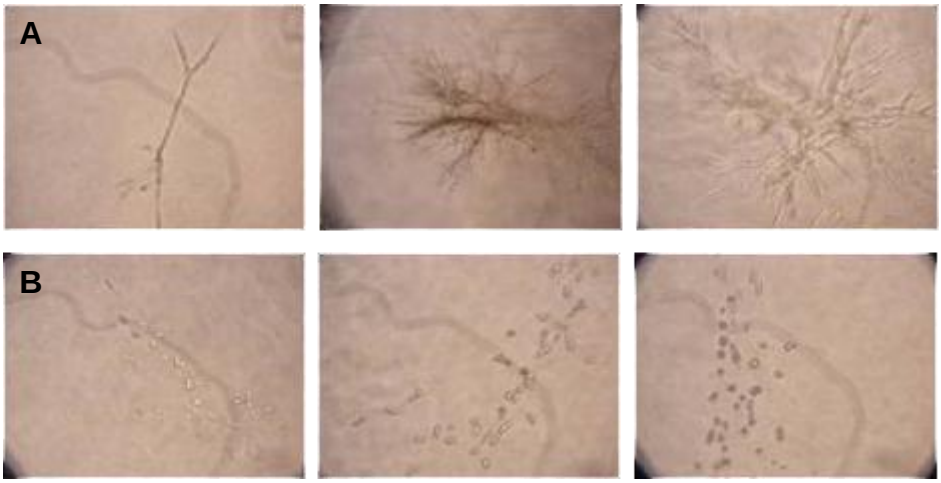


Figure 13. Collagen culture assay with antisense IFITM3 (AS)-oligos pre-treatment. LA7 cells were pre-treated for 72 hours prior to embedding them into 3D collagen, and subsequently also treated during collagen cultures. After 10 days of culture control cells show branched tubuli (A), while cells treated with AS-IFITM3 do not form tubuli (B). 4X Magnification.

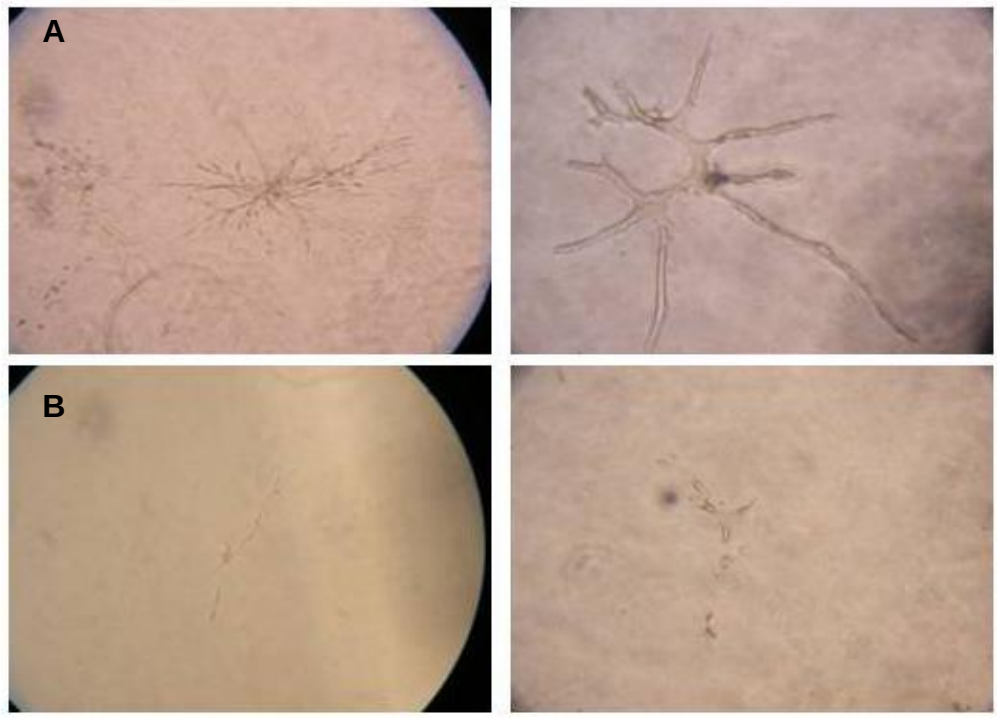


Figure 14. Collagen culture assay with no As-oligo pre-treatment. Tubuli formation. Cells were embedded in collagen in the presence of the oligos and treatment occurred after collagen solidification. Control cells show branched tubuli (A) after 7 (left, 4X magnification) and 10 days (right, 20X magnification). Cells treated with As-IFITM3 do not develop tubuli (B) after 7 days (left) or at 10 days (right) of culture.

4.2 CYSTS FORMATION ASSAY

As repression of IFITM3 is associated with impairment of dome formation in LA7 cells, and IFITM3 protein is also expressed in human mammary cells, I tested whether the inhibition of IFITM3 would also inhibit 3D structure formation of human mammary epithelial cells. We therefore used MCF7 cells, an epithelial cell line originated from a human adenocarcinoma, which are known for their ability to form cysts, alveolar like structures reminiscent of the mammary gland alveoli. To overcome the difficulty of the continuous treatment/transfection of the oligos we generated lentiviral constructs to stably down-regulate IFITM3 expression. For the constitutive inhibition of IFITM3 expression a construct containing a short hairpin RNA to target IFITM3 human transcript was generated to allow for the genomic integration and constitutive expression of the shRNA to down-regulate IFITM3 expression. shRNA expression is under the control of cytomegalovirus (CMV) promoter, whereas the GFP is under the control of Human elongation factor-1 (EF-1). We choose to use a GFP expressing vector in order to have a reporter which can be used to individuate cells positively transduced, and quantitatively estimate the efficiency of transduction.

After 15 days of culture MCF7 cells untreated or transduced with the empty GFP lentivirus formed spherical structures hollow inside (Fig.15 A to D). Conversely MCF7 cells transduced with the shRNA for IFITM3 lentivirus are unable to form cysts and the cells can only form aggregates in which a lumen is absent (Fig.15 E and F). In the experiments in which cells were transduced with the shRNA for IFITM3 lentivirus, uninfected cells uninfected and thus not expressing the construct for the down-regulation of IFITM3, were still able to form cysts (Fig.15E arrows).

Since IFITM3 is necessary for the formation of cysts-like structure also in human mammary cells, results support the role of IFITM3 in mammary gland development.

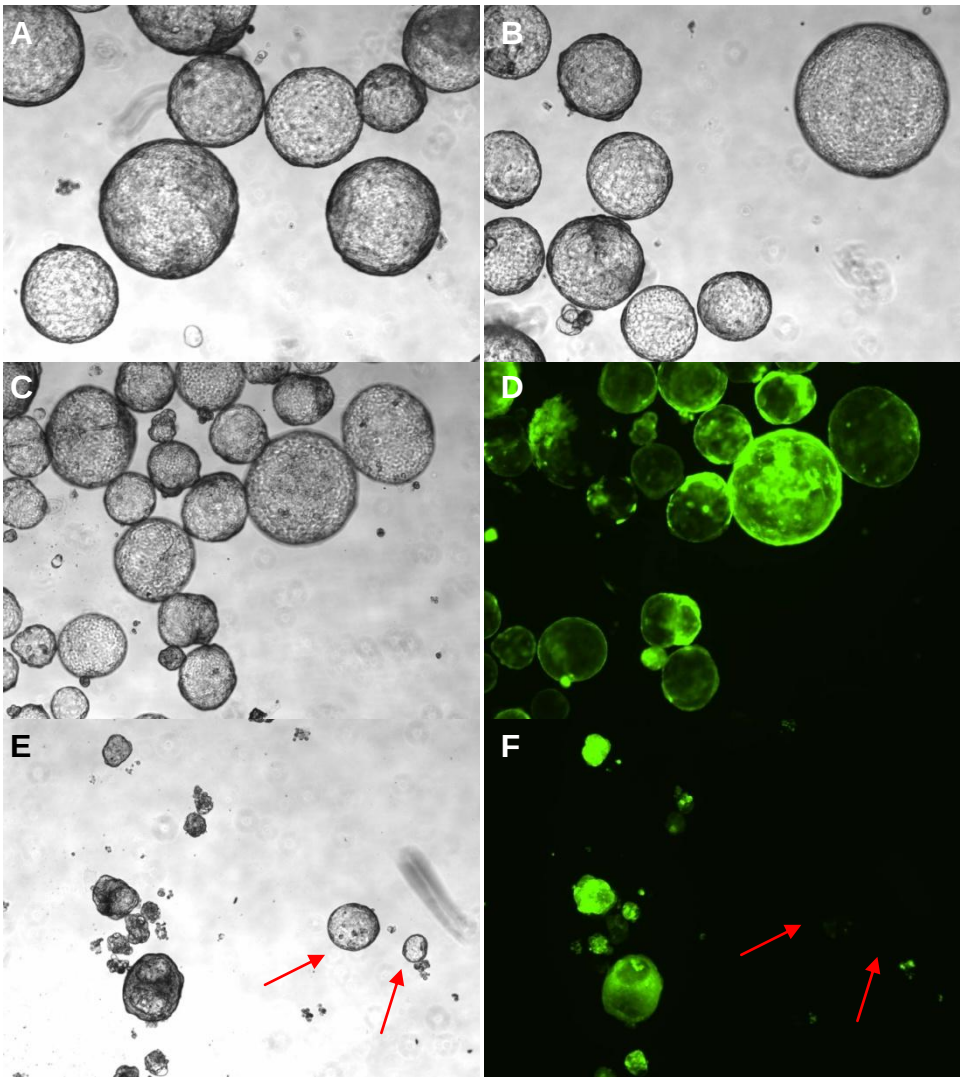


Figure 15. Cyst structure formation by MCF7 cells cultured in non adherent conditions. A and B) Un-transduced or GFP transduced MCF7 cells (C and D) form spherical structures hollow inside called cysts after 15 days of culture. E and F) MCF7 transduced with the shRNA targeting human IFITM3 lentivirus. Cells positive to GFP are expressing the shRNA and fail to develop into cysts and form aggregates. Red arrows point a cyst negative to GFP, in which there is no down-regulation of IFITM3. 4X magnification.

4.3 SPHERE FORMATION ASSAY

Our group and others have demonstrated that the capacity of cells to generate spheres in non-adherent conditions is a property that defines mammary SCs and CSCs[131]. To investigate the role of IFITM3 in MaCSs we tested the capability of LA7 cells either treated with IFITM3 antisense oligos (Fig.16) or transduced with a lentiviral construct constitutively expressing a shRNA targeting IFITM3 (Fig.17) and evaluated for their capacity to form spheres in non-adherent cultures conditions.

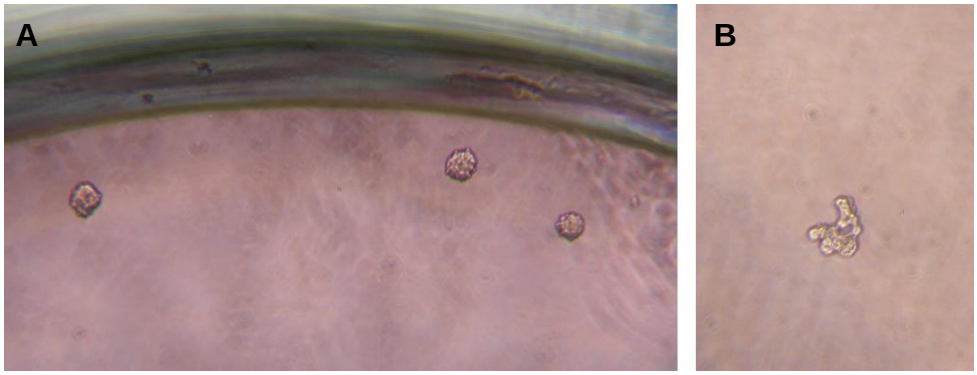


Figure 16. Spheres formation assay. Control cells (A, 4X magnification) show capability of sphere formation, while cells treated with As-IFITM3 form aggregates but no spheres (B, 20X magnification).

Control cells cultured without oligo treatment and cells treated with sense oligos (designed from the same coding sequence of IFITM3, not able to pair to IFITM3 mRNA and promote its degradation) generated spheres, while IFITM3 antisense oligo treated LA7 were unable to form spheres (Fig.16). The same experiment was repeated with LA7 cells transduced with lentivirus containing the shRNA targeting IFITM3 or the empty GFP vector and similar results were obtained. LA7 transduced with a lentivirus expressing shRNA targeting IFITM3 form aggregates but not spheres (Fig.17, A and B) while control LA7 transduced with the empty GFP vector formed spheres after 6 days of culture (Fig.17, C). As single La7 CSCs have the capacity to regenerate spheres indefinitely, lack of sphere

formation with transient or stable down-regulation of IFITM3 would help in providing insight into whether the expression of IFITM3 is required for self-renewal of LA7 CSCs. Taken together these results suggest an involvement of IFITM3 in the maintenance of stem cell properties of LA7 cells.

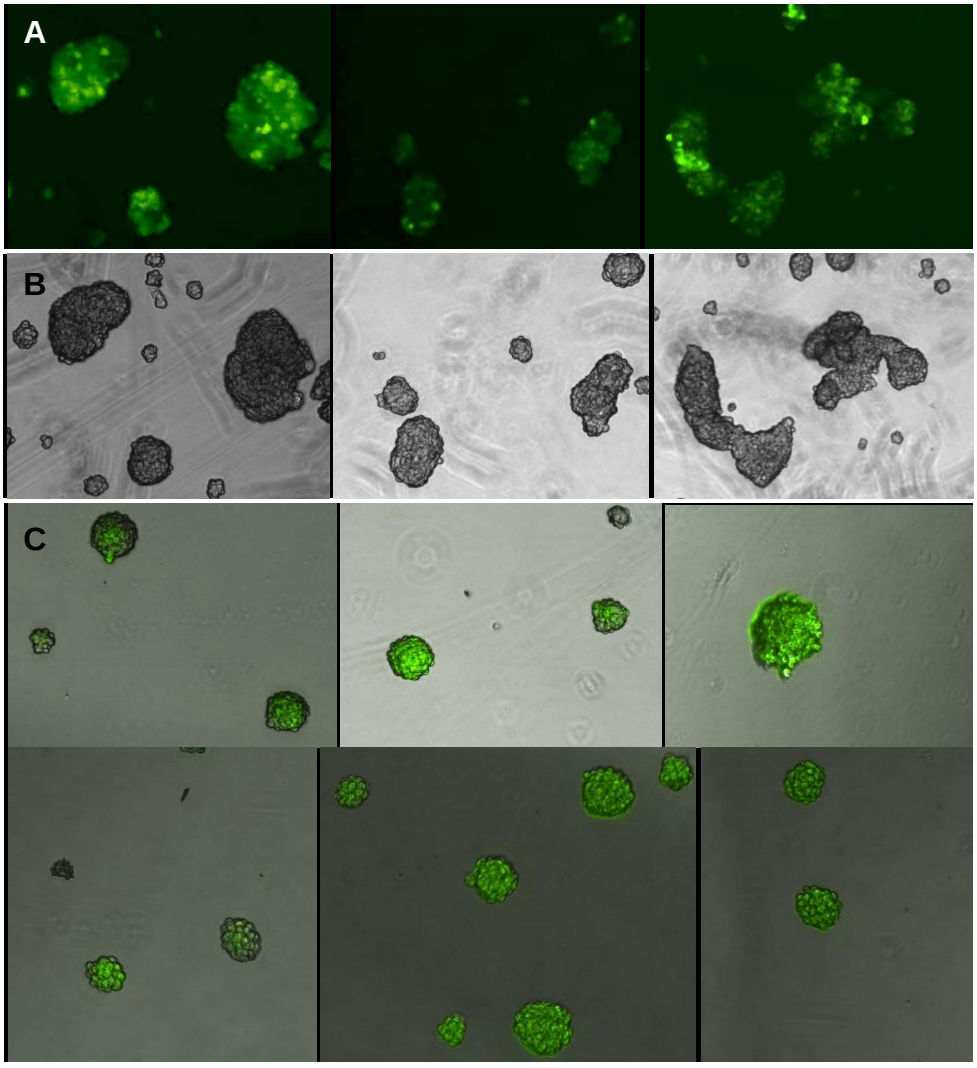


Figure 17. A,B) LA7 cells transduced with lentivirus expressing a shRNA targeting rat IFITM3. After 6 days of non adherent culture there are no spheres observable. LA7 cells in which IFITM3 is down-regulated can form only aggregates. In the upper panel the GFP filter is showing the population positive to down-regulation of IFITM3, in the above panel the phase contrast. 4X magnification.. C) LA7 cells transduced with the empty GFP lentivirus. Control cells positive to GFP are able to grow in non adherent conditions and to form spheres. 10X magnification.

Spheres generated by the LA7 cells in contrast to cysts generated by MCF7 cells (Fig.15) do not contain lumen. These results suggest that IFITM3 may have a different function in sphere formation to respect to the previously identified role in lumen formation[78] in domes or cysts (Figure 15). As previously demonstrated by Zucchi's lab LA7 spheres are generated by single cell and LA7 spheres can be regenerate indefinitely, the observed lack of sphere formation with down-regulation of IFITM3 suggest that the expression of IFITM3 is required for self-renewal of LA7 CSCs.

Data also suggest that continuous IFITM3 expression is required for maintaining CSCs as supported by the observation that transient down-regulation of IFITM3 with siRNA oligos result in the permanent loss in the ability of LA7 CSCs to regenerate spheres. In addition, in long term cultures in which IFITM3 was down-regulated in LA7 cells a significant loss of GFP positive cells was observed. While this result needs to be further investigated, an intriguing conclusion is that expression of IFITM3 may not just be required for CSC self-renewal but for cell survival. Closer investigations, suggested that the decrease in cell number observed with IFITM3 down-regulation, was concomitant with trypsin-dependent passaging step, where the cells are removed as adherent cultures from the plates and are made into single cell suspensions. With each disruption of the cells into single cells, it was observed that in the condition in which IFITM3 was down-regulated fewer cells had the ability to re-attach suggesting that re-expression of IFITM3 is essential to allow the cells to attach to the tissue plate surface. These results may also suggest that re-expression of IFITM3 after trypsinization is essential to inhibit anoikis following the disruption of cell-cell contact. Determining whether cell trypsinization combined with the down-regulation of IFITM3 resulting in decrease cell numbers is caused by induction of a cell death pathway (e.g., anoikis) or by the inability of the cells to re-attach to the tissue culture plate, needs to be further investigated.

In parallel experiments in rat myoepithelial like cells that express low levels of native IFITM3 compared to the LA7CSs (Fig.18) the down-regulation of IFITM3 resulted in no loss in cell propagation capacity (Fig.19). This control experiment supports that loss of self-renewal observed with down-regulation of IFITM3 in LA7SCs is specifically due to down-regulation of IFITM3 expression and shows the specificity of the effect of shRNA for IFITM3.

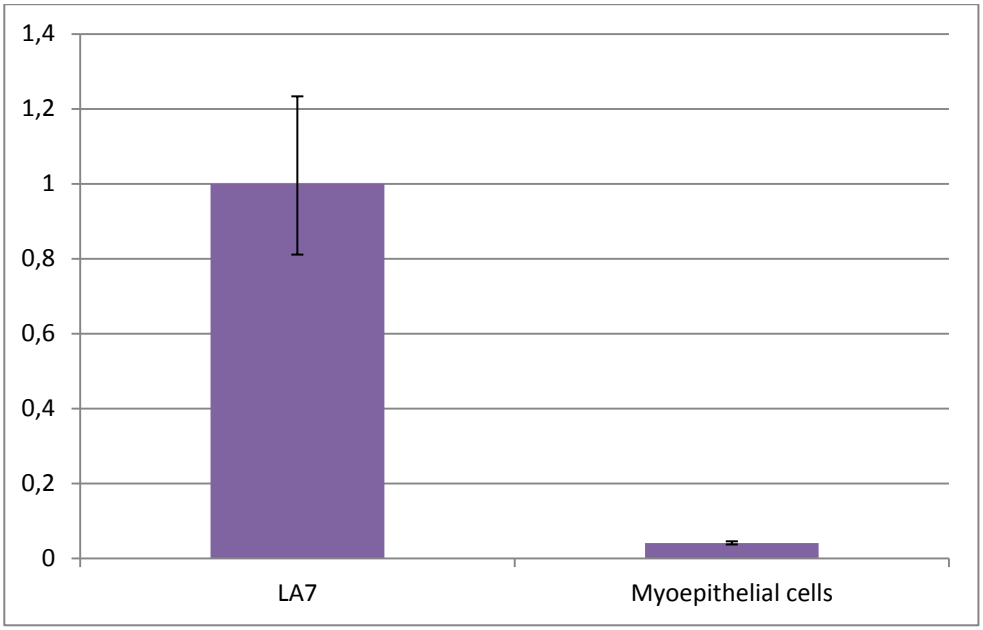


Figure 18. Real Time data of IFITM3 expression in LA7 cells in comparison to myoepithelial cells. A strong difference of IFITM3 expression approximately of 95% is evident. Vertical bars represent the standard error of the 95% level of confidence.

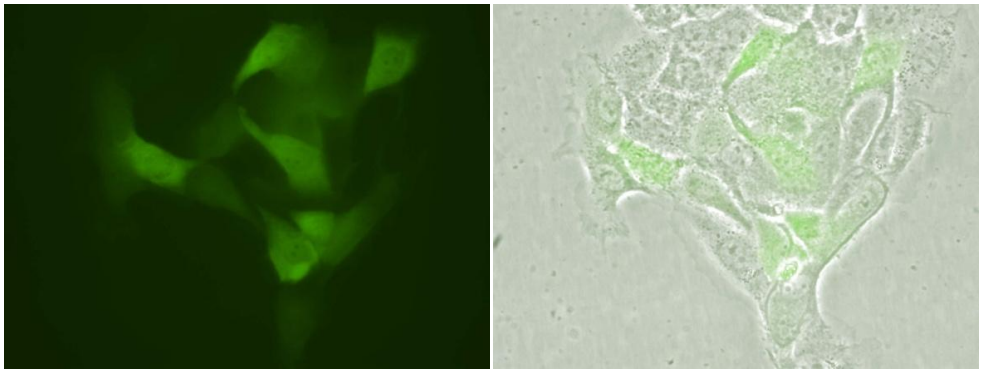


Figure 19. Rat myoepitheial cells that express low levels of IFIMT3 compared to LA7 CSCs can be propagated indefinitely with down-regulation of IFITM3. Myoepithelial like cells generated from LA7 CSC transduced with the shRNA for rat IFITM3 after 11 days of culture. The myoepitheial cells express low levels of IFIMT3 compared to LA7 CSCs. A) Fluorescent and merged fluorescent and bright field (B) images of cells showing cell positive for GFP. 40X magnification.

The intriguing conclusion is that expression of IFITM3 may not just be required for CSCs self-renewal but for cell survival. Whether IFITM3 expression is essential and how the expression functions in regulating self-renewal SC or CSC self-renewal is under investigation with on-going research by the lab of Zucchi.

4.4 eGFP FUSION PROTEIN

Since it is reported that the cellular function of IFITM3 is dependent on its ability to migrate from endosomal compartment to plasma membrane[77] we decided that following the shuttling of the protein during differentiation of the cells was of high interest for understanding IFITM3 involvement in the process.

To achieve this goal constructs expressing a chimeric protein of rat and human IFITM3 fused with the eGFP were designed and generated.
LA7 transfected with the rat IFITM3-eGFP fusion construct showed cytosolic staining of IFITM3 24 hours after transfection (Fig.20). Since down-regulation of IFITM3 suggested that LA7 CSCs displayed decreased cell survival, it was surprising to see that up-regulation of IFITM3 also

resulted in significant cell death, as indicated by the fact that cells expressing the IFITM3-eGFP fusion protein decreased over time compared to the GFP control expressing cells.
eGFP positive staining was detected in IFITM3-eGFP floating cells with fluorescent microscopic imaging (Fig.21). In contrast to the cell loss observed with down-regulation of IFITM3, which appeared to be a relavitely slow process dependent on trypsinizaiton and passaging of the of cells in adherent culture, cell death with the fusion protein in which IFITM3 was up-regulated occurred rapidly after the GFP protein was expressed. The increase cell death with up-regulation of IFIMT3 compared to cell loss with IFITM3 down-regulation may suggest that there are differet functions of IFITM3 depending on its expression level. For instance, we hypothesize, down-regulation-dependent loss of the cell number in in vitro cultures is due to a loss of a cell surface and cell-to-cell contact, while up-regulation of IFITM3 induced cell death by an unknown mechanism that is at the moment under investigation.

Cell death induced with over expression of IFITM3 was not consideredd to be due to a non-specific or off-target effect because even cells with the lowest expression of the fusion protein (as measured by eGFP expression) died relatively quickly after the fusion protein was detected by fluorescence. Many of the dead and floating cells showed a positive staining for IFITM3-eGFP (Fig.21).
In the next days positive vital cells transfected with IFITM3-eGFP were no more detectable, whereas cells transfected with the empty control vector resulted alive up to 72 hours after transfection (Fig.22).

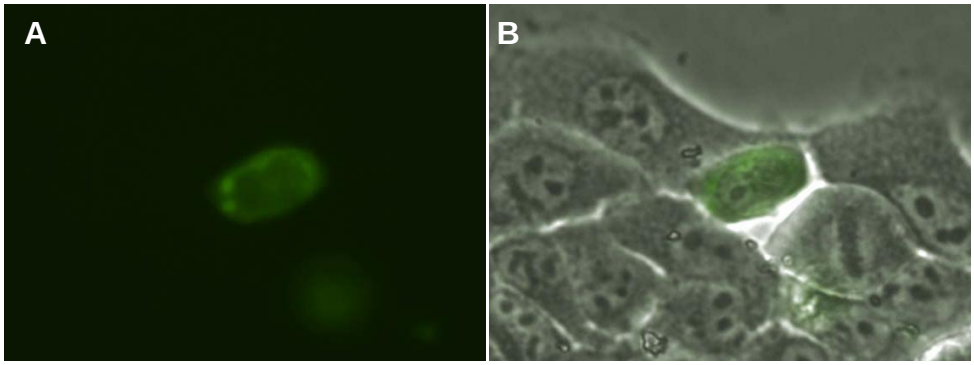


Figure 20. A) LA7 cells 24 hours from transfection with rat IFITM3-eGFP fusion protein. B) LA7 in phase contrast merged with GFP staining. 40X magnification.

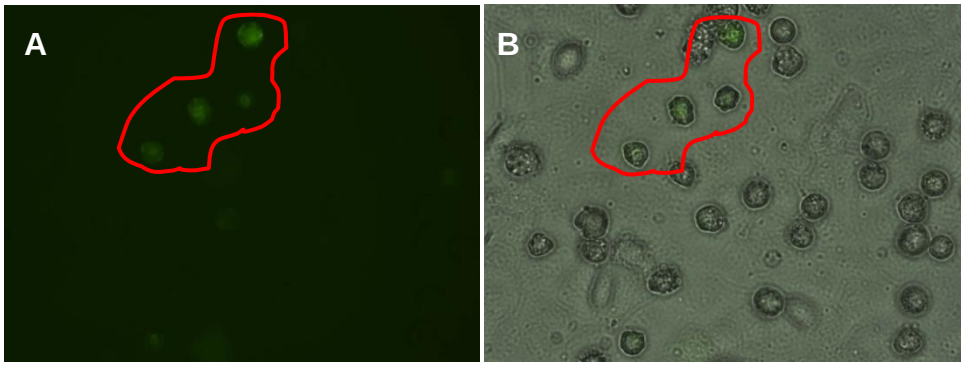


Figure 21. LA7 cells 24 hours from transfection with rat IFITM3-eGFP fusion protein. A) The majority of positive cells, highlighted in red, resulted dead. B) LA7 in phase contrast merged with GFP staining. 40X magnification.

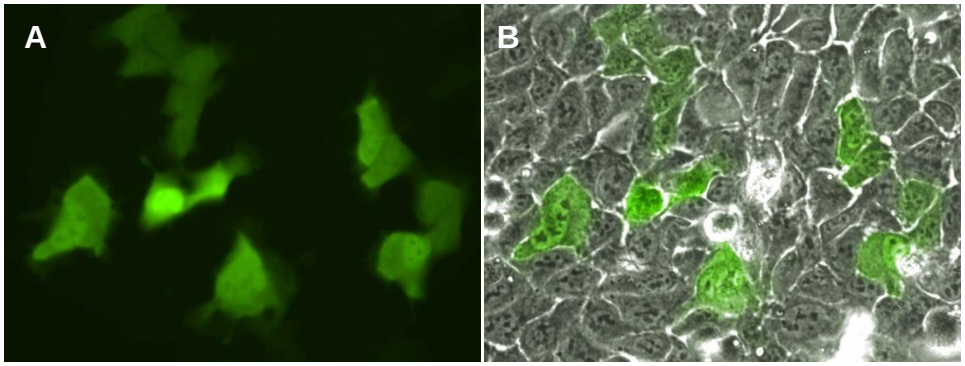


Figure 22. LA7 cells 72 hours from transfection with empty eGFP fusion protein. A) Staining of eGFP can be observed in LA7 cells. B) LA7 in phase contrast merged with GFP staining. 10X magnification.

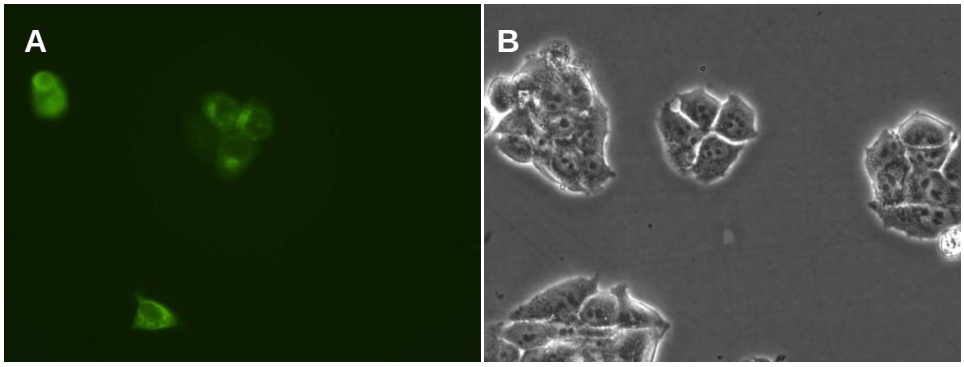


Figure 23. A) MCF7 cells 24 hours from transfection with human IFITM3-eGFP fusion protein. B) Phase contrast picture of MCF7 cells. 40X magnification.

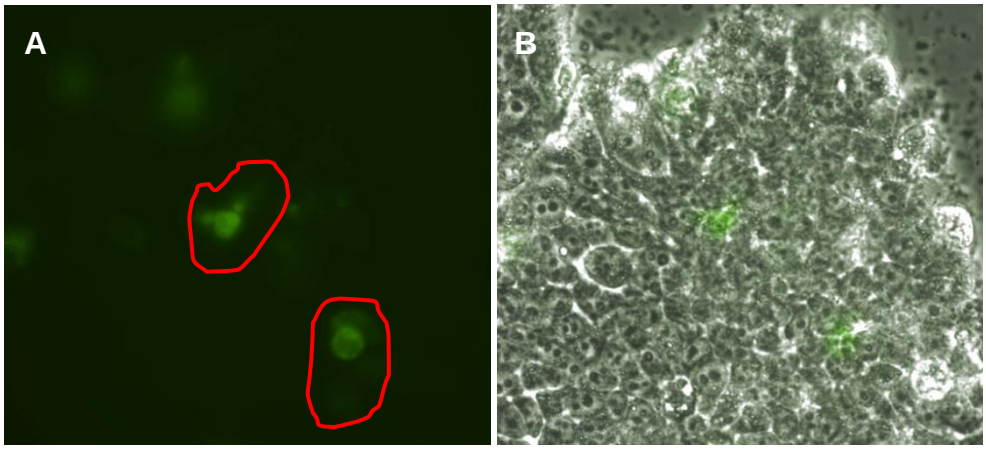


Figure 24. MCF7 cells 24 hours from transfection with human IFITM3-eGFP fusion protein. A) The majority of positive cells result dead, highlighted in red. B) LA7 in phase contrast merged with GFP staining. 40X magnification.

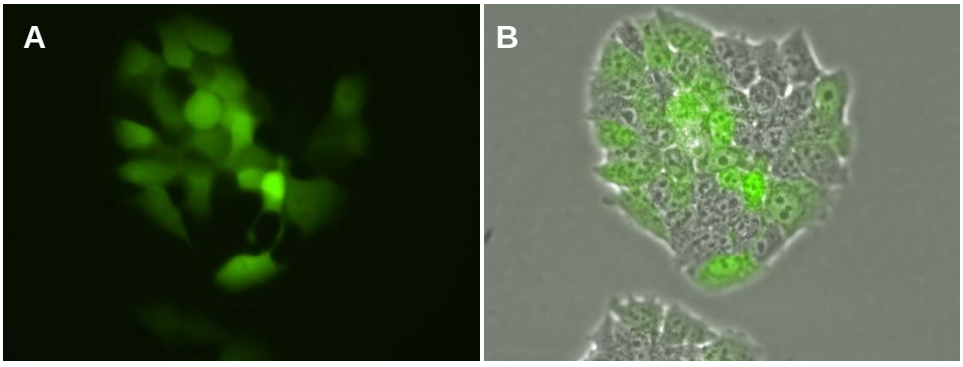


Figure 25. GFP fusion protein expression in MCF7 at 7 days after transfection. Fluorescent (A) and merged fluorescent and bright field (B) images, 40X magnification. In contrast to cells expressing the IFITM3-eGFP fusion protein the cells do not display aberrant cell morphology and do not detach from the tissue culture plate.

MCF7 were also transfected with human IFITM3-eGFP fusion protein. As for the rat IFITM3-eGFP fusion protein, perinuclear staining was observed (Fig.23). Also in MCF7 cells, expression of IFITM3-eGFP fusion protein is accompanied with aberrant cell morphology and cell detachment from the culture plates at the onset of fusion protein expression (Fig.24). In fact shortly after transfection, cells expressing IFITM3-eGFP have mostly detached from the plate surface, while control cells expressing only the GFP protein can survive even after one week (Fig.25).

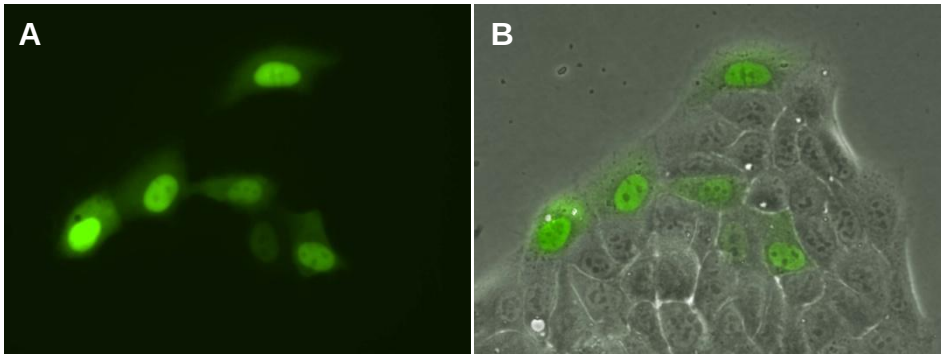


Figure 26. A)LA7 cells expressing a protein fused with eGFP localized into nuclei after 24 hours of transfection. No cell death was observed after transfection. B) LA7 in phase contrast merged with GFP staining. 40X magnification.

To assess whether the IFITM3-GFP fusion protein induces cell death due to non-specific activity or off-target effects, LA7 CSCs were transfected with a fusion protein generated from GFP and IRF1 protein used as a control transcription factor protein (Fig.26). The IRF1-eGFP fusion protein localized into nuclei as expected for a transcription factor and no cell death was observed in long term cultures (shown for 72 hours from transfection).

The expression analysis suggests that cell viability is impaired with human and rat IFITM3-eGFP expression even at the lowest detectable levels visually within the first 24-48 hours.

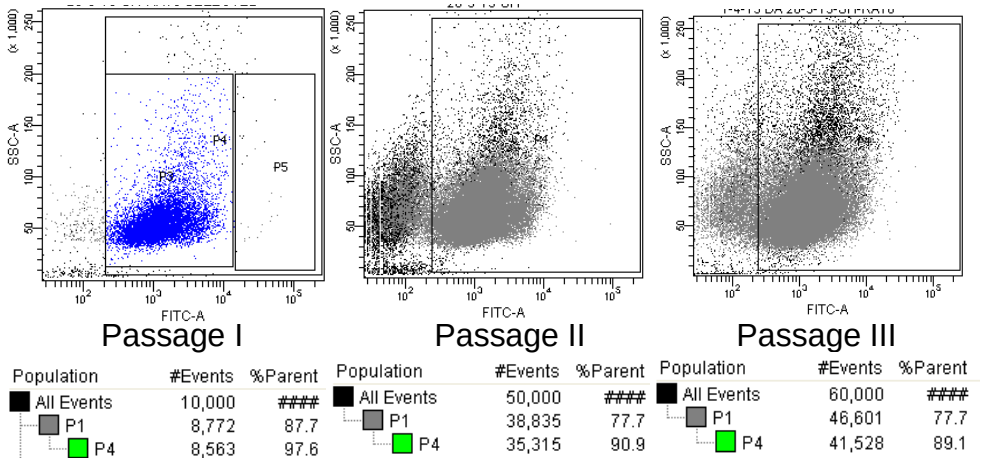
Human and rat IFITM3-eGFP seems to damage the cells in the early hours after transfection inducing the protein expressing cells to die within the first 24-48 hours.

A possible explanation of the cell induced stress is that the expression of the fusion protein induces a dominant negative effect or that IFITM3 fusion protein overexpression induces a dosage dependent blocking on the wild type IFITM3 protein activity or alters the localization of native IFITM3 protein. Further experiments are underway to gain further insight into the role of IFITM3 over expression. Early research has demonstrated that IFITM3 has a role in inhibiting cell proliferation. In agreement, the lab of Zucchi has shown that IFITM3 mRNA and protein are up-regulated in terminally differentiated cells that are maintained in a quiescent state of cell proliferation.

4.5 ROLE OF IFITM3 IN LA7 CELL PROLIFERATION

In contrast to the rapid cell loss and detachment of the cells from the culture plates with up-regulation of IFITM3-eGFP fusion protein occuring with in 72 hours of onset of induced expression (Figures 20 and 25), loss of LA7 cells with down-regulation of IFITM3 appeared to be a relavitely slow process with cell passaging. To quantify and confirm the qualitivative observation that a decrease of SC number occurred with IFITM3 down-regulation, fluorescence activated cell sorting (FACS) was utilized. LA7 CSCs transduced with lentivirus containing GFP and shRNA targeting rat IFITM3 or GFP alone were used. Transductions efficiency was assessed for both conditions and were found to be similar (Fig.27). The decrease of LA7 SC number was assessed immediately after IFITM3 down-regulation. A reduction of GFP positive population was observed with cells in which shRNA against IFITM3 was expressed (Fig.27, A), compared to control.

A



B

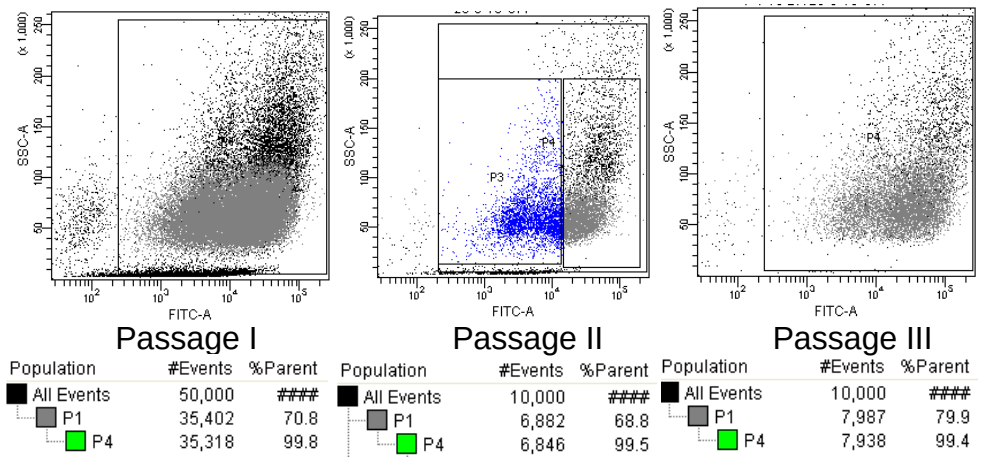


Figure 27. Dot plots obtained from LA7 transduced with a lentivirus for IFITM3 shRNA expression (A) and GFP alone (B). Relative percentage of cells for each population are shown, with P4 and P5 as the GFP positive population. Most of the GFP expressing cells are contained in P4. Increased cell passaging is associated with show a decrease in the percentage of GFP positive cells in cells transduced with a lentivirus for IFITM3 shRNA expression. The GFP population of cells transduced with GFP alone show no decrease in the percentage of GFP positive cells with passaging.

LA7 transduced with the GFP vector show no significant variation of GFP positive population in one month of observation (Fig.27, B) where as LA7 SCs in which IFITM3 was down-regulated disappeared with 3 to 6 passage cultures. The decrease of GFP positive population in LA7 transduced with

lentivirus containing shRNA targeting IFITM3 suggest that cells in which IFITM3 is down-regulated either proliferate less or have reduced SC specific self-renewal capacity. While further experiments are necessary to validate these results, microarray expression analysis supports that the expression of genes regulating cell cycle and/or SC self-renewal is specifically altered with down-regulation of IFITM3 in LA7 SCs (Fig.30).

4.6 MICROARRAY ANALYSIS

In order to better understand the IFITM3 involvement in mammary differentiation and in the stem cell property maintenance, we analyzed the transcriptome of IFITM3-depleted LA7 cells compared to LA7 control, using micro-array expression analysis. LA7 cells cultured in adherent condition were transfected with siRNA or transduced with a lentiviral construct containing a short hairpin RNA (shRNA) targeting IFITM3.

Cell in which IFITM3 down-regulation was approximately of 70% (Fig.28) were used for the microarray profile analisys.

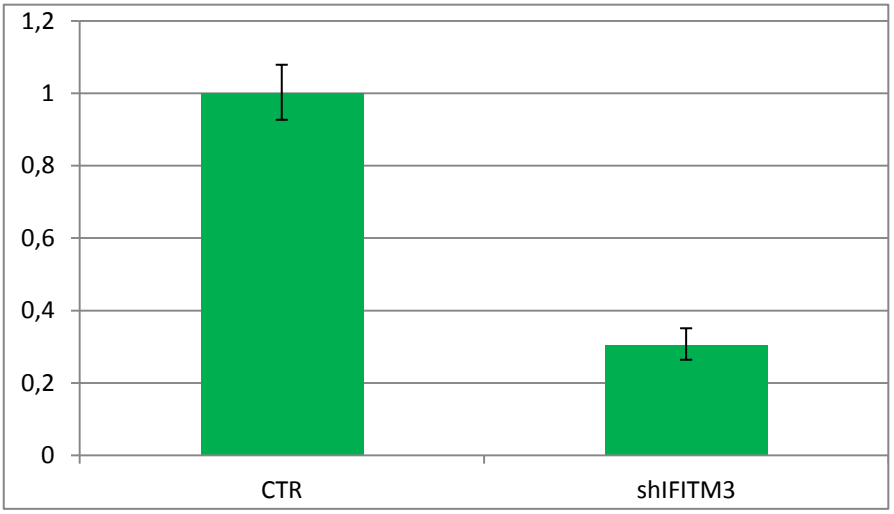


Figure 28. Real Time data of LA7 cells transduced with the shRNA for IFITM3 in comparison to untreated cells. shRNA for IFITM3 down-regulated the level of mRNA approximately of 70%. Vertical bars represent the standard error of the 95% level of confidence.

Untreated or untransduced LA7 were used as a control. The data were normalized and genes with a fold change of 1.5 were considered for analysis. The expression data was compared with two other microarray expression data sets: 1) genes differentially expressed when IFITM3 was up-regulated following LA7 CSC differentiation induced by DMSO and 2) genes differentially expressed in tumors generated in NOD-SCID mice by the injection of a single LA7 CSC[74]. Venn diagrams of overlapping biological/molecular pathways suggest the role of IFITM3 in SC/CSC differentiation and in tumor development (Fig.29). More than 470 pathways are modulated with down-regulation of IFITM3 in LA7 CSCs. The top biological pathways represented with the highest adjusted "P" value indicate that IFITM3 regulates positively or negatively cell cycle associated functions (Table 2), in agreement with observed loss of the SC population with IFITM3 down-regulation.

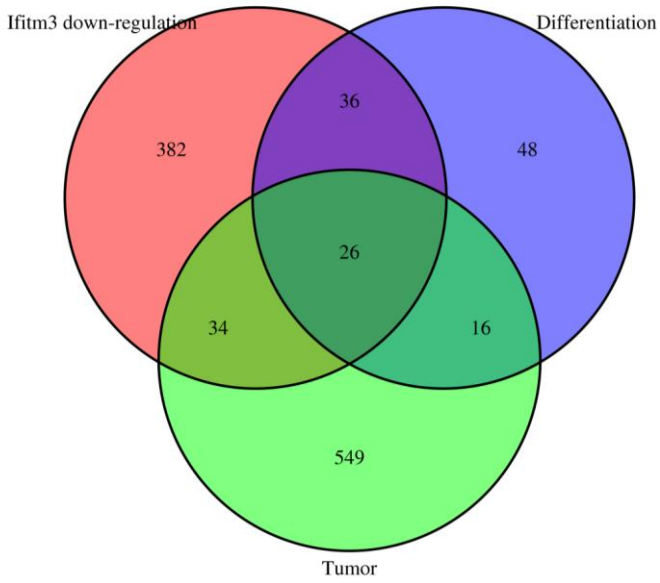


Figure 29. Venn diagrams representing overlapping pathways between LA7 depleted of IFITM3, LA7 differentiating and tumor formation of LA7 injected into NOD-SCID mouse. Of all, 26 pathways are common to all three conditions. Picture generated by Dr Ettore Mosca, modified.

	PATHWAYS		
1	Cell cycle, mitotic	14	DNA replication
2	Mitotic cell cycle process	15	Mitotic prometaphase
3	Mitotic cell cycle	16	Response to alcohol
4	Cell cycle	17	Activation of the pre-replicative complex
5	Nuclear division	18	Regulation of cell division
6	Organelle fission	19	Cell division
7	Cell cycle	20	Response to radiation
8	Response to hypoxia	21	Activation of ATR in response to replication stress
9	Response to decreased oxygen levels	22	Response to estrogen
10	Response to oxygen levels	23	Aging
11	Mitotic nuclear division	24	MicroRNAs in cancer
12	G1 to s cell cycle control	25	Regeneration
13	Regulation of cell cycle process	26	Organ regeneration

Table 2. List of the 26 pathways found common in IFITM3 down-regulated cells, tumor formation of mice injected LA7 and differentiating LA7.

More than 120 pathways were found associated with IFITM3 down-regulation in tumors generated from the injection of single LA7 CSC into the fat pads of NOD-SCID mice. The top candidate pathways regulated by IFITM3 in tumor development are associated with the regulation of cell matrix, motility and adhesion, morphological changes necessary to promote tumor development, angiogenesis and cell cycle related functions.

A total of 26 pathways were found to be in common in mammary gland normal development and tumor development and be regulated by IFITM3 (Fig.30).

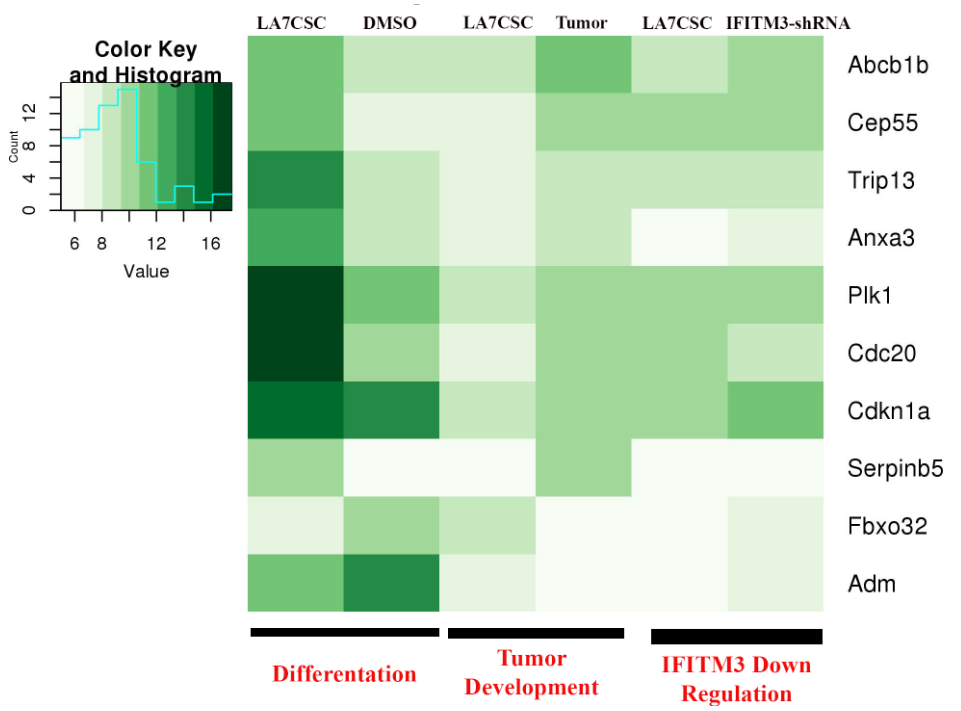


Figure 30. Top 10 responding genes differentially expressed with IFITM3 down-regulation, LA7 differentiation and LA7 dependent tumor formation. Color intensity indicates the level of gene expression in comparison to LA7 CSCs. Picture generated by Dr Ettore Mosca, modified.

A total of 1950 genes was found up-regulated and 1844 genes were down-regulated. Different analysis was performed on data with the bioinformatics tools DAVID, Panther, Kegg. From the resulting clustering of gene by a common Gene Ontology and pathways we focused our attention on 3 groups of transcripts: 1) genes involved in cell cycle, 2) genes associated with the SNARE-mediated vesicular transport, 3) olfactory epithelium associated genes, typically not expressed in the mammary gland at any stage of differentiation (Table 3, 4).

Gene ID	Gene name	LA7 control	sh IFITM3 LA7	Differential expression
NM_001000411	Olr866	5,34165	6,93785	1,59620
NM_001000413	Olr869	5,45564	6,93889	1,48325
NM_214828	Olr1378	6,36169	7,60056	1,23887
NM_001000231	Olr288	5,68993	6,90860	1,21867
NM_001000526	Olr1457	5,36608	6,57903	1,21295
NM_001000039	Olr1521	5,16541	6,37065	1,20524
NM_001000145	Olr104	5,68331	6,85548	1,17217
NM_001001114	Olr1701	5,70123	6,85524	1,15401
NM_001000009	Olr1416	5,49742	6,64621	1,14879
NM_001000748	Olr60	5,50692	6,62516	1,11823
NM_001000412	Olr867	5,19689	6,28458	1,08769
NM_001000054	Olr875	5,28214	6,35876	1,07662
NM_001000788	Olr1388	5,54433	6,61299	1,06866
NM_001000768	Olr7	5,32855	6,39490	1,06636
NM_001000264	Olr1768	5,34281	6,40504	1,06224
NM_001000538	Olr1l	5,64982	6,69759	1,04777
NM_001000017	Olr1440	6,14413	7,18763	1,04350
NM_001000425	Olr1111	5,48582	6,52859	1,04277
NM_001000771	Olr1455	5,86480	6,90605	1,04125
NM_001000583	Olr857	5,08759	6,12878	1,04120

Table 3. List of the top 20 genes of the olfactory receptors resulted up-regulated in IFITM3 depleted cells.

Cell cycle	AURKB, BOD1, CAMK2D, CDC20, CDC26, CDCA8, DLGAP5, DNAJC2, DSCC1, E2F1, EHMT2, KIFC1, LOC500342, LOC688265, MAD2L2, MIS12, MLH1, NEK6, NUDC, PINX1, RGD1309522, RGD1310778, RRS1, SGOL2, SKA1, TAF10, TCFE2A, UCHL5IP, WEE1
Vesicular transport	LOC366820, LOC366818, STX8, STX12, STX17, STX18, VAMP2, VAMP8, VAMP4, YKT6
Genes specific for the olfactory epithelium	OLR1L , OLR386, OLR104, OLR1061, OLR1076, OLR1090, OLR1095, OLR1092, OLR1093, OLR1106, OLR1107, OLR1111, OLR1227, OLR1330, OLR1334, OLR1337, OLR1339, OLR1345, OLR1346, OLR1350, OLR1352, OLR1353, OLR1355, OLR1525, OLR1365, OLR1378, OLR1387, OLR1388, OLR139, OLR1401, OLR1404, OLR1416, OLR1423, LOC686792, OLR1424,, OLR1433, OLR1434, OLR1440, OLR1448, OLR1454, OLR1455, OLR1457, OLR1458, OLR1462, OLR1509, OLR1511, OLR1512, OLR1514, OLR1521, OLR1572, OLR1584, OLR1601, OLR1607, OLR1619, OLR1637, OLR1666, OLR1667, OLR1701, OLR172, OLR176, OLR1743, OLR1744, OLR1748, OLR1746, OLR175, OLR1750, OLR1768, OLR202, OLR218, OLR286, OLR287, OLR288, OLR3, OLR303, OLR321, OLR325, OLR326, OLR37, OLR39, OLR40, OLR434, OLR44, OLR48, OLR6, OLR60, OLR61, OLR69, OLR7, OLR731, OLR733, OLR796, OLR839, OLR856, OLR857, OLR858, OLR866, LOC679789, OLR867, OLR862, OLR869, OLR875, OLR927, OLR1383, OLR1384, OLR1385, RGD1562157

Table 4. List of genes and known pathways modulated with IFITM3 down-regulation from microarray expression analysis. Cell Cycle and Vesicular Transport genes are down-regulated and Olfactory Epithelium genes are up-regulated.

4.7 RT-PCR VALIDATION

Genes of the Olfactory Receptors (ORs) are expressed in the olfactory neurons in the olfactory epithelium of the nose and are not expressed in the mammary gland at any stage of development. ORs are a very numerous genes family organized in many subfamilies and an olfactory neuron express only one single OR.

Real Time data confirmed that the ORs group is up-regulated in LA7 cells where IFITM3 is down-regulated (Fig.31 A).

The evidence that the down-regulation of IFITM3 resulted in the expression of genes that are not normally expressed in the mammary gland prompted us to investigate the molecular mechanism associated with this unexpected switch in the expression of the ORs. Since it is known that the expression of one single OR in an olfactory neuron is due to activity of epigenetic mechanisms that silence the translation of all the other ORs[132], we hypothesized that the change in expression level of ORs observed in LA7 in which the down-regulation of IFITM3 was induced, is a consequence of epigenetic modifications. We hypothesize that down-regulation of IFITM3 has a direct effect on the transcription level of gene involved ORs repression.

We analyzed the microarray data to identify genes that regulate epigenetic modification and found that genes involved in chromatin remodelling were down-regulated (Table 5).

Gene symbol	**Complete name**
Suv39h1	Suppressor of variegation 3-9 homolog 1
Setdb1	SET domain, bifurcated 1
Kdm1b	Lysine (K)-specific demethylase 1B
Prmt3	Protein arginine methyltransferase 3

Table 5. List of genes involved in chromatin remodelling found down-regulated following IFITM3 down-regulation.

SUV39H1 and SETDB1 are histone methyltransferase that trimethylate lysine 9 of histone 3, that induce gene silencing and transcriptional repression, KDM1B is an histone demethylase that regulates histone 3 lysine 9 methylation, PRMT3 is an arginine methyltransferase that may methylate histone 4.

The expression level of these genes was validated by RT-PCR and the results confirm the array data (Fig.31 B). Since as mentioned before, ORs

genes are silenced in other tissues, this suggested that IFITM3 down-regulation may induce epigenetic modification that may re-activate the transcription of these tissue specific genes.

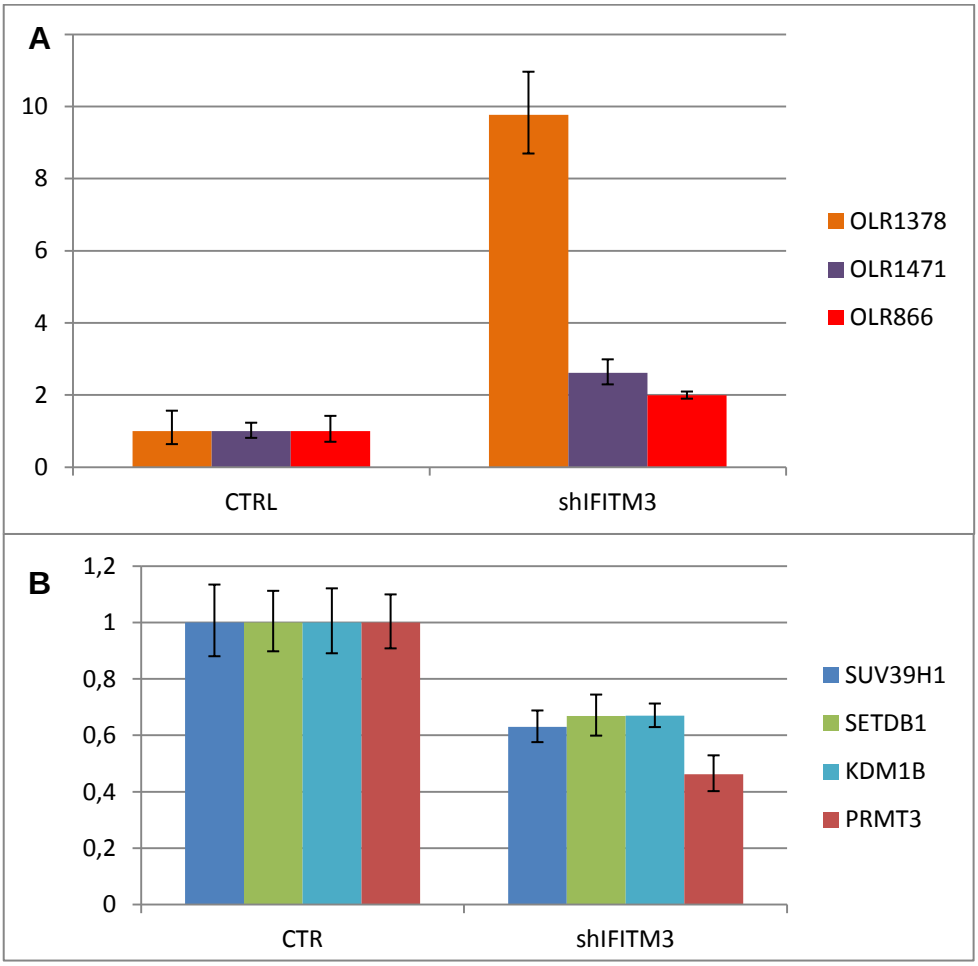


Figure 31. RT PCR: A) The representative olfactory genes up-regulated in sh-IFITM3 transduced LA7 cells. B) Representative genes associated with epigenetic machinery down-regulated in sh-IFITM3 transduced LA7 cells. Vertical bars represent the standard error of the 95% level of confidence.

Collectively these analyses provide insight into the genes and pathways that are regulated by IFITM3 in mammary cell differentiation or tumor development.

5 DISCUSSION

5.1 ROLE OF IFITM3 IN MAMMARY GLAND DIFFERENTIATION

The mammary gland is one of the few organ in which differentiation occur in the adult life and the unique organ that during the adult life, undergoes repeating cycles of regeneration. An intense proliferation program is activated at each pregnancy, followed by a multi-steps differentiation program that culminates with lactation. Subsequently an apoptosis-dependent regression process take place with involution [50]. These cycles of regeneration are maintained by resident stem cells of the mammary gland. Mammary gland stem cells therefore, represent a model system for identifying epigenomic processes, genes and factors that regulate normal cell differentiation and stem cell maintenance. Due in part to the fact that the mammary gland undergoes repeated cycles of stem cell dependent regeneration, each of these process need to be highly regulated to prevent that the gland initiate tumor development. Mammary gland is therefore also a model for understanding tumor initiation and progression.

Development of the mammary gland occurs in stages, beginning in the embryo and progressing after birth. A rudimentary system of small ducts is present in newborn that at puberty generates into an extensive network of ductal trees. At pregnancy, while further ductal growth occurs, most of the organ growth is associated with formation of lobules containing alveolar buds that secrete milk proteins during lactation. The research of Zucchi's lab. supports that terminal differentiation of alveolar epithelial cells, that is completed at parturition requires IFITM3 protein to shuttle from cytoplasm to the alveoli cell membrane for the secretion of milk[78]. Published research from the lab demonstrated that IFITM3 is necessary for the formation of blister like structures called "domes" that represent terminally differentiated mammary gland structures reminiscent of alveoli.

Since IFITM3 is already known to be fundamental in one aspect of differentiation of mammary gland we choose to investigate if IFITM3 has a role in other steps of cellular differentiation. We tested the capacity of the cells in which IFITM3 expression was modulated to grow in suspension conditions and differentiate into tubuli or cysts and we evaluated if IFITM3 could affect the stem cell properties and self renewal. Our results demonstrate that IFITM3 is necessary for LA7 to differentiate into tubuli and for MCF7 to differentiate into cysts when cultured in 3D conditions.

IFITM3 is a transmembrane protein that localize both at endosomal level and plasma membrane. At the cellular membrane it is known to associate to lipid rafts and to associate to different protein such as the kinase Fyn or the v-ATPase Atp6v0b[98].

IFITM3 hence may interact with some unknown protein involved in transduction of signal necessary for differentiation into tubuli, or with protein of the collagen matrix and directly act as a transducer of extracellular signals, promoting migration of the cells and elongation of the ducts.

5.2 ROLE OF IFITM3 IN CANCER STEM CELLS AND MAMMARY GLAND STEM CELLS

Breast cancer is the second leading cause of death of cancer for women.

Breast cancer is a very heterogeneous disease which etiology is unknown, many causes influence cancer onset, such as hereditary and environmental factors, and many genes and pathways are involved in the different types of breast tumor.

Breast cancer research is not only related to molecular mechanisms involved in the onset of the pathology but also in the process that lead to developing drug resistance and recurrence.

Breast cancer is a heterogeneous disease and to explain the origin of the many different type of cancer, two theories were formulated: clonal evolution and cancer stem cells model.
LA7 are a CSC model and can grow in non adherent condition to form mammosphere, showing self renewal properties typical of a stem cell. We decided to investigate the involvement of IFITM3 in the SC properties of LA7 as cancer and mammary gland stem cell.
LA7 cells with down-regulation of IFITM3 show no capacity to survive in non adherent conditions and to form spheres. Moreover, down-regulation of IFITM3 resulted in loss of propagation capability in LA7, but not in MCF7 or myoepithelial cells, which express low levels of native IFITM3, suggesting that IFITM3 may play a different role in cells with SC self-renewal properties and cells with no SC properties. Our results demonstrate the importance of IFITM3 in the LA7 cellular model of mammary SCs and CSCs, in which the gene is required for cells differentiation into mammary gland structures such as tubuli and for maintenance of the SC properties.

IFITM3 has been related to cancer and it has been shown to be overexpressed in different cancerous tissue, such as colon cancer and gastric cancer. In colon cancer IFITM3 seems to be under negative regulation of the KLF4 transcription factor[133], and IFITM3 overexpression is also related to metastasis and proliferation, while reduction of IFITM3 lead to decreased proliferation an migration of colon cancer cells.
Our results are consistent with literature data as IFITM3 seems to be related to cancer potential of LA7 cells.

5.3 ROLE OF IFITM3 IN PROLIFERATION

Analysis of the transcriptome of IFITM3 depleted LA7 cells unrevealed numerous genes of which the expression was altered. We focused on three group of gene with common gene ontology and similar known function.

IFITM3 has been related to cell cycle and proliferation by different authors[134–136], and finding cell cycle genes down-regulated with depletion of IFITM3 is consistent with literature data. Nonetheless literature does not agree upon the role of IFITM3, as some authors suggest an anti-proliferative effect whereas others link IFITM3 expression to a reduction of proliferation.
FACS data presented in this thesis research suggest that IFITM3 down-regulation could have an effect on LA7 cell proliferation, since the number of cells transduced with the lentiviral IFITM3 shRNA cells decrease with passages of culture.

Comparison of expression data with two other microarray data of cells in which IFITM3 is up-regulated by the differentiating agent DMSO and during tumor formation generated with LA7 injection into NOD-SCID mouse showed 26 pathways common to all three conditions. Most of these pathways are related to cell cycle regulation and cellular growth. We are currently focusing of the top 10 genes in common to all 26 pathways.

5.4 ROLE OF IFITM3 IN VESICULAR TRANSPORT

Another interestingly data emerged from microarray analysis is the down-regulation of some genes involved in the vesicular transport and fusion of small vesicles. Proteins such as vesicle-associated membrane protein 2 (VAMP2) are known to be involved in the process of formation of a fusion pore.
Since IFITM3 is also involved in blocking viral release from endosome through the inhibition of a complete fusion pore formation it is possible that it could impedes vesicle fusion of other origin, interacting with protein of the Soluble N-ethylmaleimide-sensitive factor (NSF) Attachment protein Receptor (SNARE) complex. The SNARE complex is composed of three proteins present on the plasma membrane and on the vesicular membrane

that, interacting with each other, promote membrane contact and subsequent fusion.

5.5 ROLE OF IFITM3 IN EPIGENETIC REGULATION

The most unexpected result we obtained from microarray analysis is the finding that the ORs resulted up-regulated. Since ORs are epigenetically silenced in order to have only one single OR expressed in an olfactory neuron we consequently developed the hypothesis that IFITM3 down-regulation may induce epigenetic changes of chromatin state.

The first gene, KDM1B, is an histone demethylase and is involved in sensitizing breast cancer cells to a drug inhibiting DNA methyltransferase, which then promotes cell cycle arrest and apoptosis[137].

SUV39H1 is a histone trimethylase and it was found responsible for E-cadherin promoter methylation driven by Snail[138,139], which directly interacts with SUV39H1. Snail is the main transcriptor factor involved in the epithelial to mesenchymal transition (EMT), a process in which epithelial cells acquire fibroblast-like properties that allows the cells to migrate.

EMT is a normal physiological process that takes place during embryo development allowing epithelial cells to become mesenchymal, but is also involved in tumor when cancer cells acquire motility and spread to blood vessel to form metastasis. EMT or an EMT-like process is also involved in mammary gland morphogenesis in TEBs elongation, with migration of the cap cells which lose polarity and digestion of the extracellular matrix.

Since LA7 are a cancer stem cell model with capability of metastatization, and able to develop into tubuli when properly cultured, we can hypothesize a direct effect of SUV39H1 on LA7 properties linked to the presence of IFITM3.

SETDB1 is an histone methyltransferase and has been found involved in different type of cancer, such as prostate[140] and lung cancer[141]. In lung cancer SETDB1 is found overexpressed and related to activation of the

WNT pathway[142,143], with increase in proliferation of the cells. WNT is not only fundamental for mammary gland development in all the stages of the process, but is also involved in cancer development[144] and EMT process[145–147]. In prostate cancer SETDB1 is also overexpressed and if down-regulated reduces proliferation and invasiveness of tumor cells. Moreover SETDB1 has a role in embryonic stem cell state, as it has been found involved in the repression of genes regulating development and differentiation[148], suggesting a role in preventing a destabilization or in maintenance of the ES cell state.

PRMT3, an arginine methyltransferase that can in vitro methylate a peptide of histone 4[149], may play a role in cell growth and apoptosis. DAL-1/4.1B is a human tumor suppressor which reduces cellular growth and induces apoptosis and exert his function through inhibition of PRMT3[150,151].

It may so be possible that PRMT3 must be active and methylate its targets in order for a cancerous cell to survive and proliferate.

Diminished expression of genes responsible for chromatin remodelling suggest that IFITM3 is involved in regulating the chromatin state of the cell, preventing alterations due to reduced activity of epigenetic enzymes such as histone methylase and demethylase, granting maintenance of cellular identity and properties, as for instance the self-renewal properties of LA7.

6 CONCLUSIONS

My research supports that IFITM3 is fundamental in the regulation of the development of both normal and mammary gland cancer.
IFITM3 is necessary not only for alveologensis but it is also involved in branching morphogenesis, as silencing of the gene abolishes the capability of cells to form 3D structures such as the ducts or acini of the mammary gland.
Moreover IFITM3 appears to be necessary for maintenance of mammary gland cancer stem cells.

LA7 CSCs in which IFITM3 is down-regulated lose the capability to survive or grow in non adherent conditions, suggesting that IFITM3 may inhibit apoptosis or anoikis of CSCs.

Finally preliminary evidence suggests that IFITM3, acts in mammary gland by modulating epigenetic components associated with gene expression and the chromatin state of cells.

Future studies of IFITM3 will confirm its involvement epigenenomic regulation in stem cells and in differentiated cells by determining whether re-establishment of epigenetic factors can compensate the effects observed with IFITM3 down-regulation in luminal differentiated cells and MaSCs.
Moreover we will study the various stages associated with cancer progression (including metastasis) with injection into NOD-SCID mice LA7 cells in which IFTIM3 was down-regulated or up-regulated. Lastly we will focus our attention on the other two groups of gene obtained from microarray data in order to achieve definitive answers about IFITM3's role in proliferation and in vesicle transport and fusion, functions most recently identified in immune response with viral infection.

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