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"Conditional activation of AKT1^{E17K} promotes breast tumorigenesis in a knock-in mouse model"

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ABSTRACT

Activating mutations in the PI3K/AKT pathway are present in majority of breast cancer. The gain of function mutation E17K of AKT1, was found in 8% of breast cancers, especially ductal carcinomas, but several studies performed so far have failed to define the real role of this mutation in the breast tissue trnsformation. To investigate the role of the $AKT1^{E17K}$ in breast tumorigenesis, we explored the phenotype of a new mouse model which express the mutant transgene in mammary epithelium. The expression of $AKT1^{E17K}$ enhances the activity of the kinase and the phosphorilation status of downstream substrates, such as FOXO1 and GSK3α/β. In addition, transgenic mice showed an increased cellularity 8-10 times higher than control mice breast tissues. Moreover 70% of transgenic mice expressing the mutant form of AKT1 develop ductal carcinomas from medium to high grade. We have identified also the contribution of $AKT1^{E17K}$ in the generation and maintenance of putative breast cancer stem cells. Finally, using a pharmacological study, we were able to slow down tumor formation by inhibiting downstream effect of AKT1 pathway. All together these data have allowed us to demonstrate that $AKT1^{E17K}$ is itself capable to induct the onset of ductal carcinoma in transgenic mice.

RIASSUNTO

Mutazioni attivanti nel pathway PI3K/AKT sono stati trovati nella maggior parte dei carcinomi mammari. La mutazione E17K nel gene AKT1, che ne determina un guadagno di funzione è stata trovata nell'8% dei casi di cancro al seno, specialmente in carcinomi duttali, ma i diversi studi condotti finora non sono riusciti a definire il vero ruolo di questa mutazione nel processo di trasformazione tumorale del tessuto mammario. Per studiare il ruolo di $AKT1^{E17K}$ nella tumorigenesi mammaria, abbiamo esplorato il fenotipo di un nuovo modello murino che esprime il transgene in maniera specifica nell' epitelio mammario. L'espressione di $AKT1^{E17K}$ aumenta l'attività della chinasi e dunque il grado di fosforilazione di substrati a valle, come FOXO1 e GSK3α/β.inoltre i topi transgenici mostrano una cellularità tissutale 8-10 volte superiore a quella dei topi di controllo. Il 70% dei topi transgenici che esprimono la forma mutante di AKT1 sviluppano carcinoma duttale ad alto e medio grado. Abbiamo anche identificato il contributo di $AKT1^{E17K}$ nella generazione e mantenimento delle putative cellule tumorali staminali mammarie. Infine, medinate uno studio farmacologico siamo riusciti a ritardare la formazione dei tumori inibendo il pathway a valle di AKT1. Tutti questi dati ci hanno consentito di dimostrare che $AKT1^{E17K}$ è di per sé capace di indurre l'insorgenza di carcinoma mammario duttale in topi transgenici.

INTRODUCTION

BREAST CANCER

EPIDEMIOLOGY

Breast cancer is the third most frequent cancer in the world (after lung and gastric cancer) and most common female malignancy. It is the fifth cause of death from cancer overall (after lung, stomach, colorectal and liver cancers) and leading cause of cancer death among females, accounting for 23% of the total cancer cases and 14% of the cancer deaths. [1][2] The areas of high risk are represented by the populations of North America, Europe and Australia, where 6% of women develop invasive breast cancer before age 75. The risk of breast cancer is low in the less developed regions of sub-Saharan Africa and Southern and Eastern Asia, including Japan, where the probability of developing breast cancer by age 75 is one third respect to rich countries. [3] In 2013, in USA, 232.340 new cases of invasive breast cancer have been diagnosed among women, as well as 64.640 additional cases of in situ breast cancer. In 2013, have been estimated approximately 39.620 women deaths from breast cancer and 2.240 men have been diagnosed with breast cancer and 410 men are dead from the disease. (figure 1). [4]

Age (Yrs)	In Situ Cases	Invasive Cases	Deaths
<40	1,900	10,980	1,020
<50	15,650	48,910	4,780
50-64	26,770	84,210	11,970
65+	22,220	99,220	22,870
All ages	**64,640**	**232,340**	**39,620**

Figure 1: estimated new female in situ and invasive breast cancer cases and death by age, US, 2013. [4]

HISTOLOGICAL CLASSIFICATION

Breast cancer originates from the epithelial cells of the glandular tree and may give rise to different histotypes. The most common are the lobular and ductal carcinomas, of which there are in situ and invasive forms.

IN SITU BREAST CANCER

- Ductal carcinoma in situ (DCIS) is the most common type of in situ breast cancer, accounting for about 83% of in situ cases diagnosed during 2006-2010. DCIS may or may not progress to invasive cancer; in fact, it consists of cancerous cells that proliferate within the lumens of the breast duct with no invasion. It grows so slowly that even without treatment does not affect the women health. [5][6] Clinical studies suggest, however, that about one-third, and possibly more, of DCIS cases will progress to invasive cancer if left untreated. [5]
- Lobular carcinoma in situ (LCIS, also known as lobular neoplasia) is not a true cancer or precancer, but an indicator of increased risk for developing invasive cancer. It is represented by the presence of unusual cells in the lobules of the breast. LCIS is much less common than DCIS, accounting for about 12% of female in situ breast cancers diagnosed during 2006-2010. [7]
- Other in situ breast cancers have characteristics of both ductal and lobular carcinomas or have unknown origins.[4]

INVASIVE BREAST CANCER

Invasive breast carcinoma is a group of malignant epithelial tumors characterized by invasion of adjacent tissues and a marked tendency to metastasize to distant sites. Invasive breast

carcinomas exhibit a wide range of morphological phenotypes and specific histopathological types have particular prognostic or clinical characteristics. [3]

INVASIVE DUCTAL CARCINOMA

Invasive ductal carcinoma (IDC) is the most common histologic type of invasive breast cancer and comprises 70% to 80% of breast cancer cases. Invasive ductal carcinoma starts in a milk duct of the breast, breaks through the wall of the duct, and grows into the breast fatty tissue. It is able to spread to other parts of the body through the lymphatic system and bloodstream. About 8 of 10 invasive breast cancers are infiltrating ductal carcinomas. It can occur in different histological subtypes: tubular, medullary, mucinous, papillary and cribiform. [8]

INVASIVE LOBULAR CARCINOMA

Invasive lobular carcinoma (ILC), is the second most common type of breast accounting about 8-14% of all invasive breast cancers are invasive lobular carcinomas. [9][10] It begins in the milk-producing lobules and spreads to the surrounding breast tissues, lymph nodes and possibly to other areas of the body. There are different subtypes of invasive lobular carcinoma (ILC) that are based on specific morphological properties: solid, alveolar, tubuloalveolar, pleomorphic, signet ring cell. [8]

GENETICS OF BREAST CANCER

Breast cancer is a heterogeneous disease caused by progressive accumulation of genetic aberrations, including point mutations, chromosomal amplifications, deletions, rearrangements, translocations, and duplications.[11] Germline mutations account for only about 10% of all breast cancers, while the vast majority of breast cancers occurs sporadically and is attributed to somatic genetic alterations. In familiar forms, breast

cancer susceptibility genes can be categorized into three classes according to their frequency and level of risk that they confer:

- rare high-penetrance genes, in particular *BRCA1* and *BRCA2*, which encode large proteins with multiple functions which act as classic tumor suppressor genes that maintain genomic stability by facilitating double-strand DNA repair through homologous recombination. [12]. When loss of heterozygosis (LOH) occurs via loss, mutation or silencing of the wild type *BRCA1* and *BRCA2* allele, the resultant defective DNA repair system leads to rapid acquisition of additional mutations, particularly during DNA replication. [13]. *BRCA1* and *BRCA2* mutations account for approximately half of all dominantly inherited hereditary breast cancers. These mutations confer a relative risk of breast cancer 10 to 30 times higher that of women in the general population, resulting in a nearly 85% lifetime risk of breast cancer development. [14]. Other high-penetrance genes are *TP53*, *PTEN*, *STK11/LKB1*, and *CDH1*. These high-penetrance genes confer an eight-ten fold increase in risk of breast cancer as compared to non- carriers, but they collectively account for less than 1% of cases of breast cancer. Like *BRCA1* and *BRCA2*, these genes are inherited in an autosomal dominant manner and function as tumor suppressors. [15].

- rare intermediate-penetrance genes. Four genes that confer an elevated but moderate risk of developing breast cancer have been identified, namely *CHEK2*, *ATM*, *BRIP1*, and *PALB2*, involved in signal transduction and DNA repair. Each of these genes confers approximately a two-three fold relative risk of breast cancer.[13].

- common low-penetrance genes and loci, include approximately ten different alleles and loci in 15% to 40% of women with breast cancer. [14] Despite their frequency, the relative risk of breast cancer conferred by any one of these genetic variants alone is minimal, less than 1.5 fold.[16] Nevertheless, these alleles and loci may become clinically relevant in interaction

with other high-, moderate-, and low-risk genes; these additive or multiplicative relationships could account for a measurable fraction of population risk.

For example, association studies of FGFR2 and MAP3K1 within *BRCA* families showed that these single nucleotide polymorphisms (SNPs) conferres an increased risk in the presence of *BRCA2* mutations. Recent studies suggest that microRNA (miRNA) SNPs may also contribute to breast cancer susceptibility, and miRNAs appear to regulate many tumor suppressor genes and oncogenes via degradation of target mRNAs or repression of their translation. [13]

The vast majority of breast cancers are sporadic, caused by accumulation of several somatic genetic alterations. Recent data suggest that a typical individual breast cancer harbors anywhere from 50 to 80 different somatic mutations. [11] Many of these mutations occur as a result of erroneous DNA replication; others may occur through exposure to exogenous and endogenous mutagens. [13] Among gene amplifications, the most frequent in breast cancer regard HER-2/Neu, growth receptor that activates the Ras-MEK and PI3K pathway, amplified in about 13% of the breast cancer. Cyclin D1, amplified in about 10-12% of the breast cancers, WIP1 (13%) and GASC1, amplified in about 5-10% of total breast cancers and in the 20-25% of the basal breast cancer. [17]

Inactivation of gene functions by deletion or other mechanisms commonly occurs in PTEN and p53 in HER2/neu positive breast cancers, triple negative breast cancers, and BRCA-associated breast and ovarian cancers. PI3K amplifications and activating mutations are common in breast cancers and several genes such as AKT and STAT3 are often expressed at high activities but without detectable amplifications of those genes. [18] Epigenetic alterations, such as methylation of cytosine residues in CpG dinucleotides, can bring about gene inactivation, for example p16 gene in breast cancers. [17] A substantial number of these somatic mutations sort out among a much smaller number of biological groups and cell

signaling pathways that are known to be pathogenetic in breast cancer, thereby vastly reducing the complexity of the genomic landscape. Examples of such pathways include interferon signaling, cell cycle checkpoint, BRCA1/2- related DNA repair, p53, transforming growth factor-β (TGF-β) signaling, Notch, epidermal growth factor receptor (EGFR), FGF, ERBB2, RAS, and PI3K-AKT. [13]

MOLECULAR CLASSIFICATION

The accumulation of different mutations has significant effects on the expression of important tissue-specific genes. Distinct molecular subtypes of breast cancer have been identified using biological markers, including the presence or absence of estrogen receptors (ER+/ ER-), progesterone receptors (PR+/PR-), and human epidermal growth factor receptor 2 (HER2+/HER2-). [4][13]

We can distinguish four different types of breast cancer: luminal A, luminal B, Basal-like and HER2 enriched. (figure 2)

Luminal A tumors have high expression of ER and ER-regulated genes, low expression of the HER2 cluster and proliferation-associated genes. Luminal B tumors tend to be highly proliferative, express mutant form of TP53, show lower expression of ER and ER-regulated genes and can be HER2+ or HER2-. [19][20] Basal-like breast cancer are referred to as “triple negative” because they are ER-, PR-, and HER2-. The basal- like subtype is characterized by low expression of the luminal genes, low expression of the HER2 gene cluster, high expression of the proliferation cluster, and high expression of a unique cluster of genes called the basal cluster (cytokeratins 5, 6, 14,17, c-Kit; Vimentin; P-Cadherin). Several risk factors for developing basal-like tumors have been identified, among which the most interesting being the link between the basal-like subtype and BRCA1 mutation carriers. [19]

The HER2-enriched subtype is relatively infrequent (10% of all breast cancers). This subtype shows elevated expression of HER2 and many other genes that reside near HER2 in the genome (GRB7) and do not express hormone receptors (ER- and PR-). [4] [19]

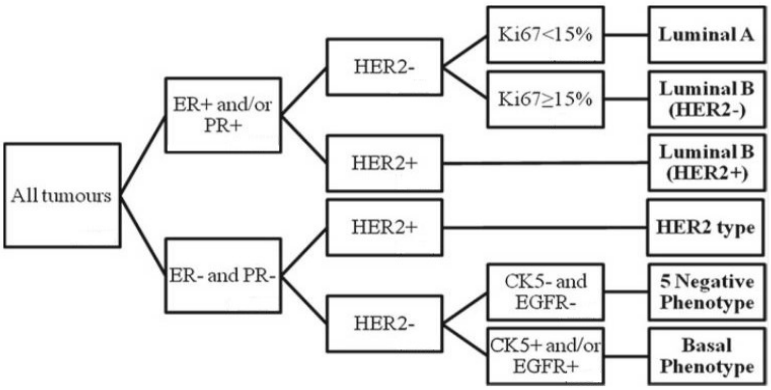


Figure 2: Classification algorithm for molecular subtyping. [20]

BREAST CANCER METASTASIS

Metastatic breast cancer is defined by tumor spread beyond the breast, chest wall, and regional lymph nodes. Tumor dissemination can occur through blood and lymphatic vessels and via direct extension through the chest wall. The most common sites for breast cancer metastasis include the bone, lung, liver, lymph nodes, chest wall, and brain. [13] (figure 3)

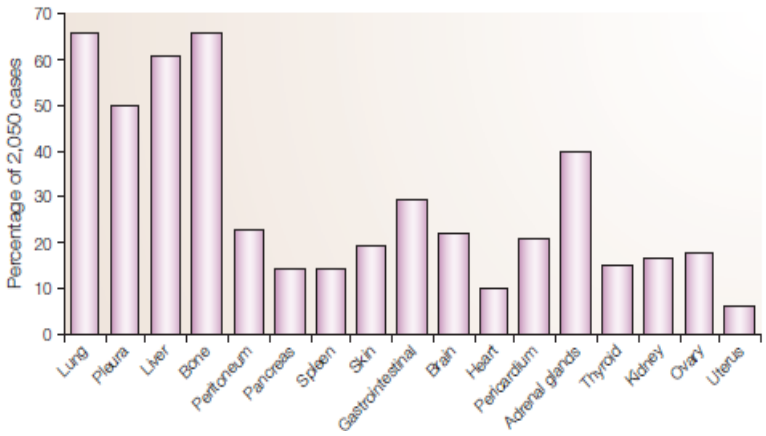


Figure 3:Most common metastasis sites of breast cancer at autopsy. Primary breast cancer cells metastasize through the blood vessels to various distant organs, preferentially, to the lung, liver and bones. Patients frequently develop metastases at multiple sites. [21]

An increased likelihood of visceral metastases and a particularly poor prognosis are associated with the lack of estrogen receptor (ER) and progesterone receptor (PR) expression in breast cancer while triple-negative breast cancer is accompanied by distant, hematogenous metastasis that usually occur in the first five years after the initial diagnosis and are associated with relatively short relapse-free and overall survival times. [22]

Hormone receptor–positive tumors are more likely to spread to bone as the initial site of metastasis; hormone receptor–negative and/or *HER2*-positive tumors are more likely to recur initially in viscera. [13][22] Lobular (as opposed to ductal) cancers are more often associated with serosal metastases to the pleura and abdomen.[13] Anyway, bone is the most commonly observed site for distant metastasis, around 70% of patients have lesions at bone and is the location of 30–40% of first tumor recurrence. [22] [23] Has been recently suggested that a cellular subpopulation with stem cell (SC)-like features, known as cancer SCs (CSCs), is critical for tumor generation and maintenance, and responsible for breast cancer metastasis. Indeed, it is conceivable that several of the traits ascribed to CSCs may provide them with the potential to occupy and prosper at distant sites. [24]

BREAST CANCER STEM CELLS

Cancer stem cells (CSCs) are tumor cells with enhanced capacity for tumor generation. CSCs possess several fundamental attributes similar to normal adult stem cells. They are capable of dividing asymmetrically to produce one stem cell, characterized by self-renewal, and one progenitor cell, which allows to produce phenotypically diverse cancer cells that constitute tumors. [25] In breast cancer has been isolated a small population of tumorigenic cells with stem cell (SC)-like features, capable of regenerating the phenotypic heterogeneity of the original tumor when injected subcutaneously into NOD/SCID mice. [26][27] These breast cancer stem cells (BCSCs) are characterized by the cell-surface markers $ESA^{+}/CD44^{+}/CD24^{-/low}$, devoid of the expression of the lineage markers CD2, CD3, CD10, CD 16, CD18, CD31, CD64, and CD140b (Lin^{-}) and bear high ALDH1 activity. [25][26][28] Putative breast CSCs have also been isolated from patient samples after *in vitro* propagation and from breast cancer cell lines, through their ability to proliferate in suspension as non adherent spheres (mammospheres). Because the capacity to form mammospheres is increased in early progenitor/stem cells, this system has been widely used as an indirect measurement of the number of cells with self-renewal capability. [29] The origin of breast CSCs is controversial. Current experimental evidence supports two different, but not exclusive, theories (figure 4).

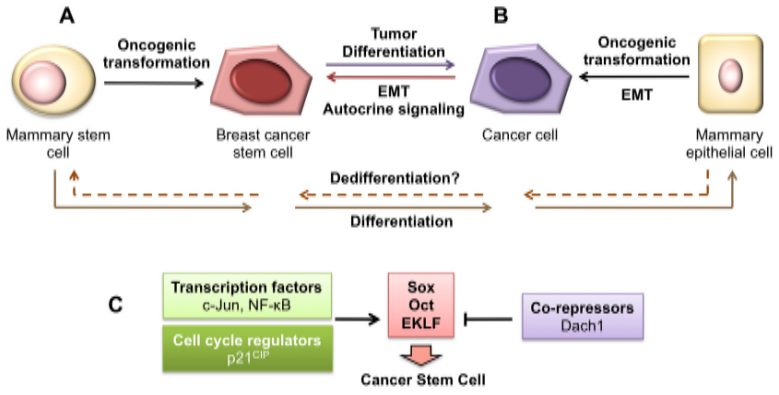


Figure 4. Origin of the breast cancer stem cells (BCSCs). BCSCs may arise from normal mammary stem cells (A) or from non-stem tumor cells that have gained the ability for self-renewal (B) by epithelial to mesenchymal transition (EMT) and oncogenic transformation. Both of these hypotheses consider that the phenotypic characteristics of BCSCs are caused by genetic alterations

and/or EMT. As result, BCSCs display alterations in signaling pathways controlling the cell cycle, differentiation, and survival (C). [25]

One theory proposes that CSCs resulted from the deregulation of normal stem cell self-renewal and differentiation pathways, resulting in cancer cells with both self-renewal and differentiation capabilities. A second theory suggests that BCSCs develop from epithelial-mesenchymal transition (EMT). Cells that have undergone EMT are susceptible to transformation and have many characteristics and behaviors similar to those of normal and neoplastic stem cells. [25]

BCSCs are characterized from the deregulation of different pathways implicated in the regulation of BCSCs self-renewal and differentiation. [30] A number of signaling pathways have been found to play a role in mammary stem cell self-renewal, including Wnt, Notch, and Hedgehog. In addition, the PTEN (phosphatase and tensin homolog deleted on chromosome 10) tumor suppressor gene, one of the most frequently mutated genes in human malignancies, has also been suggested to play a role in stem cell self-renewal. In particular, in vitro and in vivo evidence revealed the importance of PTEN/PI3-K/Akt/Wnt/β-catenin pathway in BCSCs biology, in fact have been demostrated that active AKT phosphorylates GSK3β and thereby the Wnt pathway. [25][31] BCSCs have a great importance for the clinic and therapy, several studies have demonstrated that these cells are responsible for resistance to chemo- and radiotherapy, (es. for overexpression of Notch-1) and metastatic dissemination of tumors (es. for overexpression of genes that promote cell motility, invasion). The chemio- and radio-resistance is not a universal characteristic of BCSCs, in fact, BCSC population within a triple (estrogen receptor (ER), progesterone receptor (PR) and HER-2) negative cell line can be depleted by radiotherapy and several drugs tested clinically have shown activity against BCSCs. [32]

RISK FACTORS FOR BREAST CANCER

Multiple factors are associated with an increased risk of developing breast cancer, including increasing age, exposure to female reproductive hormones (both endogenous and exogenous) and reproductive history, dietary and lifestyle factors, environmental factors and familial factors and inherited predisposition. The majority of these factors convey a small to moderate increase in risk for any individual woman. [13]

- increasing age: The age-specific incidence of breast cancer increases steeply with age until menopause. After menopause, when ovarian synthesis of estrogens and progesterone ceases and ovarian androgen production gradually diminishes, although the incidence continues to increase, the rate of increase decreases to approximately one-sixth of that seen in the premenopausal period. The dramatic slowing of the rate of increase in the age-specific incidence curve suggests that ovarian activity plays a major role in the etiology of breast cancer. [3]
- Exposure to female reproductive hormones and reproductive history: The development of breast cancer in many women appears to be related to female reproductive hormones. Early age at menarche (before age 12), nulliparity or late age at first full-term pregnancy (> 30 years), and late age at menopause (after age 55) increase the risk of developing breast cancer. Age at menarche and the establishment of regular ovulatory cycles are strongly linked to breast cancer risk. Earlier age at menarche is associated with an increased risk of breast cancer; there appears to be a 20% decrease in breast cancer risk for each year that menarche is delayed. Furthermore, most studies suggest that breastfeeding for a year or more slightly reduces a woman's risk of breast cancer. As regards the exposure to exogenous hormones, two major type of hormonal compounds have been evaluated in relation to breast cancer: oral contraceptives and

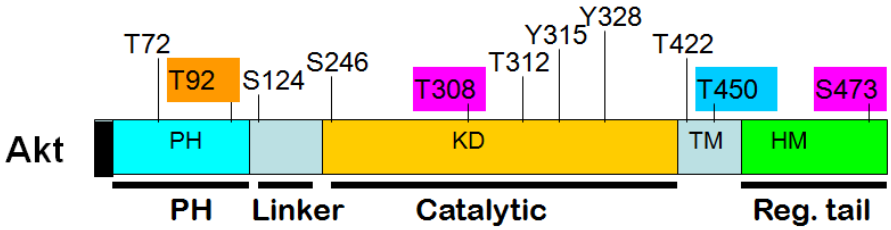
menopausal replacement therapy. The evidence suggests a small increase in the relative risk associated with the use of combined oral contraceptives and postmenopausal hormone replacement therapy (HRT). [3][4]

- dietary and lifestyle factors: Observational studies suggested that high-caloric diets rich in animal fat and proteins were associated with higher rates of breast cancer , combined with consumption of alcohol, a lack of physical exercise, overweight and obesity (in particular for postmenopausal breast cancer). [3][4][13]
- environmental factors: only limited data are available on specific exposures in relation to breast cancer. Long-term follow-up of women exposed to the Hiroshima or Nagasaki nuclear explosions indicates an increased risk of breast cancer, in particular for women exposed around puberty. Similarly, exposure as a result of treatment and surveillance of tuberculosis is associated with risk. Other environmental factors, including exposure to electromagnetic fields and organochlorine pesticides, have been suggested to increase breast cancer risk. [3][13]
- familial factors and inherited predisposition: Women (as well as men) with a family history of breast cancer, especially in a first-degree relative (mother, sister, daughter, father, or brother), are at increased risk of developing breast cancer; this risk is higher if more than one first-degree relative developed breast cancer. Compared to women without a family history, risk of breast cancer is 1.8 times higher for women with one first-degree female relative who has been diagnosed, nearly 3 times higher for women with two relatives, and nearly 4 times higher for women with three or more relatives. Risk is further increased when the affected relative was diagnosed at a young age. It is important to note that the majority of women with one or more affected first-degree relatives will never develop breast cancer and that most women who develop breast

cancer do not have a family history of the disease. It is estimated that 5% to 10% of breast cancer cases results from inherited mutations, including those in the breast cancer susceptibility genes *BRCA1* and *BRCA2* .[4][13] Other inherited conditions associated with smaller increased breast cancer risk include Li-Fraumeni and Cowden syndromes and a number of more common genetic mutations [4]

THE PROTEIN SERINE / THREONINE KINASE B (PKB / AKT) IN BREAST CANCER

AKT, a downstream effector of phosphatidylinositol-3 kinase (PI3K), is one of the most frequently hyperactivated protein kinase in human cancer .[33] It is a serine/threonine protein kinase and its hyperactivation is associated with resistance to apoptosis, increased cell growth, cell proliferation and cell energy metabolism. In mammalian cells AKT comprises three highly homologous members (>80% protein sequence identity) termed AKT1/PKBα, AKT2/PKBβ and AKT3/PKBγ, encoded by three different genes located on chromosomes 14q32, 19q13 and 1q43. AKT kinases share the same structural organization, containing an N-terminal pleckstrin homology (PH) domain, a central catalytic domain and a C-terminal regulatory domain that contains the hydrofobic motif (HM). (Figure 5)



Chr.	Homology (%):			
(14q32)	Akt1/Akt2:	80	46	90	66
(19q13)	Akt2/Akt3:	76	17	87	70
(1q44)	Akt3/Akt1:	84	40	88	76

Figure 5: Akt domains and comparison of Akt isoforms (% of homology). Chromosome location of each Akt isoform in human and phosphorylation sites in Akt1. [35]

The PH domain of AKT can bind specifically to D3- phosphorylated phosphoinositides with high affinity and mediates AKT activation. [34][35] Analysis of mice lacking either individual AKT isoforms or various combinations of AKT isoforms has indicated that the AKT1 isoform has a dominant role in embryonic development, fetal growth, and fetal survival, whereas AKT2 and AKT3 have non-redundant functions in glucose homeostasis and postnatal brain development, respectively. [35] Also the expression of AKT1, AKT2 and AKT3 apparently contribute to the different roles of AKT isoforms. AKT1 and AKT2 are widely expressed while tissue distribution of AKT3 seems to be more restricted, being primarily expressed in brain and testis. [34]

AKT is activated through receptor tyrosine kinase pathways, through a multi-step PI3K dependent process, that involves membrane binding and phosphorilation. Upon activation, PI3K produces increased levels of PIP3 (phosphatidylinositol- 3,4,5-trisphosphate) from PIP2 (phosphatidylinositol-3,4-trisphosphate) which contribute to recruit AKT and PDK1 (phosphoinositide-dependent kinase 1) to the inner plasma membrane through the pleckstrin homology (PH) domain. The interaction of the AKT PH domain with 3'- phosphoinositides is thought to impose conformational changes in AKT, exposing its two main phosphorylation sites at the kinase domain (T308 for AKT1) and the HM of the C- terminal (S473 for AKT1). The direct homodimerization of the two PH domains between AKT and PDK1 might also mediate protein proximity and subsequently phosphorylate Thr-308 in AKT, which stabilizes the activation loop in an active conformation and renders Ser473 phosphorylation by the rapamycin-insensitive mTORC2, resulting in full activation of AKT kinase. [34][35][36]

Conversely, this activation cascade can be blocked by cellular inhibitors including the phosphatase and tensin homolog (PTEN) and INPP4B which directly antagonize PI3K function via dephosphorylating PIP3, thereby abrogating PIP3-mediated activation of

downstream signaling events such as PDK1 and AKT. However, in vitro engineered AKT kinase can override this regulatory mechanism and maintain it in a "supercharged" stage. This can be done by insertion of myristoylated (Myr) tag at its N-terminus which results in AKT anchoring in plasma membrane and constitutive AKT activation independently of PI3K activity. [36]

Upon activation, AKT leaves the plasma membrane and phosphorylates a number (≈ 9000 [33]) of substrates both in the cytoplasm and in the nucleus, which mediate AKT-dependent regulation of cell growth and survival, mitogenesis, migration, glucose metabolism and protein translation. [34]

Among those substrates are: regulators of cell survival or cell death, such as Bad, caspase-9, ASK1, apoptosis signal-regulating kinase 1 (ASK1), forkhead box O transcription factors (FoxOs), Bim1, FasL, inhibitor of nuclear factor-κB kinase (IKK-NFκB), and p53; regulators of cell cycle progression; such as p21, p27, cyclin D1, and glycogen synthase kinase-3 (GSK-3α and β); regulators of protein synthesis or cell growth, such as tuberous sclerosis complexes 1 and 2 (TSC1/2), mTOR, elongation-initiation factor 4E binding protein-1 (4E-BP1), and S6K; regulators of angiogenesis, such as mTOR and hypoxia-inducible factor-1 (HIF-1); and regulators of cell metabolism, such as glucose transporter 1 (Glut1), GSK3, and a Ras homologue enriched in brain (RheB). [34][35] (figure 6)

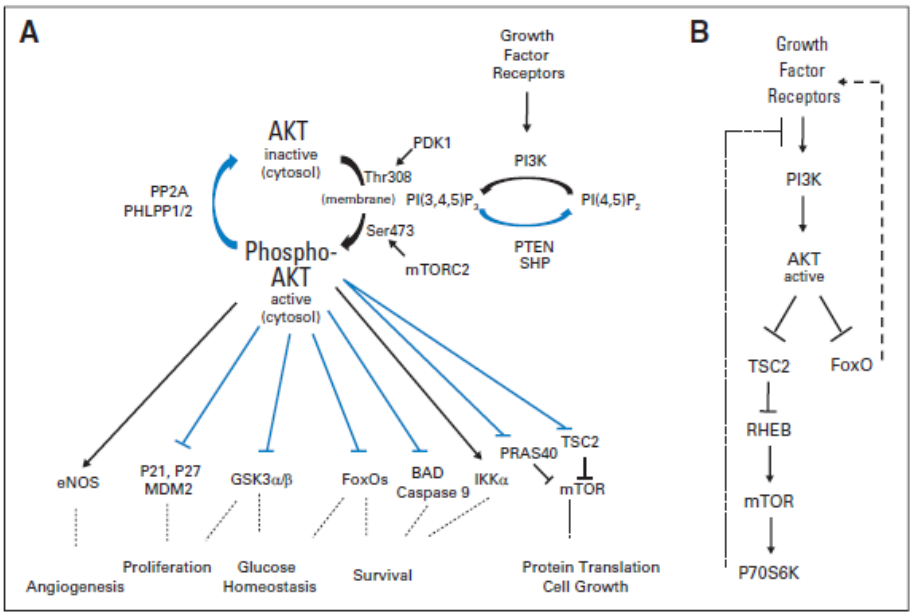


Figure 6: Akt signaling pathway. [37]

Mutations in AKT genes are rarely found in human cancers. Activation of AKT acts as a survival/proliferative signal; however, activation of AKT alone is generally insufficient to induce cancer unless combined with a transforming lesion in a second pathway. For example, overexpression of constitutively active human AKT1 in the mouse prostate induces precancerous intraepithelial lesions. However, these lesions do not progress even after 78 months. Overexpression of AKT1 alone in mouse mammary epithelium causes defective mammary gland involution, but when expressed with a mutant polyoma virus middle T antigen unable to signal through PI3K, the combination produces a marked increase in mammary neoplasia. Hyperactivation of AKT signaling occurs in a wide variety of human precancerous and cancerous lesions.[37] (figure 7)

Target	PI3K/AKT pathway alteration
Lung	↑ AKT activity
	PI3K catalytic subunit amplification
Prostate	↑ AKT activity
	↑ AKT expression
Melanoma	↑ AKT activity
Colon	↑ AKT expression
Endometrium	*PTEN* mutation
Cervix	↑ AKT activity
	↓ PTEN expression
	PI3K catalytic subunit amplification
Breast	↑ AKT activity
Stomach	↓ PTEN expression
Head and neck	↑ AKT activity

Figure 7: Alteration in the PI3K/PTEN/AKT pathway in human precancerous lesions. [38]

Overexpression and/or activation of AKT in tumor cells causes resistance to traditional chemotherapeutics and molecularly targeted drugs, including trastuzumab, gefitinib, retinoic acid, and tamoxifen. [38]

All three AKT isoforms have been associated with tumorigenesis. Overexpression of AKT2 transforms NIH3T3 fibroblasts and increases the invasive and/or metastatic capacity of human cancer cells in vitro and in vivo. [38] Ablation of AKT2 in either HER-2/NEU or polyoma middle T transgenic mice decreases metastatic spread. [39] AKT3 activity and expression are up-regulated in estrogen receptor-negative breast carcinomas and androgen-insensitive prostate cancer cell lines. [40] Selective knockdown of AKT3, but not of other isoforms, inhibits melanoma development driven by PTEN loss. In transgenic mice, constitutively active AKT1 induces precancerous prostatic lesions and accelerates oncogene-dependent mammary tumor formation. [38]

Deletion of AKT1 reverses the survival phenotype in PTEN null cells and abrogates its growth advantage. Similarly, inactivation of AKT by dominant negative mutants inhibits the survival advantage provided by activated class I PI3K. Disruption of AKT1 inhibits ErbB2-induced

mammary tumorigenesis. AKT1 deficiency delays tumor growth and reduces metastasis. AKT1 null mammary epithelial tumor cells have also reduced proliferative capability with reduced cyclin D1 and increased p27. These data suggest that AKT1 plays an important role in mammary tumorigenesis. [41]

The oncogenic activation of AKT1 can be induced by several means, most commonly occurring either due to the compromise in its membrane-targeting by PH domain, or due to the pathological conformational changes occurring in the mutant structure. Genetic mutations in the PH domain alter AKT1 localization and sensitivity to the PtdIns bringing serious consequences for its activity.[42]

A dominant hotspot mutation at nucleotide 49 (G>A) of the gene encoding AKT1 results in the substitution of a lysine for glutamic acid at the amino acid 17 (Akt1-E17K). [34][42][43] In the apo conformation, Glu 17 occupies the phosphoinositide-binding pocket and forms a network of hydrogen bonds. The Lys 17 substitution results in a shift in the surface charge around the pocket from negative with Glu 17 to effectively neutral in the mutant. [42][43] The $AKT1^{E17K}$ mutation alters the electrostatic interactions of the pocket, activates AKT1 in a PI3K-independent manner, increasing level of AKT1 phosphorylation on Thr 308 and Ser 473 as compared to wild-type. [42][44] $AKT1^{E17K}$ kinase activity was shown to be approximately four fold higher than that of AKT1 wild type, suggesting that the mutation alters AKT1 regulation and hence it enhances cellular activity. [42][43] Furthermore, it has also been proposed that the mutation induces large affinity increase for PI(4,5)P2 which is essential to the constitutive plasma membrane targeting of the mutant PH domain and thus to the oncogenic nature of the full-length $AKT1^{E17K}$ protein. [42] Moreover, it was also suggested that the E17K PH domain mutation causes structural changes in the PH domain, which further hinders its interaction with AKT1/2 inhibitor VIII. [43] All these observations strongly

suggest that the damaging conformational changes in mutant PH domain might cause such pathological outcomes. Functionally, the AKT1^{E17K} mutation stimulates AKT signaling, induces cellular transformation and produces leukaemia in mice. [44][45] The AKT1^{E17K} mutation was found in 5 out of 61 (8.2%) breast cancers, 3 out of 51 (5.9%) colorectal cancers, 1 out of 50 (2.0%) ovarian cancers [43] and 3 out 105 (2.9%) lung cancer. [46]

GENETICALLY ENGINEERED MOUSE MODELS OF PI3K/AKT SIGNALING IN BREAST CANCER

The role of PI3K/AKT pathway mutations in human breast cancer can be studied using genetically engineered mouse models (GEMMs), developed to mimic human genetics of breast cancer, with special attention to the role of PI3K/AKT signaling in oncogenesis, response and resistance to therapy and metastatic capability. (figure 8) The gene of interest (GOI) can be inserted into the host genome in a construct containing a tissue-specific promoter to achieve tissue specific expression, such us acidic protein (WAP), beta-lactoglobuli (BLG) and mouse mammary tumor virus (MMTV) LTR promoters that are more or less selectively active in the mammary epithelial cells. [47]

GEMM	Type	Comments
Pten$^{+/-}$	Knockout	Targeted disruption of *Pten*
MMTV-Cre; *Pten*$^{loxp/loxp}$	Conditional knockout	
Pten$^{hy/+}$	Hypomorphic allele	Transcriptional interference of *Pten* by insertion of CMV-neo^{R} in *Pten* intron 3
MMTV-myrAkt1	Transgenic	Constitutive activation
R26-Pik3ca^{H1047R}; *MMTV-Cre*	Conditional transgenic	*Rosa26-lox-stop-lox-Pik3ca*^{H1047R}
WapiCre; *Pik3ca*^{H1047R}	Conditional transgenic	*CAGS-lox-stop-lox-Pik3ca*^{H1047R}
MMTV-Cre; *Pik3ca*^{H1047R}		
MMTV-Cre; *Pik3ca*^{H1047R}	Conditional knock-in	*lox-stop-lox-Pik3ca*^{H1047R} in endogenous *Pik3ca* locus
MMTV-Cre; *Pik3ca*^{H1047R}	Conditional knock-in	*lox-stop-lox-Pik3ca*^{H1047R} in endogenous *Pik3ca* locus
MMTV-rtTA; *TetO-PIK3CA*^{H1047R}	Inducible transgenic	Transgenic *MMTV-rtTA* Transgenic *TetO-PIK3CA*^{H1047R}

Figure 8: GEMMs of PI3K-driven breast cancer. [47]

Several GEMMs with tissue-specific mutation of PIK3CA, which encodes the phosphoinositide-3-kinase (PI3K) catalytic subunit p110α have been published. Several groups have shown that expression of the H1047R PIK3CA mutant in luminal mammary epithelium results in the formation of mammary tumors of several phenotypes, in particular adenosquamous carcinoma or adenomyoepithelioma phenotype. Genetic interaction between $PIK3CA^{H1047R}$ and p53 loss-of-function mutations in R26-$PIK3CA^{H1047R}$;$p53^{loxP/+}$;MMTV-Cre mice leads to the reduction of survival of double-mutant animals, which developed lymphoma and mammary tumors with rapid kinetics. R26-$PIK3CA^{H1047R}$;$^{p53loxP/+}$;MMTV-Cre mammary tumors were mostly adenosquamous carcinoma or spindle cell/EMT indicating that double-mutant mice develop a distinct spectrum of mammary tumors. [47][48]

In Pten heterozygous knockout ($Pten^{+/-}$) the loss of PTEN expression is associated with basal-like tumors. [49] Deletion of the *Pten* gene in mammary epithelium in conditional *Pten* gene knock-out mice, generated by flanking exon 5, which encodes the phosphatase domain of PTEN, with LoxP sequences, causes increased cell proliferation, hyper-branched ductal structure, precocious development, delayed involution and severely impaired apoptosis. PTEN-deficient mammary epithelium also displays remarkable neoplastic changes. [50]

To examine the role of AKT1 in the etiology of mammary tumorigenesis, transgenic mice were generated that express human AKT1 under the control of the mouse mammary tumor virus (MMTV) LTR. Ackler et al. have demonstrated for the first time that AKT1 expression during lactation results in a pronounced delay in involution, associated with hyperplasia and marked expression of cyclin D1. [51]

Addition of a myristoylation signal to the murine AKT1 gene, which results in AKT1 anchoring in plasma membrane and constitutive AKT1 activation independently of PI3K

activity, increases the incidence of benign lesions, delay in mammary involution and susceptibility to epithelial mammary tumor (ER-positive adenocarcinomas or adenosquamous tumors) formation induced by the carcinogen 9,10-dimethyl-1,2 benzanthracene (DMBA). [52]

Finally, in double myrAKT;p53(R172H) mice p53 inactivation by R172H point mutation combined with myrAKT transgenic expression significantly increases the percentage and size of mammary carcinoma, but was not sufficient to promote full penetrance of the tumorigenic phenotype. [41]

PROJECT'S AIM

Data from the literature have shown a significant involvement of the PI3K/Akt pathway in the onset of breast cancer. The role of AKT1 is controversial, the kinase seems to have a minor role, and limited to the ductal breast carcinoma. The purpose of this research project is to identify the oncogenic role of $AKT1^{E17K}$ in the mammary tumorigenesis by generating a mouse model that expresses $AKT1^{E17K}$ specifically in the breast, with particular attention to the effect on cell proliferation and breast cancer stem cells (BCSCs) maintenance. Furthermore this project comprises a pharmacological study in order to identify AKT1 as a putative target in breast cancer therapy.

MATERIALS AND METHODS

RESTRICTION DIGEST OF PLASMID DNA

Digestion of plasmid DNA was performed using different NEB enzymes. It is preferable to digest 0.2-1.5 µg DNA with a 2-fold to 10-fold excess of enzyme in a total volume of 20 µl. In the case of double digestion use the most compatible buffer with all the enzymes and it is important that the total volume of enzymes add to reaction is not more than 1/10 of the total reaction volume. A

typical restriction enzyme digestion protocol is below:

- In a 1.5mL tube combine the following:
    - DNA
    - Restriction Enzyme(s)
    - 10X Buffer
    - 10X BSA (if recommended by manufacturer)
    - dH_2O up to total volume (20 µl)
- Mix gently and spin down briefly
- Incubate at the optimal reaction temperature (usually 37°C) for 2 hours

The samples were run in an 1% agarose gel electrophoresis with ethidium bromide, using the non-digested plasmid as negative control.

PURIFYING DNA FROM AGAROSE GEL

Gel purification allows to isolate and purify DNA fragments based on size. The procedure starts with standard agarose gel electrophoresis, which separates DNA by their length in base pairs. Following electrophoresis, cut DNA bands out of the agarose gel and purify the DNA

samples. In our case, for this purpose, we used the QIAGEN QIAquick Gel Extraction Kit, using the standard protocol provided by the manufacturer.

DNA LIGATION

The final step in the construction of a recombinant plasmid is connecting the insert DNA (gene or fragment of interest) into a compatibly digested vector backbone. This is accomplished by covalently connecting the sugar backbone of the two DNA fragments. This reaction, called ligation, is performed by the T4 DNA ligase enzyme. The DNA ligase catalyzes the formation of covalent phosphodiester linkages, which permanently join the nucleotides together. This experiment was carried out using NEB T4 DNA Ligase (M0202).

Before setting up the ligation reaction itself, it is important to determine the amount of cut insert and vector to use for the ligation reaction. The volume of vector DNA and insert DNA used in the ligation will vary depending on the size of each and their concentration. However, for most standard cloning (where the insert is smaller than the vector) a 3 insert : 1 vector ratio will work just fine.

To calculate the volume of the insert for the ligation reaction must use the following formula:

X ng of insert = (3) (bp insert) (50 ng linearized plasmid-) ÷ (size of plasmid in bp)

- Set up the typical ligation reaction as follows:
    - 10X T4 DNA ligase buffer
    - vector DNA
    - insert DNA
    - dH_2O up to total volume (20 µl)
- Gently mix the reaction by pipetting
- Incubate at 16°C overnight

- Chill on ice and transform 1-5 µl of the reaction into 50 µl competent cells.

TRANSFORMATION OF ELECTROCOMPETENT BACTERIAL CELLS

The electrical transformation refers ingestion of foreign DNA in competent bacterical cells, that are able to take exogenous DNA, through the creation of pores in the bacterial cell walls using an electrical pulse.

The transformation protocol requires several steps:

- Thaw competent cells on ice for defrosting and mix 1 to 5µl of DNA (usually 10pg to 100ng) into 50µL of competent cells;
- Add cell/DNA mixture to the electroporation cuvette;
- Place cuvette in electroporator and shock cells at 2500 V;
- Remove cuvette from the chamber and immediately add SOC (SOB + glucose 2M). This step should be done as quickly as possible to prevent cells from dying off;
- Transfer SOC-cell mixture to an eppendorf tube and incubate tube in 37°C shaker for at least 1 hr to permit expression of antibiotic resistance gene;
- Centrifuge at 4500 rpm, discard the supernatant and resuspend the bacterial pellet in 100 µl of LB;
- Plate transformation onto prewarmed LB-agar plate supplemented with appropriate antibiotic and Incubate overnight at 37°C.

RECOVERING PLASMID DNA FROM BACTERIAL CULTURE (MINIPREP)

After liquid bacteric culture, at 37°C for overnight in a shacking incubator, bacterial cells were pelleted by centrifugation at 5000 rpm for 10 minutes. Afterwards, the QIAprep Spin Miniprep kit by QIAGEN was used to extract plasmid DNA as manufacturer's

recommendations and resuspended in 30 µl of H_2O milliQ.

SITE DIRECTED MUTAGENESIS

In vitro site-directed mutagenesis has been used to insert the specific mutation in the gene of interest. This experiment was performed using stratagene QuikChange Lightning Site-Directed Mutagenesis Kit, following the protocol provided and specific oligonucleotides specially designed, whose sequences are shown below:

Mut hAKT1 Fow	TGCACAAACGAGGGAAGTACATCAAGACCTG
Mut hAKT1 Rev	CAGGTCTTGATGTACTTCCCTCGTTTGTGCA

SOUTHERN BLOT

- digest 30µg of genomic DNA overnight with desired enzyme;
- prepare a 0,7% agarose gel with EtBr 0,25µg7µl;
- load samples with Sounthern Dye;
- run overnight at 20-50V;
- take a picture using a ruler;
- cut marker and wasteful agarose gel;
- place gel in a bowl with DEPURNATION BUFFER (0,2 N HCl) for the time necessary to make the color of the BBF turn from blue to yellow;
- wash with H_2O (2 x 2 min);
- cut BBF and measure the gel;
- wash with TRANSFER BUFFER 1 (0,4 NaOH-1 M NaCl) (2 x 15 min);
- cut a nitrocellulose membrane (Hybond N+) of same dimension of gel, 3MM paper and blotting paper;

- set up transfer apparatus:elettroforetic apparatus, glass, 3 MM paper;
- up side down agarose gel and place on nylon membrane, blotting paper and weight;
- transfer overnight in TRANSFER BUFFER 2 (10X SSC- NaOH 0,1 M);
- disassemble the blot and mark with a pencil well location on membrane;
- wash 15 min with NEUTRALIZATION BUFFER (0,5 M Tris-Hcl pH 7,5);
- dry membrane with 3MM paper;
- pre-hybridize using dig easy hyb granules roche kit cat. No. 11796895001, for 30 min at 55°C;
- hybridize overnight in agitation at 55°C with labeled and denatured probe;
- wash with WASH BUFFER 1 (2X SSC-0,1% SDS) (2 x 5 min) RT in agitation;
- wash with WASH BUFFER 2 (0,5X SSC-0,1% SDS) (2 x 15 min) at 65°C in agitation;
- blocking 30 min with BLOCKING SOLUTION (Blocking Reagent Roche Kit Cat. No. 11096176001 in 1X Maleic Acid 1:10);
- hybridize 30 min with anti-digoxigenin antibody alkaline phosphatase conjugated;
- wash with WASH BUFFER (1X Maleic Acid-TRITON) (2 x 15 min);
- equilibrate with DETECTION BUFFER (0,1 M Tris-HCl-0,1 M NaCl pH 7,5);
- detect hybridization by chemioluminescence witn CDP-Star kit Roche;

Previous probe labeling require PCR reaction with PCR DIG Probe Synthesis Kit Roche Cat. No. 11636090910, and digoxigenin-conjugated dideoxynucleotides (Dig-ddUTP) and probe denaturation is carried out at 100°C for 5 minutes.

ISOLATION OF GENOMIC DNA FROM MOUSE TAILS

- Cut tail pieces (5mm);
- Add 750 µl lysis buffer (0,05M Tris pH8.0, 1M EDTA, 0,1M NaCl, 1% SDS) and

Proteinase K (0,5 mg/ml);

- Incubate at 60°C overnight;
- Shake the samples at 1000 rpm at room temperature for 10 minutes;
- Add 250 µl of 6M NaCl and shake the samples at 1000 rpm at room temperature for 10 minutes;
- Centrifuge at 4° C for 10 minutes at full speed;
- Recover the liquid phase and add dropwise 500 µl of isopropanol;
- Precipitate DNA by inverting the tube;
- Spin down DNA at 4° C for 10 minutes at full speed and remove supernatant;
- Wash pellet with 1 ml of cold 70% ethanol;
- Spin down genomic DNA at 4° C for 5 minutes at full speed and remove supernatant;
- Allow DNA to dry for 1-2 minutes;
- Resuspend DNA in 100-200 µl depending on size of pellet;
- Place tube at 55°C for 1 hour to facilitate dissolution of DNA.

PCR AMPLIFICATION FOR GENOTYPING

To determine the genotype of the transgenic mice, we used a PCR protocol with 3 oligonucleotides.

The reaction mixture contains:

- 100 ng DNA;
- 10X TAQ buffer ;
- DMSO 2%;
- dNTP 0,3 mM;
- TAQ 0,2 U/µl ;

- primer mE17K 5’ Arm Fow (SIGMA-ALDRICH) 0,5 μM;
- primer mE17K Rev New (SIGMA-ALDRICH) 1 μM;
- primer mE17K 3’ Arm Rev (SIGMA-ALDRICH) 0,4 μM.

The sequences of the oligonucleotides used are following listed:

	5'-3' SEQUENCE
mE17K 5'ARM Fow	AACTGCAGACTTGTGGGATAC
mE17K 3'ARM Rev	ATATTAGTCCACCTCACTCCT
mE17K 3'ARM Rev New	GCCAACCCTCCTTCACAATA

and the amplification program was carried out as below:

	TEMPERATURE	**TIME**	
1)	**95°C**	**5 min**	
2)	**95°C**	**30 sec**	**35 cycles**
3)	**60°C**	**30 sec**	
4)	**72°C**	**1min 30 sec**	
5)	**72°C**	**7 min**	
6)	**4°C**	**Forever**	

FIXATION AND PARAFFIN EMBEDDING OF TISSUE

- Collect the tissue into a tube containing cold 1X PBS;
- wash in 1X PBS;
- Fix the tissue in 10% formalin overnight and proceed with the successive steps:

SOLUTION	**TIME**
saline solution 1X	3x1h
saline solution/EtOH 95%	1x30’ +1h

EtOH 70%	1h
EtOH 70%	o/n
EtOH 85%	2x1h
EtOH 95%	2x1h
EtOH 100%	1h
EtOH 100%	o/n
Xilene	3x1h
Xilene/paraffin	2x1h
Paraffin I	2x30’
Paraffin II	50’
Paraffin III	1h

Perform paraffin steps at about 60° C and transfer tissue to a mould with paraffin to submerge the tissue. Cool at room temperature and store at 4° C. Cut sections (5 μm), mount them on coated slides and dry overnight at 37˚ C for subsequent experiments.

HEMATOXYLIN AND EOSIN STAINING PROTOCOL

- Deparaffinize in Xylene I and II for 10 minutes;
- Rehydrate:
    - EtOH 100% (5 minutes)
    - EtOH 100% (2 minutes)
    - EtOH 95% (2 minutes)
    - EtOH 95% (2 minutes)
    - EtOH 70% (2 minutes)
- Rinse in distilled water for 5 minutes;
- Stain in hematoxylin Harris for 30 seconds;
- Decolorize in running tap water for 10 minutes;

- EtOH 70% for 3 minutes;
- EtOH 95% for 10 minutes;
- Counterstain in alcoholic Eosin for 30 seconds;
- Dehydrate
    - EtOH 95 % (5 minutes)
    - EtOH 100% (10 minutes)
- Clear in Xylene I and II for10 minutes;
- Mount with Eukitt BIO-OPTICA.

RNA EXTRACTION

To extract RNA from mammary tissue we lysate samples using QIAGEN Tissuelyser for 2 min at 30 Hz by adding 1 ml of TRIzol Reagent (Invitrogen) and following the standard protocol provided by the manufacturer. The RNA concentration was determined using the NanoDrop spectrophotometer.

RT-PCR

The reverse transcription reaction is performed using Quantitect Reverse Transcription Kit (Qiagen). The protocol used requires the following steps:

- Mix 1 µg of total RNA with gDNA Wipeout Buffer 7X in a final volume of 14 µl;
- Incubate at 42° C for 2 minutes;
- Add 1 µl di Quantiscript Reverse Trascriptase, 4 µl di Quantiscript RT Buffer 5X and 1 µl RT Primer Mix;
- Incubate at 42°C for 30 minutes and inactivate reaction at 95°C for 3 minutes;

REAL-TIME PCR

The cDNA obtained from the previous reaction of reverse transcription was diluited and used to perform a Real-Time quantitative PCR using Sybr Green PCR Master mix (Applied Biosystems) to evaluate AKT1 expression levels.

The reaction mixture contains:

- 20ng cDNA
- 0,2 μM primers
- 2X Sybr Green PCR Master mix
- MilliQ H_2O to 20 μl

The reaction program is performed using Applied Biosystem 7900 Real-Time PCR System and SDS Enterprise Database software and consists of the following steps:

	TEMPERATURE	**TIME**	
1)	**50°C**	**2 min**	
2)	**95°C**	**10 min**	
3)	**95°C**	**15 sec**	**40 cycles**
4)	**60°C**	**1min**	

The primers sequence used are represented below:

	5'-3' SEQUENCE
GAPDH Fow	CCGGGTTCCTATAAATACGGACTGC
GAPDH Rev	CGGCCAAATCCGTTCACACCG
AKT1 Fow	CACACCACCTGACCAAGATG
AKT1 Rev	AATCAAGGGTCCCCAAACTC

TOTAL PROTEIN EXTRACTION

The mammary tissue protein extraction is performed using a specific lysis buffer that allows to extract the proteins from the lipid component typical of the breast tissue. Tissue fragments were lysed using QIAGEN Tissuelyser for 2 min at 30 Hz by adding a volume of lysis buffer such as to cover the tissue.

The lysis buffer is consists of the following components:

- Urea 7 M;
- Thiourea 2 M;
- CHAPS 2%;
- DTT 50mM;
- Protease inhibitor SIGMA*FAST*™ 1X;
- NaF 1 mM;
- PMSF 1mM;
- Na_3VO_4 1mM;
- Okadaic Acid 15 nM;

After breaking up with Tissuelyser and short spin to remove tissue debris, the samples were incubated on ice for 30 minutes and then centrifuged in a refrigerated centrifuge at 4ºC for 30 minutes at 13200 rpm. The supernatant was recovered to a microcentrifuge tube and protein concentration was determined by a standard Bradford Assay (BioRad) in a Beckman DU 530 spectrophotometer.

WESTERN BLOT

This technique was used to analyze the protein levels in different samples.

Polyacrylamide gels were prepared at 10% acrylamide concentrations, according to the molecular weight of the proteins in study. After complete polymerization of the gel, it is transferred to a electrophoresis tank that was filled with a running buffer containing 25 mM of TRIS, 250 mM of Glicine (pH 8.3) and 0.1% of SDS. 50 µg of protein were dispensed in the wells, and the gels were run at 100 V. When the proteins were separated between them, the run was stopped and fractionated proteins were transferred from the gel to a nitrocellulose membrane by Trans-Blot Turbo Transfer System (BIORAD).

After transferring, the membranes were colored with 1X Red Ponceau (ATX Ponceau S Red staining solution, FLUKA) for 1 minute at room temperature to validate the transferring homogeneity and quality. Next, the membranes were washed in TTBS (1X TBS and 0,1% Tween) and were pre-hybridized for 45 minutes in a solution of TTBS containing 5% Nonfat dried milk (AppliChem) to block the non-specific hybridization sites of the primary antibody. Subsequently 3 washes of 5 minutes each were made before incubating with the primary antibodies diluted in red solution (1X TBS, 3% BSA, 0.02% NaAzide, 100 mg Phenol Red), overnight at 4ºC, with agitation.

The next day, the primary antibody was recovered and the membranes were washed for 5 minutes, 3 times with TTBS. After that, the membranes were incubated with the respective secondary antibody (rabbit or mouse), diluted at 1:2500 in a solution of TTBS with 5% of nonfat dried milk, for 1 hour, followed by 3 washes of 5 minutes each with TTBS. Next the membranes were incubated for 1 minute with an amplified chemiluminescence kit, ECL (Amersham Inc.), which allows the operator to see the chemiluminescence on High

performance chemiluminescence film (Amersham Inc.) due to the reaction between the substrate present on ECL and the peroxidase covalently bound to the secondary antibody.

The primary antibodies used in this thesis were the following:

- Anti-P-AKT (Ser473) antibody (rabbit), 1:1000 (Cell Signaling TECHNOLOGY®, #4058);
- Anti-AKT1 antibody (rabbit), 1:1000 (Cell Signaling TECHNOLOGY®, #2938);
- Anti-P-FoxO1 (Ser256) antibody (rabbit), 1:1000 (Cell Signaling TECHNOLOGY®, #9461);
- Anti-FoxO1 antibody (rabbit), 1:500 (Cell Signaling TECHNOLOGY®, #2880);
- Anti-P-GSK3α/3β antibody (rabbit), 1:1000 (Cell Signaling TECHNOLOGY®, #9331);
- Anti-GSK3α antibody (rabbit), 1:1000 (Cell Signaling TECHNOLOGY®, #9338);
- Anti-GSK3β antibody (rabbit), 1:1000 (Cell Signaling TECHNOLOGY®, #9315);
- Anti-β-Actin antibody (mouse), 1:10000 (Sigma-Aldrich, A2228)

IHC-PARAFFIN PROTOCOL (IHC-P)

Before proceeding with the staining protocol, the slides must be deparaffinized and rehydrated. Incomplete removal of paraffin can cause poor staining of the section.

Proceed as follows:

- incubate slides at 60 overnight.

- Deparaffinize in Xylene I and II for 10 minutes.
- Rehydrate:
    - EtOH 100% (5 minutes)
    - EtOH 100% (2 minutes)
    - EtOH 95% (2 minutes)
    - EtOH 95% (2 minutes)
    - EtOH 70% (2 minutes)
    - PBS 5 minutes
    - PBS+Tween20 5 minutes
    - PBS 5 minutes

- Perform antigen retrieval to unmask the antigenic epitope, using the citrate buffer Epitope Retrieval 1X pH=6 Novocastra Leica and incubate slides, immersed in this buffer, in the microwave at 900 WATT for 5 minutes and 300 WATT for 5 minutes, 3 times;
- Cool at 4° C for 30 minutes;
- Air dry the slides and wrap with pap pen;
- Wash in TTBS for 5 minutes, 2 times;
- Incubate sections in 3% hydrogen peroxide in methanol, for blocking of endogenous peroxidase, for 15 minutes;
- Wash in TTBS for 5 minutes, 2 times;
- Incubate slides with the primary antibody diluted in Bond Primary Antibody Diluent (Leica AR9352), overnight in humidifying chamber;
- Wash in TTBS for 5 minutes, 2 times.

Continue using the kit Novocastra Novolink Polymer Detection Systems (RE7140-K) as follows:

- Novocastra Post Primary Block (RE7111) for 30 minutes;
- Wash in TTBS for 5 minutes, 2 times;
- Novolink Polymer (RE7112) for 30 minutes;
- Wash in TTBS for 5 minutes, 2 times;
- Develop peroxidase activity with DAB working solution (Ratio 1:20 DAB Chromogen RE7105/DAB Substrate Buffer RE7106) maximum for 5 minutes;
- Rinse slides in water;
- Counterstain with Hematoxylin RE7107 for 10 minutes;
- Rinse slides in water for 5 minutes;
- Dehydrate (EtOH 70%, 80%, 90%, 100%, each from 5 minutes);
- Clear (xylene 10 minutes, 2 times);
- Mount sections with Eukitt BIO-OPTICA.

ISOLATION OF VIABLE EPITHELIAL CELLS FROM MURINE MAMMARY TISSUE AND MAMMOSPHERE CULTURES

- mechanically dissociate mammary tissues into small pieces using a surgical blade and placed in a digestion medium (DMEM/F12) supplemented with 200 U/ml collagenase (Sigma) and 100 U/ml hyaluronidase (Sigma) for about 5h at 37° C;
- filtering the cell suspensions through 100, 70, 40 and 20 μm meshes;
- Centrifuge at 1200 rpm for 5 minutes;
- Wash the pellet with PBS and centrifuge at 1200 rpm for 5 minutes;

Resuspend the pellet in 1-5ml of RBC buffer (NH_4Cl 155mM; $KHCO_3$ o $NaHCO_3$ 10mM; EDTA 0,1mM) to eliminate the possible presence of red blood cells;

- Incubate at RT for 5 minutes;
- Centrifuge at 1200 rpm for 10 minutes;
- Wash the pellet with PBS and centrifuge at 1200 rpm for 5 minutes;
- Count the cells preparing a 1:1 of the cell suspension using a 0,4% Trypan Blue solution. Non-viable cells will be blue, viable cells will be unstained.

Resulting cells were plated onto Corning®Ultra-Low Attachment Surface plates at a density of 400,000 viable cell/ml (to obtain primary mammospheres) in a serum-free mammary epithelial basal medium (MEBM, LONZA), supplemented with 5 μg/ml insulin (Sigma), 0.5 μg/ml hydrocortisone (Sigma), B27 (Invitrogen), 20 ng/ml EGF and bFGF (PEPROTECH), and 4 μg/ml heparin (Sigma). Mammospheres were collected after 14 days and dissociated with trypsin, to obtain single cells derived from mammospheres. For serial passage experiments, 5,000 cells from disaggregated primary-mammospheres were plated in 24 multiwell plates and, after 14 days, disaggregated and re-plated at the same density.

EVEROLIMUS TREATMENT

Everolimus (Afinitor®, Novartis), inhibitor of mammalian target of rapamycin (mTOR), was dissolved in 12,5% DMSO at a concentration of 1,25 mg/ml (5mg/kg). Virgin $Akt1^{E17K/E17K}$;MMTV-Cre female mice, 20-week-old, were treated with two weekly doses of EVEROLIMUS, for 8 weeks, via oral gavage. Oral gavage consisted of inserting a curved blunt tipped needle attached to a 1 ml syringe into the mid-throat of a firmly grasped mouse and injecting 100 μl of a EVEROLIMUS solution. The control group of virgin $Akt1^{E17K/E17K}$;MMTV-Cre female mice was treated with 12,5% DMSO. The mice were maintained in the absence of males and were checked by palpation for mammary tumor

formation. Mice were sacrificed by CO2 inhalation at the end of the period established for the experiment and mammary tissue was explanted.

RESULTS

GENERATION OF A Cre-INDUCIBLE AKT1^{E17K} MAMMARY MOUSE MODEL

To investigate the contribution of AKT1^{E17K} mutation in mammary tumorigenesis, we generated a mouse model that, through a Cre/Lox system, expresses the mutated form of AKT1 specifically in mammary gland tissue, in time and tissue specific manner. The gene targeting strategy allowed us to insert the transgene into the mouse Rosa26 locus, preferentially selected for the knock-in strategies as it has a high recombination frequency and allows the ubiquitous expression of the inserted gene both in embryonic development and in adult mice. The pRosa26 plasmid, containing 5 ' and 3' homology arms for the specific locus, has been suitably modified by adding the following elements:

- splice acceptor site;
- Neomycin resistance expression cassette, flanked by two Frt sites;
- triple SV40 polyadenylation sequence, LoxP- flanked, that prevents the transcription of the locus until the elimination of the sequence by the Cre recombinase;
- β-globin intron and SV40 polyA.

The human Akt1 cDNA (hAkt1) was inserted into the pBLUScript-KS vector and G>A nucleotide substitution was introduced by site-specific mutagenesis. The hAkt1E17K cDNA was then inserted into the pRosa26 shuttle vector generating the final construct pRosa26hAkt1E17K. The obtained plasmid was linearized and electroporated into mouse embryonic stem cells R1/129/Sv. G418-resistant clones were isolated, expanded and screened by PCR and Southern Blot. Targeted clones were transiently transfected with Flp recombinase, to eliminate the NeoR and allow the expression of the gene. (Figure 9)

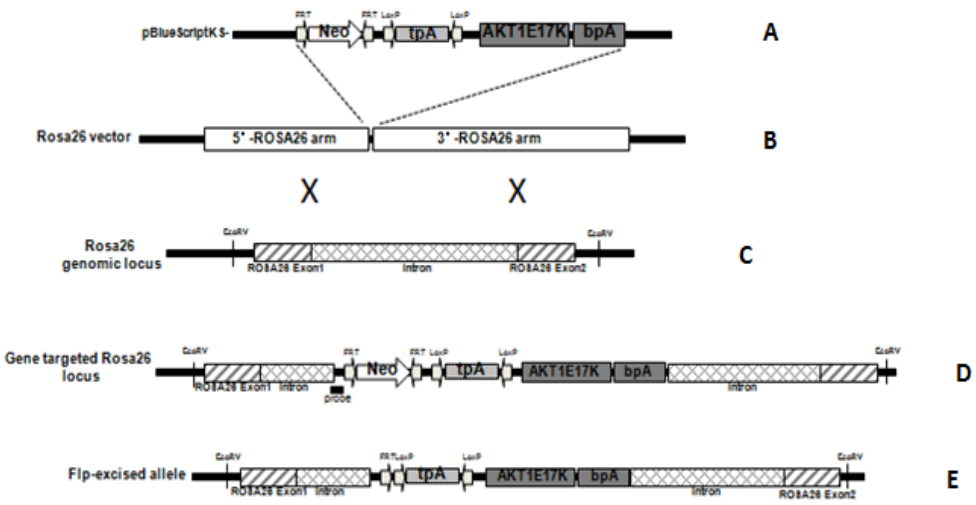


Figure 9: gene targeting strategy for pRosa26hAkt1E17K construct. A: insertion of the cDNA of interest into the vector pBLUScript-KS, B: hAkt1E17K insertion of the cDNA into the Rosa26 locus, C: final recombination of the construct with the genomic locus of the murine Rosa26, D: representation of the genomic locus Rosa26 with the insert of interest; E: excision of the neomycin resistance cassette by Flp.

The recombined clones were microinjected into C57BL/6 blastocysts for the generation of chimeric mice which were crossed with C57BL/6 mice. The presence of the transgene has been evaluated by PCR on DNA extracted from tail biopsies. (Figure 10)

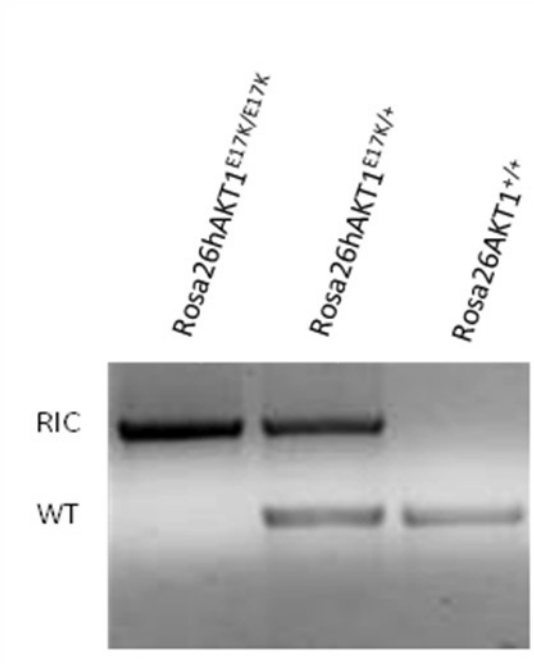


Figure 10: Genotyping PCR for Rosa26hAKT1$^{E17K/+}$ mice. The presence of the transgene is represented by the band of 1300bp.

To activate the expression of AKT1^{E17K} mutant allele in mammary glands, we bred this mouse with an MMTV-Cre line (B6129-Tg(MMTV-cre)1Mam/J, #003551; Jackson Laboratory), that expresses P1 Cre recombinase under the control of the MMTV-LTR promoter. This mating allows high levels of recombination in the virgin and lactating mammary gland. In double transgenic mice the deletion of STOP cassette, flanked by two loxP sites, occurs almost exclusively at the level of the breast. The occurrence of deletion was verified by PCR on DNA extracted from breast biopsies, the mice that have this deletion show a 350-bp PCR product, compared to wild-type mice. (Figure 11)

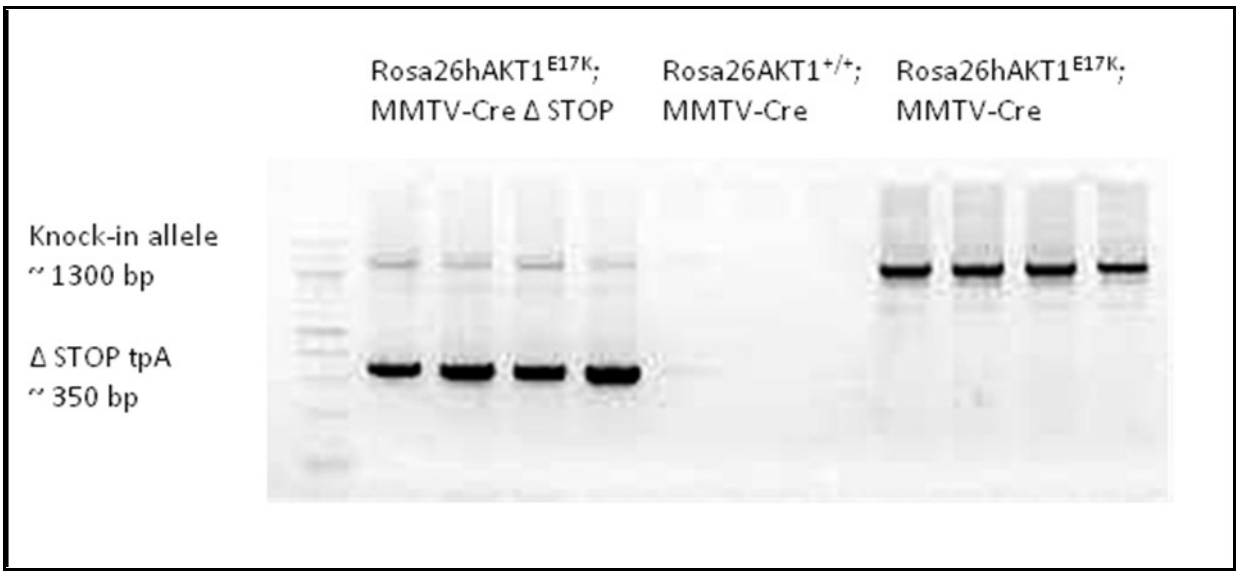


Figure 11: PCR screening to assess the successful deletion of the STOP cassette

CHARACTERIZATION OF AKT1^{E17K} EXPRESSION IN MAMMARY TISSUE

In order to characterize the generated mouse model for the effective expression of the transgene and the activity of AKT1 in Rosa26hAKT1^{E17K};MMTV-Cre mice, we performed a Real-Time PCR in which we analyzed the expression of specific transcript, on RNA extracted from breast biopsies from Rosa26hAKT1$^{E17K/E17K}$;MMTV-Cre (n=5),

Rosa26hAKT1$^{E17K/+}$;MMTV-Cre (n=5) mice. In figure 12 we reported the results of the AKT1^{E17K} mRNA quantification in the different mice groups. We observed an increased expression of 1.5 times in homozygous respect to the heterozygous transgenic mice. (Figure 12)

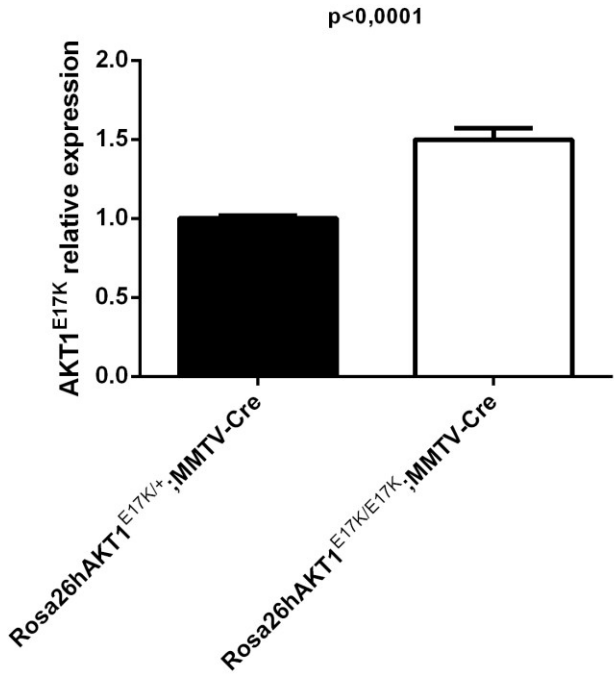


Figure 12: Relative expression of transegene in the different genotype groups of mice

This result was confirmed by analyzing the protein level of AKT1 and, in particular, its activation by phosphorylation of residue Ser473, compared to control mice. (Figure 13)

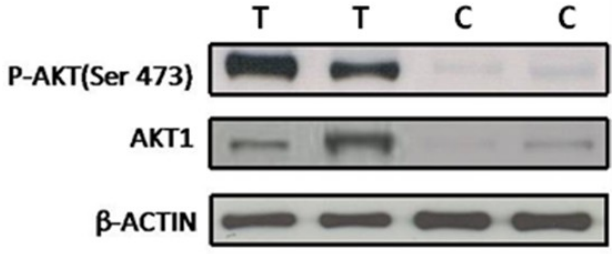


Figure 13: Western blot analysis of protein extracts derived from mammary gland tissue of Rosa26hAKT1^{E17K};MMTV-Cre mice and Rosa26AKT1$^{+/+}$;MMTV-Cre mice (T =transgenic mice mammary tissue , C =control mice mammary tissue)

The Western Blot analysis shows that in breast tissue derived from transgenic mice AKT1 is more expressed compared to control mice and we observed also an elevated accumulation of phospho-AKT1^{Ser473}. In order to evaluate the effect of AKT1^{E17K} on epithelial breast cells proliferation we performed a trypan blue viability assay on single cells suspension derived from transgenic and control mice. We observed that the presence of AKT1^{E17K} causes a 5 fold increased tissue cellularity in heterozygous transgenic mice compared to control mice while in homozygous transgenic mice the cellularity of breast tissue is almost 10 fold increased compared to control mice. (Figure 14)

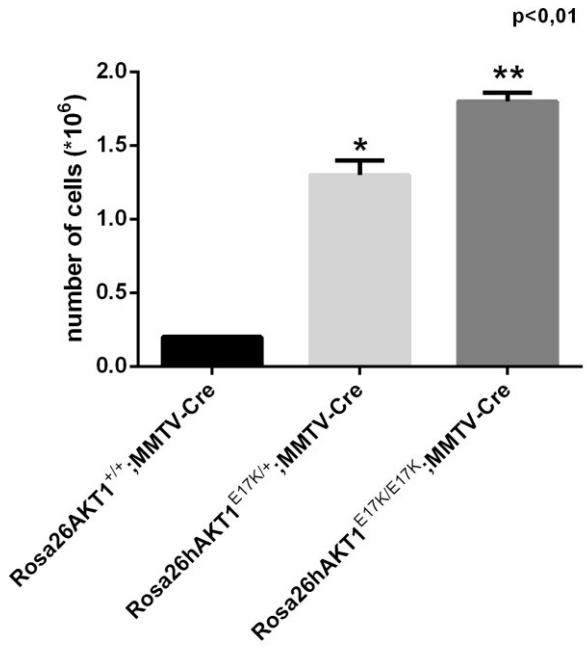


Figure 14: Analysis of mammary gland tissue cellularity derived from the different genotype groups of mice

AKT1^{E17K} EXPRESSION IS ONCOGENIC IN MAMMARY TISSUE

To asses the potential role of AKT1 as oncogene in mammary epithelium, we examined the consequences of AKT1^{E17K} expression in mammary gland in Rosa26hAKT1^{E17K};MMTV-Cre mice. First of all, we evaluated the possible appearance of tumors in Rosa26hAKT1^{E17K};MMTV-Cre virgin females compared to Rosa26AKT1$^{+/+}$;MMTV-Cre, for

a period of 15 months. At the end point (15 months), we observed that 70% (15/21) of Rosa26hAKT1^{E17K};MMTV-Cre mice developed mammary tumors, single or multiple, indicating a high incidence of cancer that is strictly dependent on the presence of the transgene. This data has been correlated with a survival analysis, which allowed us to monitor temporally the occurrence of tumors. The mice are monitored by fat pad palpation and were sacrificed when the tumors have achieved certain sizes. The data obtained have allowed us to perform a statistical analysis of survival, through a Kaplan-Meier curve. The data show a reduction in the overall survival of mice expressing the mutation AKT1^{E17K}, with a peak of mortality between 10 and 12 months. (Figure 15)

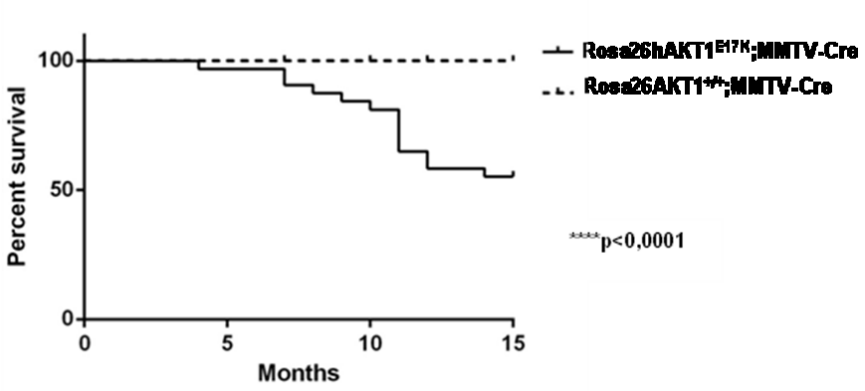


Figure 15: Survival curve of Rosa26hAKT1^{E17K};MMTV-Cre and Rosa26AKT1$^{+/+}$;MMTV-Cre mice, computed using the Kaplan-Meier method.

Tumors harvested from mice, that may affect both thoracic that inguino-abdominal mammary glands, and the counterpart of healthy breast tissue, if present, were histologically characterized. The tissue biopsies were fixed in formalin, paraffin embedded and subjected to hematoxylin/eosin staining. Histological analysis of tumor sections of Rosa26hAKT1^{E17K};MMTV-Cre mice shows the presence of ductal carcinomas from medium to high grade. Furthermore, transgenic mice that do not have a visible tumor show an alteration of mammary epithelium which goes from hyperplastic lesion to dysplasia. The figure (Figure 16) shows representative images of mammary tumors derived from

Rosa26hAKT1^{E17K};MMTV-Cre mice compared with the mammary epithelium of Rosa26AKT1$^{+/+}$;MMTV-Cre control mice, which present the typical morphology of breast tissue, consists of a lipidic reticulum with glandular units composed by a lumen surrounded by a single cell layer.

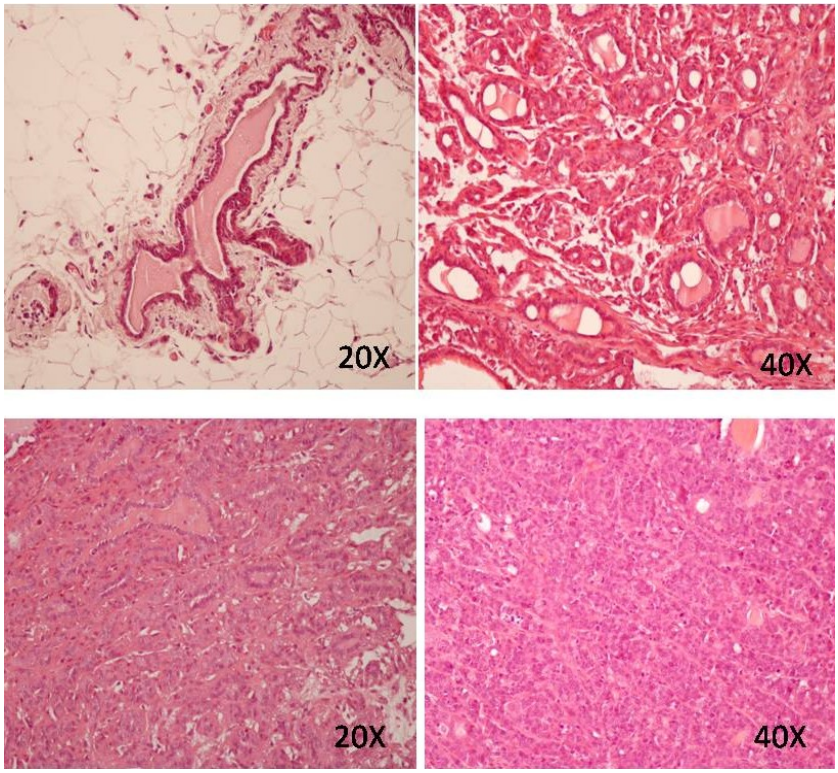


Figure 16: hematoxylin-eosing staining of representative normal (up) and tumoral (down) breast tissue

Analysis of the tumors from Rosa26hAKT1^{E17K};MMTV-Cre mice allowed us to confirm literature data that report the presence of the mutated form AKT1^{E17K} especially in human ductal breast carcinomas.

To test the PI3K pathway activation, lysate from representative tumors of Rosa26hAKT1^{E17K};MMTV-Cre mice were analyzed for phosphorilation status of AKT1 and its downstream effectors, such as FOXO1 and GSK3α/β and cyclin D1. Total protein extract from tumors were compared with those derived from healthy counterparts and breast tissue of

control mice. In the proteins derived from tumors is observed an increased phosphorylation of AKT1 than control tissues. In all tumors analyzed we observed elevated levels of phospho-FOXO1^{Ser256} and phospho-GSK3α/β$^{Ser21/9}$ and high levels of cyclin D1. (Figure 17)

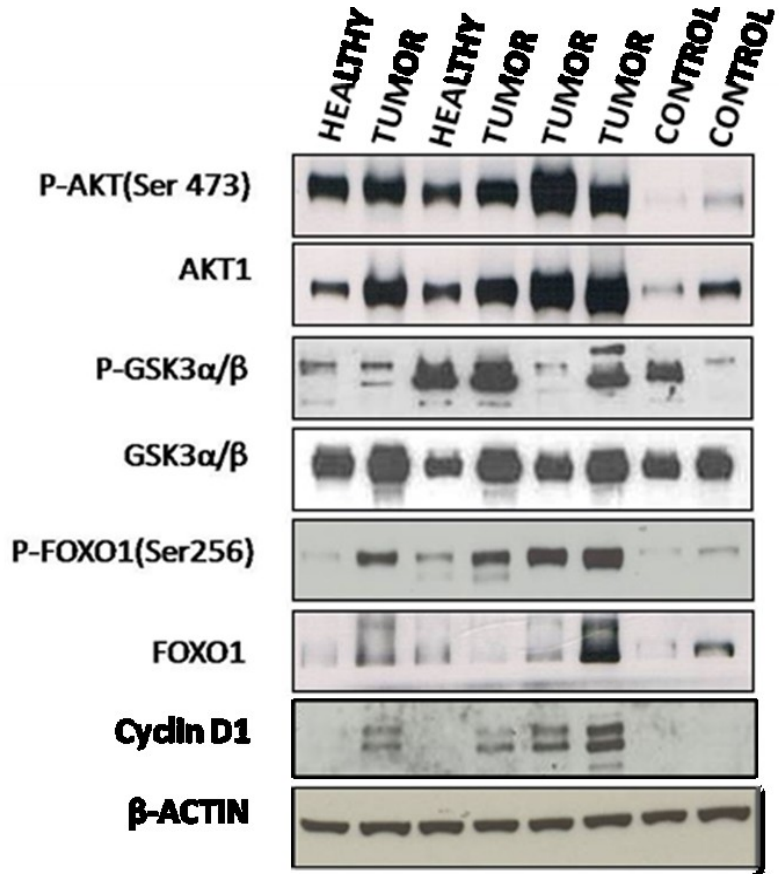


Figure 17: Western blot of AKT1 and downstream effects in tumor derived from Rosa26hAKT1^{E17K}; MMTV-Cre mice compared to healthy counterparts and normal breast tissue from control mice.

The elevated level of AKT1 phosphorylation was confirmed by immunohistochemistry on paraffin embedded sections of breast cancer from Rosa26hAKT1^{E17K};MMTV-Cre mice compared with their healthy counterparts. This histological analysis showed that tumors from transgenic mice were characterized by hyperactivation of AKT1. (Figure 18)

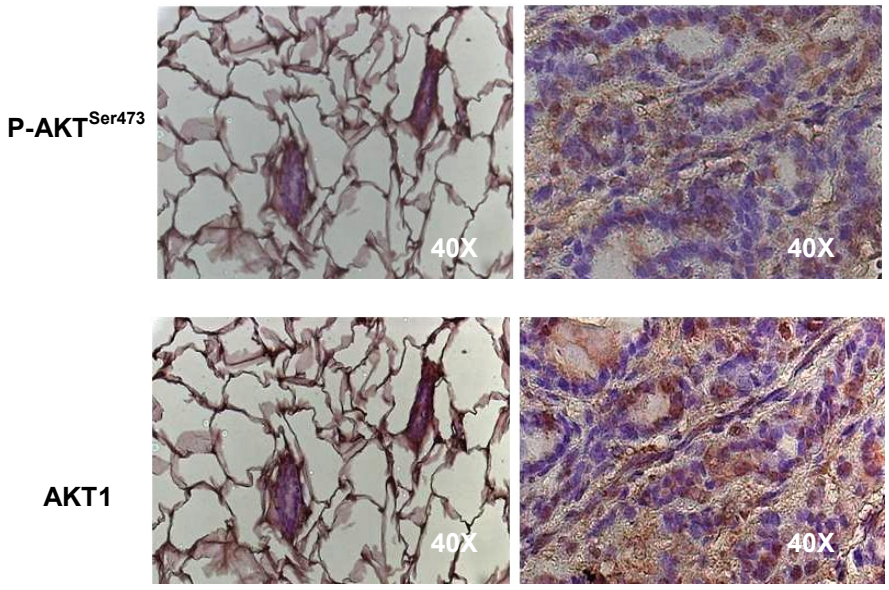


Figure 18: anti p-AKT^{Ser473} and AKT1 staining of paraffin embedded sections of breast cancer from Rosa26hAKT1^{E17K};MMTV-Cre mice compared with their healthy counterparts.

AKT1^{E17K} CONTRIBUTION IN THE GENERATION AND MAINTEINANCE OF PUTATIVE BREAST CANCER STEM CELLS

In tumors has been identified a subpopulation of cells, called cancer stem cells (CSCs) with stem cell characteristics, probably responsible for the formation and maintenance of the tumor, metastases and chemoresistance. This cell subpopulation has also been identified in breast cancer (breast cancer stem cells, BCSCs) and several studies have demonstrated the implication of PI3K/AKT pathway in BCSCs biology. [29][31] Putative BCSCs can be identified through their ability to proliferate as spheroid structures in non-adherent conditions. To analyze the AKT1^{E17K} role in BCSCs we performed a mammosphere formation assay. Homogeneous cell suspension derived from non cancerous mammary tissue of Rosa26hAKT1$^{E17K/E17K}$;MMTV-Cre, Rosa26hAKT1$^{E17K/+}$;MMTV-Cre and

Rosa26AKT1$^{+/+}$;MMTV-Cre mice were seeded in non-adherent condition, serum-free medium supplemented with B27, EGF and FGF. After about 10 days the single cell suspension generated floating colonies called "mammospheres". (Figure 19)

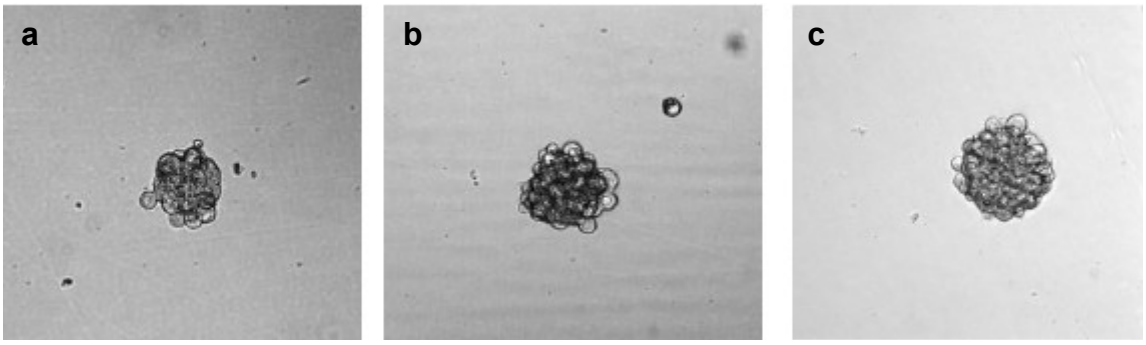


Figure 19: representative images of mammospheres derived from Rosa26AKT1$^{+/+}$;MMTV-Cre,(a) Rosa26hAKT1$^{E17K/+}$;MMTV-Cre(b) and Rosa26hAKT1$^{E17K/E17K}$;MMTV-Cre mice(c).

We observed that cells derived from Rosa26hAKT1$^{E17K/+}$;MMTV-Cre and Rosa26hAKT1$^{E17K/E17K}$;MMTV-Cre mice generated a number of mammospheres 2.5 and 8 fold increased, respectively, compared to Rosa26AKT1$^{+/+}$;MMTV-Cre mice. Size analysis of the mammospheres revealed that the spheroids derived from Rosa26hAKT1$^{E17K/+}$;MMTV-Cre and Rosa26hAKT1$^{E17K/E17K}$;MMTV-Cre mice showed a diameter of 1.5 and 2 folds increased, respectively, compared to spheroid generated by single cells from Rosa26AKT1$^{+/+}$;MMTV-Cre mice (Figure 20)

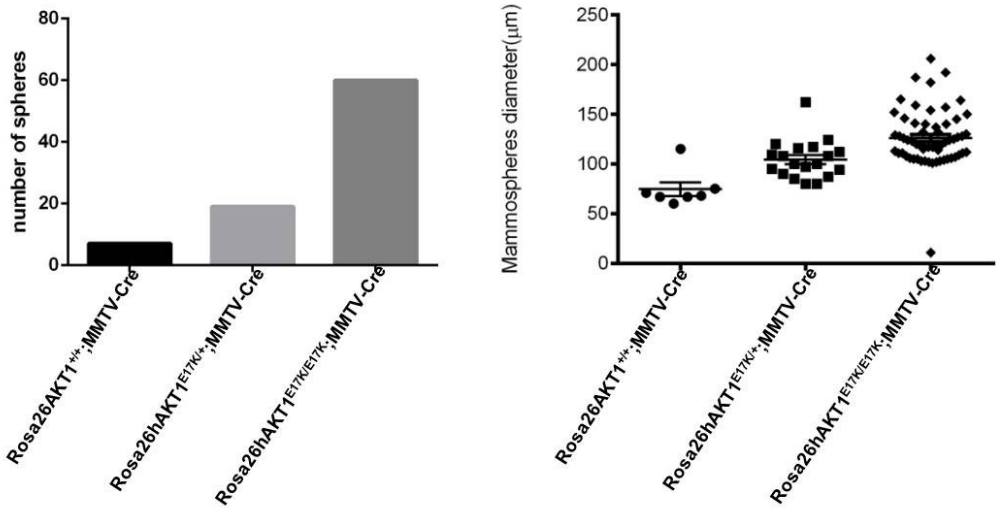


Figure 20:cumulative number and size measurement of mammospheres derived from the different mice groups.

To test the self-renewal capability of the mammosphere-forming cells during generations, the primary mammospheres were dissociated into single cells and replated in the same conditions. Mammospheres derived from Rosa26AKT1$^{+/+}$;MMTV-Cre mice were regenerated for only four generations, while those derived from Rosa26hAKT1$^{E17K/E17K}$;MMTV Cre are capable to regenerate the spherois until eight generations. (Figure 21)

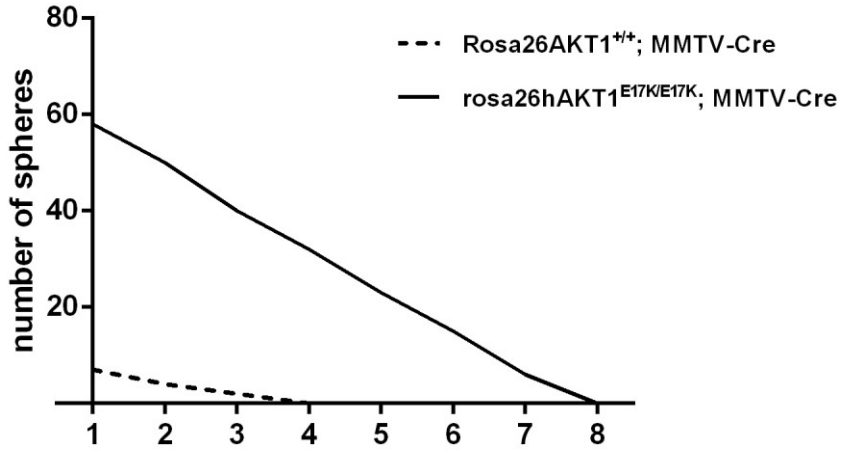


Figure 21: graphic representation of capability to regenerate spheroids of putative BCSCs from Rosa26AKT1$^{+/+}$;MMTV-Cre and Rosa26hAKT1$^{E17K/E17K}$;MMTV Cre mice.

AKT1^{E17K} IS A PUTATIVE TARGET IN BREAST CANCER THERAPY

We performed a pharmacological study to identify a therapeutic strategy in order to target PI3K/AKT pathway for breast cancer treatment. Recently has been developed a new drug, Everolimus, for the treatment of advanced stage ER+, HER2- mammary carcinomas. The Everolimus is a selective inibitor of mTOR (*mammalian target of rapamycin*), a serine-threonine kinase, whose activity is upregulated in several cancers. The activation of the mTOR complex-1 (mTORC1) is downstream of AKT signaling. Everolimus binds the intracellular protein FKBP-12, forming a complex that inhibits mTORC1. Four-five months-old Rosa26hAKT1$^{E17K/E17K}$;MMTV-Cre mice (n=8) are treated with Everolimus (5mg/kg,

gavage), for 8 weeks (2 doses in week), as control we treated a group of mice (n=9) with the vehicle. At the end of treatment, the breast tissue of both groups was collected and subjected to histological analysis. We observed that 66.7% (6/9) of the control mice developed tumors and 11,1% (1/9) severe dysplasia, while only 37.8% (3/8) of treated mice with Everolimus showed medium-grade dysplasia.

DISCUSSION

Breast cancer is the most common female malignancy and the leading cause of cancer death in women. It is caused by the combination of genetic and environmental factors. Specific inherited mutations in *BRCA1* and *BRCA2* increase the risk of breast cancers. Together, *BRCA1* and *BRCA2* mutations account for about 20-25% of hereditary breast cancers and about 5-10% of all breast cancers. [54][55] In the onset of sporadic breast cancer are involved gene alterations in oncogenes and tumor suppressors genes. Among these, the phosphoinositide-3 kinase (PI3K) pathway has been identified to have an important role. The importance of this pathway in tumorigenesis is represented by the high frequency of his activativation in cancer, as illustrated in Figure 7. In the figure are showed the tumors in which this pathway is upregulated by mutations. In addition some of the components have an intrinsic inhibitory effect, such as phosphatase and tensin homologue deleted on chromosome ten (PTEN). [56] In particular in breast cancer mutations in the components of the PI3K/AKT pathway occur in the 25% of the cases. The majority of mutations are in PIK3CA, encoding the catalytic p110α subunit, and are nonrandomly localized in three "hot spots," resulting in single amino acid substitutions: E545K and E542K in the helical domain (exon 9) and H1047R in the kinase domain (exon 20). These mutations increase enzymatic function, enhance downstream signaling elements, including AKT, and promote oncogenic transformation. [18]

In hormone receptor–positive tumors, these mutations occur in >30% of cases. Also, in HER2+ disease, mutations are evident in about one quarter of tumors. Meanwhile, it seems that mutations in triple-negative breast cancer may be less frequent. [3] More recently a single hotspot mutation, G49A:E17K, in the pleckstrin homology domain of AKT1 was described, predominantly in human breast tumors. [43] The reported frequency of this mutation has

ranged from 1.8%–8%. [18] [43][44][45] Importantly, mutant AKT1 is able to activate PI3K signaling pathway and to transform rodent fibroblasts and bone marrow cells . [43] Most studies have found PTEN, PIK3CA, and AKT1 mutations to be mutually exclusive in individual tumors [43][44][49], suggesting that mutational activation of the PI3K/AKT pathway by any one of the components are biologically equivalent.

Until now the role of AKT1 in mammary tumorigenesis represents a secondary event. *In vivo* studies have shown that its hyperactivated form, myrAKT1, which results in AKT anchoring to the plasma membrane and constitutive activation, delay the mammary involution and increases the incidence of benign lesions. In addition myristoylation signal increases the susceptibility to epithelial mammary tumor formation after induction by the carcinogen 9,10-dimethyl-1,2 benzanthracene (DMBA). [52] Somewhat surprisingly, published data demonstrated that overexpression of AKT1 is able to transform cells *in vitro*[43], but the knock-in of mutant $AKT1^{E17K}$ in MCF10A cells failed to recapitulate this capacity. [53] These findings are similar to what was observed with knock in of mutant KRAS and may reflect differences between knock in and transgenic overexpression as well as differences between cell types in their susceptibility to transformation. However, were not made in vivo studies to analyze the effect of mutant form of AKT1 in mammary tumorigenesis. For this reason the aim of this study is the evaluation of the role of $AKT1^{E17K}$ in the mammary tumorigenesis in a mouse model that expressed $AKT1^{E17K}$ specifically in mammary gland tissue. These mice are derived by crossing $Rosa26AKT1^{E17K}$ transgenic mice with an MMTV-Cre mouse line. The resulting mice, $Rosa26hAKT1^{E17K}$;MMTV-Cre, drive the expression of $AKT1^{E17K}$ almost exclusively in the mammary tissue.

The data from this study showed that the transgene was expressed primarily in the mammary gland. This is consistent with the findings that increase in MMTV-LTR transcriptional activity

is through mammary development and during pregnancy. [57] The hAKT1^{E17K} specific transcript was expressed in mammary gland of trangenic mice compared to the control littermates. In addition, the analysis of the expression of AKT1^{E17K} in breast tissue derived from transgenic mice demonstrates that the transgene expression is increased from heterozygous to homozygous mice. These results are confirmed by the analisys of downstream pathway activation status, phosphorylation of AKT1 is higher than that wild-type control mice.

The activation of AKT1 by overexpression or myristoylation signal has been shown to delay mammary gland involution and induce hyperplasia [51]. This process accelerates mammary tumorigenesis in MMTV-c-ErbB2 mice [58] but overexpressing or activated AKT1 in mammary gland alone is not able to induce dysplasia and neoplasia [59][60]. Activation of AKT1 induces oncogenic transformation upon exposure to carcinogens [52]. Surprisingly, in our model, we demonstrated that transgenic mice with active AKT1 led to a significant higher incidence of mammary carcinoma without exposure to the carcinogen insults. In particular tumor incidence was of about 70%, starting from four months of age, with a peak of mortality between 10 and 12 months. The histological analysis conducted on the explanted mammary tumor masses revealed that the tumors are ductal carcinomas from medium to high grade, confirming the literature data from breast cancer patients showing the presence of the mutant AKT1^{E17K} in ductal and lobular carcinomas. [43] The tumor tissues from transgenic mice had increased phosphorylated AKT1 at Ser473 and the tumors derived from Rosa26hAKT1^{E17K};MMTV-Cre mice showed hyperactivation of the PI3K/Akt pathway. Increased pAKT^{Ser473} was associated with strong phosphorilation levels of downstream substrates as FOXO1 and GSK3α/β. Moreover tumor tissues from transgenic mice overexpress cyclin D1. Cyclin D1 plays role in tumor cell proliferation, migration and was

inversely correlated with tumor size in human breast cancer [61] Overexpression of cyclin D1 has been reported between 40% and 90% of cases of invasive breast cancer, while gene amplification is seen in about 5–20% of tumors [35]. Increase in protein level of cyclin D1 was apparent in hyperplasia and further increased with malignancy. [62][63] Cyclin D1 has also been shown as an essential oncogenic intermediary for Neu pathway inducing mammary carcinoma in transgenic mice. [64][65]. In our model, we observed that cyclin D1 expression was higher in Rosa26hAKT1^{E17K};MMTV-Cre mice driven mammary carcinoma, compared to control mice. The histological analysis revealed also the presence of preneoplastic lesions, from hyperplasia to dysplasia. These results, associated with the activation of AKT pathway, correlates the presence of mutant AKT1 to the cell proliferation, *in vivo*. Mammary gland derived from 8-12 weeks Rosa26hAKT1^{E17K};MMTV-Cre mice showed increased cellularity, ten fold increased compared to mammary tissue derived from control littermates.

It has been recently suggested that a cellular subpopulation with stem cell (SC)-like features, known as Cancer Stem Cells (CSCs), is critical for tumor generation and maintenance, and responsible for breast cancer metastasis. [24] *In vitro* and *in vivo* evidences revealed the importance of PTEN/PI3K/Akt/Wnt/β-catenin pathway in breast cancer stem cells (BCSCs) biology. Increased AKT Ser473 phosphorylation in suspension culture of putative breast cancer stem cells (mammospheres) as compared with monolayer cultures, determines increased levels of GSK3-β phosphorylation and β -catenin activation. β-catenin has been demonstrated to play an important role in the development of mammary stem cells in mouse models, suggesting that this pathway may also be active in human mammary stem/progenitor cells in mammospheres. [31] To assess the role of AKT1^{E17K} in the context of BCSCs, we exploited his ability to regulates the growth of cells suspension, derived from Rosa26hAKT1^{E17K};MMTV-Cre an control mammary gland tissue, in spheroid conditions.

Performing a mammospheres assay we observed that cells derived from Rosa26hAKT1^{E17K};MMTV-Cre mice generates increased number of mammospheres compared to those derived from control mice. Furthermore the mammospheres derived from transgenic mice showed a diameter two fold increased compared to the wild-type mice. The self-renewal assay revealed that cells derived from Rosa26hAKT1^{E17K};MMTV-Cre mice are capable to renew until 8 generations the spheroid culture. The obtained data allow us to identify an important role of AKT1^{E17K} in mammary tumorigenesis and in the maintenance of the tumor through its involvement in the biology of the BCSCs. We therefore conducted a pharmacological study in which we evaluated the antitumor activity of the drug Everolimus on tumor induction and / or progression of breast cancer in Rosa26hAKT1$^{E17K/E17K}$;MMTV-Cre mice. This drug has recently been developed for the treatment of breast cancers in advanced phase, positive estrogen receptor and HER2 negative. Everolimus is a selective inhibitor of mTOR (mammalian target of rapamycin) a serine-threonine kinase whose activity is known to be upregulated in several human cancers. The activation of this complex is downstream of AKT, which can act in a direct manner on mTOR, through phosphorylation at the residue Ser2448, and in indirectly, by inactivating phosphorylation at residue Thr246 of PRAS40, a inhibitory component of the mTORC1 complex and at the level of 5 residues (S939, S981, S1130, S1132 and T1462) of TSC2, which together with TSC1 constitutes a complex that inhibits mTOR via RHEB protein. [35] Treatment with this drug has allowed to inhibit, or at least slow down, the complete transformation of breast tissue in Rosa26hAKT1^{E17K};MMTV-Cre mice.

In summary this project led us to these results: i) the generation of a new mouse model for breast cancer, AKT1-dependent; ii) the analysis of the mouse models revealed that the activation of AKT1 in mammary tissue increases the risk of development of mammary tumors

iii) the involvement of $AKT1^{E17K}$ in the generation and maintenance of BCSCs; iiii) Rosa26h$AKT1^{E17K}$;MMTV-Cre transgenic mouse is a valuable resource for understanding the mechanisms of breast cancer target therapy.

Future experiments will be needed for the molecular characterization of mammary tumors induced by $AKT1^{E17K}$. We will also confirm the stemness of mammospheres derived cells, testing their positivity to stem marker (such us Sox2, Oct4, Nanog), their symmetric and asymmetric division and their in vivo tumorigenic capability.

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