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FOXP3 EXPRESSION IN BREAST CARCINOMA CELLS AND METASTATIC SPREAD

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ABSTRACT

Forkhead box P3 (FOXP3), a gene member of the forkhead/winged-helix family of transcription regulators, is implicated in regulating immune system development and function. This gene has been found to be of crucial importance for the generation of CD4+CD25+ regulatory T cells (Tregs). Tregs express both the full-length FOXP3 and Δ2FOXP3 natural splice variant. In addition to its expression in the lymphocyte lineage, studies have recently described FOXP3 expression in non-hematopoietic-derived cells, including cancerous or normal epithelial cells of multiple lineages and origins.

The role of FOXP3 in cancer cells is still unclear. Our immunohistochemical (IHC) and statistical analyses of archival material from two old series of breast cancer patients indicated that the expression of FOXP*3* in tumor cells is an independent strong prognostic factor for distant metastases. The impact of FOXP3 on patient survival has been confirmed by our IHC analysis on chemotherapy-treated breast cancer patients. In fact, FOXP3 positive patients had poorer disease-free survival compared to FOXP3-negative patients.

To investigate FOXP3 role in breast cancer, its expression was assessed in a panel of breast carcinoma cell lines and in human primary breast carcinoma samples by Western blot analyses. Full lenght FOXP3 was detected in all human breast cancer samples and breast cancer cell lines analyzed, whereas the Δ2FOXP3 isoform was visible solely in human breast tumors. The involvement of Δ2FOXP3 in breast cancer and the possibility that this isoform could have a different role from that of full length FOXP3 in breast cancer progression has been investigated. WTFOXP3 or Δ2FOXP3 overexpression was induced in MDA-MB-231 breast cancer cell line. Both WTFOXP3 and Δ2FOXP3 overexpression significantly increased *in vitro* migration and invasion capability of breast cancer cells, whilst inhibiting breast cancer cell proliferation. Taking advantage of these *in vitro* results and to further investigate *in vivo* role of FOXP3 in breast cancer metastasis, the metastatic capability of WTFOXP3- or Δ2FOXP3-overexpressing MDA-MB-231 breast cancer cells was investigated. The mean number of spontaneous lung metastases was superimposable in WTFOXP3- and Δ2FOXP3-overexpressing tumor bearing mice (mean±SD: 12.5±27.4 and 10.3±18.1, for WTFOXP3- and Δ2FOXP3-MDA-MB-231-injected mice, respectively). These findings do not support a role of Δ2FOXP3 isoform in promoting breast cancer metastasis.

In our IHC analyses of breast carcinoma specimens subcellular staining of FOXP3 was observed to be cytoplasmic or cytoplasmic/nuclear. Since the role of FOXP3 is transcription

regulation, which mainly occurs in the nucleus, a cytoplasmic FOXP3 localization could affect its biological role. Thus the hypothesis that FOXP3 in tumor cells may have distinct biological activities and prognostic values according to its subcellular localization was investigated. Metastatic capability of two breast cancer cell clones with inducible FOXP3 expression and with different FOXP3 subcellular localization was evaluated. FOXP3 overexpression in breast cancer cells with a predominantly nuclear FOXP3 localization led to a significant reduction in the number of both spontaneous and experimental lung metastases compared to controls (mice in which FOXP3 overexpression was not induced). Contrarily, in mice injected with breast cancer cells which showed a predominantly cytoplasmic FOXP3 localization, FOXP3 overexpression in tumor cells led to a significant increase in the number of lung metastatic lesions compared to control group.

These results suggested that nuclear FOXP3 localization enable its transcriptional activity, resulting in an onco-suppressive effect, while its cytoplasmic localization unable this transcription factor to perform its biological functions, resulting in an opposite in *vivo* effect.

Our findings indicate that FOXP3 subcellular localization in breast tumor cells is an important determinant of prognosis, supporting the involvement of this transcription factor in breast cancer metastasis. Additional studies are in progress to confirm these data and to better understand the molecular mechanisms involved in FOXP3 role in driving breast cancer metastasis.

Introduction

1. Breast cancer

1.1 Rational for the proposed research

Breast cancer is the most common non-skin cancer in women and the second most common cause of cancer-related death in women. Multiple factors ranging from age, family history, and obesity to lifestyle factors such as alcohol consumption, smoking and exposure to carcinogens have been shown to contribute to susceptibility to breast cancer [Gray et al., 2009]. Breast cancer causes 450,000 deaths each year worldwide, mainly as a result of metastatic spread of the disease. Metastasis is the process in which the cancer cells dissociate from the primary tumor and localize to distant or secondary sites within the body. The five year survival rate is 98% in cases where breast cancer remains localized; however, when a breast cancer has metastasized the five year survival rate reduces to 26% [Jemal et al., 2007]. Thus, a better understanding of the molecular mechanisms underlying metastasis is one of the most important issues in cancer research. The detection of molecular markers for prediction of metastatic potential of cancer cells will contribute to the creation of new principles for prevention, diagnosis and therapy of metastasis.

1.2 Breast cancer epidemiology

Breast cancer is the most common cancer among women worldwide and it is also the leading cause of cancer-related mortality [American Cancer Society, 2011; Jemal et al., 2011]. An estimated total of 1,384,000 females were diagnosed with breast cancer globally in 2008 [Ferlay et al., 2010], corresponding to 42.3 new cases per 100,000 population [Ahmad et al., 2001]. This represented the 23% of all invasive cancers diagnosed amongst females that year, and compared to the 2002 estimates [Parkin et al., 2005] there was an increase in terms of both the number of cases and the incidence rate (1,152,000 and 40.4/100,000 in 2002, respectively). Although the overall number of new diagnoses were similar in more developed countries compared to less developed countries, incidence rates were almost two and a half times higher in the former (71.7/100,000 and 29.3/100,000, respectively) [Ferlay et al., 2010]. Breast cancer had the highest incidence of any cancer amongst females in most regions of the world, with the exception of several countries in Eastern and Western Africa as well as parts of Central and South America and Southern Asia. The differences in incidence are due to variations in environmental factors rather than genetic factors [Hoover, 2012], as demonstrated by migrant studies which show an increased risk for migrants moving from a low-risk to a high-risk country [Tyczynski et al.,

1994] and that the risk increases from generation to generation [Ziegler et al., 1993]. Thus, parallel with the progress of the developing countries and the adoption of a more westernized lifestyle, incidence of breast cancer is also increasing in these countries [Bray et al., 2004].

The range of mortality rates is much less (approximately 6-19 per 100,000) because of the more favorable survival of breast cancer in high-incidence developed regions.

As a result, breast cancer ranks as the fifth cause of death from cancer overall, but it is still the most frequent cause of cancer death in women in both developing and developed regions.

1.3 Breast cancer classification

Breast cancer can be classified by different aspects each of one influences treatment, response and prognosis. A full classification of breast cancer includes histopathological type, grade, stage, receptor status, and gene expression patterns.

- **Histopathology**

Breast cancer is divided into non-invasive and invasive (infiltrating) carcinoma.

Breast carcinoma in situ (CIS) comprise a heterogenous group of lesions, covering a wide spectrum of clinical conditions and histopathological changes. With respect to biological behavior, CIS range from lesions with a very low malignant potential to biologically aggressive lesions with a substantial risk of progression into invasive carcinoma (IC). Studies of CIS indicate that approximately a third will subsequently develop IC. Autopsy studies indicate that CIS is frequently occurring and it was estimated that about 20% of all women will develop CIS during lifetime [Ottesen, 2003]. Two main types of CIS are described: ductal carcinoma in situ (DCIS) and lobular carcinoma in situ (LCIS). Ductal carcinoma in situ (DCIS) is considerably more common than its lobular carcinoma in situ (LCIS) counterpart and encompasses a heterogeneous group of tumors. DCIS has traditionally been further sub-classified based on the architectural features of the tumor which has given rise to five well recognized subtypes: Comedo, Cribiform, Micropapillary, Papillary and Solid.

Invasive carcinomas are a heterogeneous group of tumors differentiated into histological subtypes. The major invasive tumor types include infiltrating ductal (70–80% of all invasive lesions), invasive lobular, ductal/lobular, mucinous, tubular, medullary and papillary carcinomas [Li et al., 2005].

- **Grade**

The neoplastic grading is a measure of tumor cell anaplasia and indicated how closely the tumor cells resemble the tissue from the organ of origin. The grading method in most widespread clinical use presently is the Nottingham combined histologic grading system (NHG). In this system, the degree of tubule formation, nuclear grade, and mitotic rate are each assigned a value of 1 to 3; these values are then added together to produce assigned scores from 3 to 9. Tumors with total scores of 3 to 5 are categorized as grade 1; those with scores of 6 and 7 are grade 2, and those with scores of 8 and 9 are grade 3.
Pathologists describe tumor cells as well differentiated (grade 1), moderately differentiated (grade 2), and poorly differentiated (grade 3) as the cells progressively lose the features seen in normal breast cells. Grade 1 tumors have better prognosis than tumors of grade 3.
NHG correlates well with 10-year survival, and has been shown to be an independent factor in prediction of prognosis [Rakha et al., 2008; Razavi et al., 2005].

- **Stage**

The most well-established clinically used prognostic marker in breast cancer is the Tumor, Node and Metastasis staging system (TNM) (Table 1). TNM staging is a measure of how far the tumor has spread and summarizes information about three important features of the tumor, namely size, lymph node status and distant metastasis. These variables are scored, and the tumor is classified into one of five different stages (0; *in situ*, I, II, III and IV), with the highest stage correlating to a worse prognosis. Lymph node involvement is the single most powerful predictor of prognosis in patients who do not have distant metastasis [Carter et al., 1989]. In fact, the number of metastatic lymph nodes strongly and negatively influences survival [Goldhirsch et al., 2001].

- **Receptor status**

Breast cancers can be categorized based on estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2) status.
The hormone receptor ERα, is expressed in 70-80% of all breast tumors (ER+) the majority of which are also positive for PR (PR+), since this receptor is upregulated in response to ER-signalling. ER-status is the most important factor influencing response to endocrine therapy. However, has been recently demonstrated that PR was a stronger predictor of treatment response than the ER. In fact, both recurrence-free and overall survival improved significantly on tamoxifen treatment in patients with tumors showing >75% PR-positive

nuclei [Ferno et al., 2000; Stendahl et al., 2006]. Apart from predicting response to endocrine therapy, hormone-receptor status also influences prognosis; hormone receptor-positive cancers conferring a survival advantage compared to hormone receptor-negative cancers [Mansour et al., 1994].

Human epidermal growth factor receptor 2 (HER2) (also known as HER2-neu and ERBB2) is amplified in 10-30% of all breast cancer cases. HER2 mediates growth, differentiation, and survival of cells. Women whose tumors overexpress HER2 have a more aggressive disease than women whose tumors do not overexpress HER2, both with respect to disease-free and overall survival [Slamon et al., 1987].

Tumors that are ER-, PR-, and HER2-negative are referred to as triple-negative tumors and account for 15-20% of all newly diagnosed breast cancers [Metzger-Filho et al., 2012]. Triple-negative breast cancers (TNBC) have a higher probability of relapse and poorer overall survival in the first years after breast cancer diagnosis compared to other types of breast cancer [Metzger-Filho et al., 2012].

- **Molecular subtypes of breast cancer**

A discovery which has had a major impact in directing breast cancer research is the existence of molecularly distinct breast tumor subtypes with pervasive differences in their gene expression patterns (figure 1) [Perou et al., 2000]. The molecular portrait that classify the different subtypes reflect differences in the intrinsic biology, such as growth rate, activation of intracellular pathways and cellular composition, clinical presentation, histopathological feature, outcome, and response to systemic therapies [Perou et al., 200; Reis-Filho et al., 2011; Sorlie et al., 2001, 2003, 2004;].

The five original subtypes were the luminal A, luminal B, basal-like, HER2, and the normal breast-like subtype [Perou et al., 2000, Sorlie et al., 2001]. The claudin-low and molecular apocrine subtypes were identified later on [Farmer et al., 2005; Herschkowitz et al., 2007].

Luminal A tumors are mostly ER-positive, they have a low proliferation rate, and they are of low grade.

Luminal B tumors are also mostly ER-positive but may express low levels of hormone receptors. They are usually of high grade and have a high proliferation rate.

The basal-like subtype is often characterized by triple-negative tumors, high levels of expression of proliferation-related genes, and expression of genes associated with basal and myoepithelial cells.

The HER2-enriched group displays amplification and high expression of the HER2 gene and it is generally represented by ER-negative breast cancers.

The normal breast-like subtype shows expression of many genes expressed by adipose tissue and other non-epithelial cell types, strong expression of basal epithelial genes, and low expression of luminal epithelial genes.

The claudin-low tumors are ER/negative carcinoma characterized by down-regulation of genes involved in cell adhesion, are more enriched in epithelial-to-mesenchymal transition features, and express stem cell-associated genes [Reis-Filho et al., 2011].

The apocrine subtype is usually ER-negative but it expresses androgen receptors and androgen receptor-associated genes, and has histological features suggestive of apocrine differentiation [Doane et al., 2006; Farmer et al., 2005].

The molecular subgroups harbor prognostic value; patients with luminal A tumors have better prognosis than patients with other tumor subtypes (particularly luminal B and basal-like). On the contrary, basal-like and ERBB2+ subtypes associated with the shortest survival times and the shortest relapse-free survival [Hu et al., 2006; O'Brien et al., 2010; Perou et al., 2000; Sorlie et al., 2001] (figure 2).

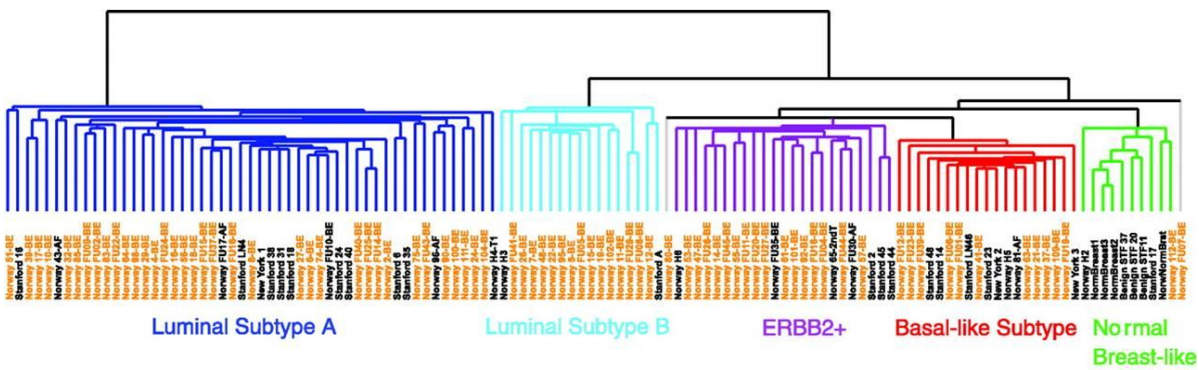

Figure 1. Molecular classification of breast cancers
Hierarchical clustering of 115 tumor tissues and 7 nonmalignant tissues using the intrinsic gene set.
A scaled-down representation of the entire cluster of 534 genes and 122 tissue samples based on similarities in gene expression. Gene expression profiling divided breast cancers into 5 distinct molecular classes or intrinsic subtypes, termed luminal A, luminal B, HER2-enriched, basal-like and normal-like subtypes.
Adapted from Sorlie et al., 2006.

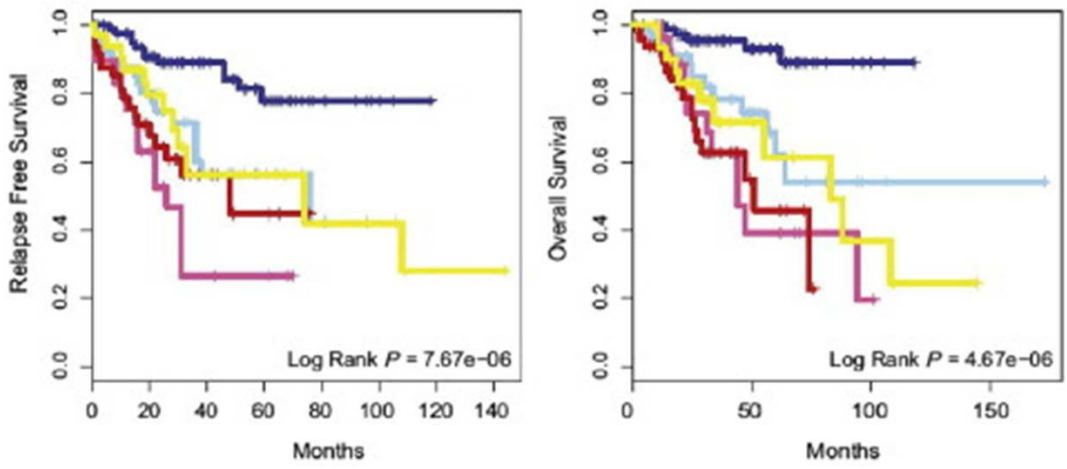

Figure 2. Survival probability according to breast cancer subtype
Kaplan-Meier curves of disease-free survival and overall survival based on the UNC337 database. Dark blue, luminal A; light blue, luminal B; red, basal-like; pink, HER2-enriched; yellow, claudin-low. Adapted from Cadoo et al., 2013.

1.4 Risk factors associated with breast cancer

- **Age**

Breast cancer incidence increases with age and it is relatively rare in women less than 40 years of age, after which the incidence increases greatly [Adami et al., 2008]. Conversely, the rate of the age-specific incidence rises sharply until around 50 years of age (i.e. around menopause), after which the increase is less pronounced, suggesting that hormones are important to breast cancer development [Adami et al., 2008; Pike et al., 1983].

- **Endogenous hormones**

Breast cancer is influenced by many hormonally related factors and it has therefore long been assumed that high levels of endogenous sex hormones are partially at fault. This was also corroborated by the Endogenous Hormones and Breast Cancer Collaborative Group which showed that postmenopausal women who had increased levels of endogenous sex hormones, also were at higher risk of breast cancer and there was a dose-response relationship [Key et al., 2002].

A relationship has been observed between higher levels of free estradiol during the follicular phase and breast cancer risk in premenopausal women [Eliassen et al., 2006].

- **Hereditability**

A family history of breast cancer in a first-degree relative increases risk of breast cancer by approximately 2-fold [Pharoah et al., 1997], and a family history of more than one first-degree relative confers an even greater risk. Approximately 16% of breast cancers in women with a positive family history are thought to arise due to mutations in the high penetrance susceptibility genes BRCA1 and BRCA2 [Breast Cancer Study Group, 2000]. High penetrance susceptibility genes are characterized by the fact that carriers have a high likelihood of developing disease. The lifetime risk of developing breast cancer is 45-65% for carriers of BRCA1 and BRCA2 mutations [Antoniou et al., 2003]. There are four other known high penetrance genes associated with breast cancer, TP53, PTEN, STK11, and CDH1, but mutations in these genes are very rare. Together all six genes are only believed to account for 20% of the familial risk of breast cancer. Four, intermediate penetrance genes have also been identified: CHEK2, ATM, BRIP1 and PALB2. These genes increase breast cancer risk by 2-4 fold but are also believed to be uncommon [Stratton et al., 2008].

- **Reproductive factors**

Age at menarche influences breast cancer risk, where older age decreases risk [Kelsey et al., 1993]. The opposite is true for age at menopause, where older age at menopause increases risk of breast cancer [Collaborative Group on Hormonal Factors in Breast Cancer, 1997]. Hence, the longer a woman has menstrual cycles, the higher her risk of breast cancer. An early age at first full-term pregnancy and number of full-term pregnancies both have protective effects on breast cancer risk, independent of each other [Ewertz et al., 1990]. The reason for this is thought to be that mammary gland cells are undifferentiated until first pregnancy, and that each pregnancy decreases the number of undifferentiated cells. However, the effect of pregnancy is dual; a full-term pregnancy increases breast cancer risk immediately after birth, but the risk then gradually diminuishes, and in the long-term, imparts a protective effect. Breast-feeding also has a protective effect on breast cancer risk independent of age at first birth and parity [Collaborative Group on Hormonal Factors in Breast Cancer, 2002; Key et al., 2002].

- **Exogenous hormones**

The use of combined oral contraceptives is related to an increase in breast cancer risk of about 25% but is only associated with current or recent use. 10 years after cessation there

seems to be no increased risk [Key et al., 2002]. Hormone-replacement therapy, given as a combination of estrogen and progesterone or estrogen alone, increases breast cancer risk, where combination treatment increases risk the most [Narod et al., 2011]. Risk also increases with duration, e.g. in women on combination therapy, breast cancer risk increases by 7.6%/year [Lee et al., 2005].

- **Lifestyle factors**

Two recent International Agency for Research on Cancer (IARC) Monographs considered the effect of alcohol drinking in cancer etiology, and concluded that female breast cancer is causally related to alcohol consumption [IARC, 2010; Secretan et al., 2009]. A small but significant increase of the order of 4% in the risk of breast cancer is already present at intakes of up to one alcoholic drink/day. Heavy alcohol consumption, defined as three or more drinks/day, is associated with an increased risk by 40–50% [Mezzetti et al., 1998].

Although not initially considered to be a tobacco-related cancer [IARC, 2004; United States Public Health Service, 2004], breast cancer has been the subject of well over 100 studies of tobacco-related risk. Consensus has been building that active smoking is likely to pose a modest risk for breast cancer. Contributing to that view is evolving evidence for biologic plausibility, risk estimates from large well designed human health studies, evidence from pooled and meta-analyses, and evidence for higher risk in more susceptible subsets of the population. Highlighted from the literature on active smoking and breast cancer is the importance of timing, with evidence for increased risk among women who initiated smoking at an early age and/or who smoked heavily prior to a first full term pregnancy. This is complemented by increasing evidence for host susceptibility associated with genetic polymorphisms involved in tobacco metabolism [Reynolds, 2013].

A decrease in the risk of breast cancer of approximately 25% among the most physically active women compared with the less active women. All types of activity and both moderate and vigorous intensity activity were associated with a reduction in the risk of breast cancer. Physical activity reduced the risk for breast cancer among all categories of Body Mass Index except obese women [Friedenreich et al., 2008].

1.5 Management of breast cancer

Management of breast cancer depends on tumor stage, tumor size, the expression of hormone and growth factor receptors on the tumor cells, and patient factors.

- **Surgery**

Surgery is considered the primary treatment for breast cancer. The aims of surgery include complete resection of the primary tumor, with negative margins to reduce the risk of local recurrences, and pathologic staging of the tumor and axillary lymph nodes (ALN) to provide necessary prognostic information. Lumpectomy (partial or segmental mastectomy) is defined as complete surgical resection of a primary tumor with a goal of achieving widely negative margins (ideally 1 cm). A total mastectomy involves complete removal of all breast tissue. Complete mastectomy is used less frequently, since breast-conserving surgery in combination with radiotherapy has been shown to be an adequate alternative [Wapnir et al., 2011]. ALN dissection is the gold standard as part of initial management to detect regional nodal metastasis. ALN status can serve as an indicator to likely presence of systemic disease, assist in locoregional control of disease and determine the need for further adjuvant therapy [Ahmed et al., 2013]. Over the last few decades, ALN dissection has changed from therapeutic clearance of the ALN to selective sentinel lymph node dissection (SLND). The sentinel lymph node is defined as the first node that receives lymphatic drainage from the affected breast. It is the node most likely to harbor metastases. Sentinel node biopsy (SNB) allows accurate axillary staging of patients with invasive breast cancer and a clinically negative axilla. SNB has less morbidity and fewer complications than traditional axillary lymph node dissection (ALND) [Wilke et al., 2006]. According to ASCO guidelines [Lyman et al., 2005] patients with a sentinel lymph node biopsy result indicating that they are free from metastatic disease may avoid ALND. SLN can be used in conjunction with both mastectomy and breast conservation. ALND should be performed when sentinel node mapping fails to identify the sentinel node or when non sentinel nodes are clinically suspicious. SLND alone has shown excellent locoregional control of disease in patients with breast cancer [Giuliano et al., 2000; Reitman et al., 2003; Schrenk et al. 2001].

- **Radiotherapy**

Radiation is the international standard treatment in order to reduce the risk of local relapse. Radiotherapy is applied to all patients who have gone through breast-conserving surgery

and a majority of mastectomized patients with affected lymph nodes. A meta-analysis by the Early Breast Cancer Trialists' Collaborative Group in 2011 showed adjuvant radiotherapy after breast-conserving therapy to reduce the relative risk of recurrence by 50%, the largest effect being seen on local recurrences. The proportional benefit was similar regardless of other prognostic factors such as lymph node status, tumor size or the patient's age. The benefit of radiotherapy is thus dependent on the patient's inherent risk of recurrence.

- **Systemic treatment**

Systemic treatment includes chemotherapy, endocrine treatment and targeted drugs, such as antibodies. Treatment can be given neo-adjuvantly, adjuvantly or for palliation. In the neo-adjuvant setting the primary goal is to reduce the size of the tumor, making it operable. Adjuvant therapy is given postoperatively to eradicate micro-metastases and to reduce the risk of recurrence. Generally, adjuvant therapy is considered if the risk of recurrence is higher than 20-30%. In the palliative setting, the purpose of treatment is to shrink metastases, reduce symptoms and prolong life.

With the purpose to reduce the risk of undetected deposits of disease to develop into a clinical recurrence, adjuvant chemotherapy is administered and results in improved relapse-free and overall survival in the general breast cancer population.

Adjuvant chemotherapy is today recommended for most patients with lymph-node-positive disease [Swedish Breast Cancer Group, 2011]. Furthermore, adjuvant chemotherapy is being offered increasingly over time to node-negative patients, with grade, tumor size, ER status, and younger age the most significant factors influencing chemotherapy recommendations [Elder et al., 2011]. Adjuvant treatment with poly-chemotherapy resulted in about 36% breast cancer mortality rate reduction versus no chemotherapy [Early Breast Cancer Trialists' Collaborative Group, 2012]. Poly-chemotherapy has also proven to be more effective than single-agent regimes in neo-adjuvant and adjuvant settings [Early Breast Cancer Trialists' Collaborative Group, 1992]. The reasons for this are the potential synergetic effects and the different toxicity profiles, allowing more intense treatment. Taxanes, such as paclitaxel and docetaxel, are well established drugs for metastatic breast cancer and are comparable to anthracyclines when administered as monotherapy. However, the addition of a taxane to anthracycline-based therapy has been shown to improve distant recurrence rates as well as overall survival in the adjuvant setting [De Laurentiis et al., 2008].

- **Targeted therapy**

The term “targeted therapy” is generally used to describe treatment disrupting specific molecules involved in carcinogenesis and tumor growth, rather than generally affecting rapidly dividing cells, as is the case in most traditional forms of chemotherapy. Current treatment options for breast cancer are moving toward nontoxic, potent targeted therapies that can be tailored to an individual patient’s tumor. There are now targeted therapeutic options available for the majority of breast cancer subtypes, exploiting the differing drivers of carcinogenesis within these individual tumors. A greater understanding of the underlying biology of breast cancer has resulted in the identification of a number of molecular targets and development of novel therapeutics. Among them are tyrosine kinase inhibitors (TKIs) directed at a number of targets (HER1, HER2, HER3, IGF receptor [IGFR], C-MET, FGF receptor [FGFR]), inhibitors of intracellular signaling pathways (PI3K, AKT, mammalian target of rapamycin [mTOR], ERK), angiogenesis inhibitors, and agents that interfere with DNA repair. Some of these agents have shown remarkable activity and have already become part of the standard of care in patients with breast cancer (exemplified by the anti-HER2 agents Trastuzumab and Lapatinib). Others have shown clinical activity but are not yet approved for clinical practice.

ER- and PR-positive breast cancers have been the prime example of cancer amenable to targeted drug approaches. Estrogen-focused therapies remain pivotal to the treatment of this disease. The ER pathway can be targeted by inhibiting the ER (Tamoxifen), or by removing the ligand estrogen (oophorectomy or aromatase inhibitors (AIs)).

The ER modulator Tamoxifen has been demonstrated to improve survival among women with early and advanced breast cancer and further improvements have been provided by aromatase inhibitors and the ER-degrading agent Fulvestrant [Gibson et al., 2009; Gradishar, 2010; Robertson et al., 2009]. In premenopausal patients the ovaries are the main source of estrogen, while in postmenopausal women estrogen is predominantly produced by aromatization of adrenal and ovarian androgens in the liver, muscle and fat tissue. AIs block this aromatization in peripheral tissue, although they do not affect the production of estrogen in the ovaries. Hence, AIs are only effective in postmenopausal patients, while Tamoxifen can be used for all women regardless of menopausal status. Long-term efficacy of Tamoxifen and AIs is limited by relapse of disease and development of resistance.

The monoclonal antibody Trastuzumab is directed against the HER2 oncogene. Patients overexpressing HER2 have a poorer prognosis and an increased risk of metastasis. Trastuzumab in combination or sequence with cytotoxic chemotherapy significally improves the prognosis of patients with HER2-overexpressing early breast cancer and is now routinely offered to this population mostly in combination with adjuvant chemotherapy [Piccart-Gebhart et al. 2005; Romond et al., 2005]. Similarly, the dual HER1 and HER2 TKI Lapatinib has been approved for the therapy of patients whose disease has progressed on Trastuzumab [Baselga and Swain, 2009]. In addition to the approved agents, there are a number of novel strategies against HER2 that have shown activity in tumors that have progressed during treatment. Pertuzumab is a recombinant humanized monoclonal antibody directed against the dimerization domain II of HER2 extracellular domain that is required for ligand-dependent dimerization with HER3 [Baselga and Swain, 2009]. The combination of Pertuzumab and Trastuzumab among patients with metastatic HER2-positive breast cancer who had experienced progression during prior Trastuzumab therapy demonstrated a clinical benefit rate of 50%.

To date, unlike other molecular subtypes of invasive breast cancer, validated targeted therapies are unavailable for triple negative breast cancer patients.

1.6 Triple negative breast cancer

Triple-negative breast cancer (TNBC) is defined by the lack of estrogen receptors, progesterone receptors and by human epidermal growth factor receptor 2–negative status and accounts for 15% to 20% of newly diagnosed breast cancer cases [Bauer et al., 2007]. TNBC is associated with African American ethnicity, younger age, advanced stage at diagnosis, and poorer outcome when compared to other breast cancer subtypes. The majority of TNBCs are invasive ductal carcinoma, but less common histologic subtypes (ie, medullary, metaplastic, and adenoid cystic) share the TNBC phenotypic characteristics [Jacquemier et al., 2005; Livasi et al., 2006].

TNBC is a heterogeneous disease and can be further classified on the molecular level into two main subgroups, basal-like and claudin-low. 80% of basal like breast cancers are also triple negative breast cancers and 80% of triple-negative breast cancers are also basal like breast cancers [Foulkes et al., 2010; Lehmann et al., 2011]. At the morphology level, TNBC and basal-like tumors share similar characteristics [Rakha et al., 2009]. Larger tumor size,

high grade, presence of geographic necrosis, pushing borders of invasion, and stromal lymphocytic infiltrate are characteristics commonly reported [Fulford et al., 2006; Livasy et al., 2006]. Moreover, an important phenotypic overlap is present between *BRCA1*-associated tumors and TNBC/basal-like cancers. *BRCA1* is an important breast-cancer susceptibility gene. IHC-based studies also classify 80% to 90% of *BRCA1*-associated tumors as TNBC and/or basal-like breast cancers [Arnes et al., 2005; Foulkes et al., 2003; Lakhani et al., 2005]. In addition, both triple-negative and basal-like breast cancers are more likely than other breast cancer types to metastasize to viscera, particularly to the lungs and brain, and are less likely to metastasize to bone [Foulkes et al., 2010].

A minority subtype of TNBC, termed claudin-low, has recently been identified in non-basal TNBCs. Claudin-low TNBC cells are uniquely characterized by low to absent expression of epithelial cell–cell adhesion proteins, differentiated luminal cell surface markers, and enrichment of epithelial-to-mesenchymal transition markers, immune response genes and cancer stem cell-like features [Prat et al., 2010].

TNBCs have a poor outcome compared to the other subtypes of breast cancer [Chavez et al., 2010]. The shape of the survival curve for patients with triple-negative or basal-like breast cancer differs from that for patients with other types of breast cancer: there is a sharp decrease in survival during the first 3 to 5 years after diagnosis, but distant relapse after this time is much less common (figure 3) [Foulkes et al., 2010].

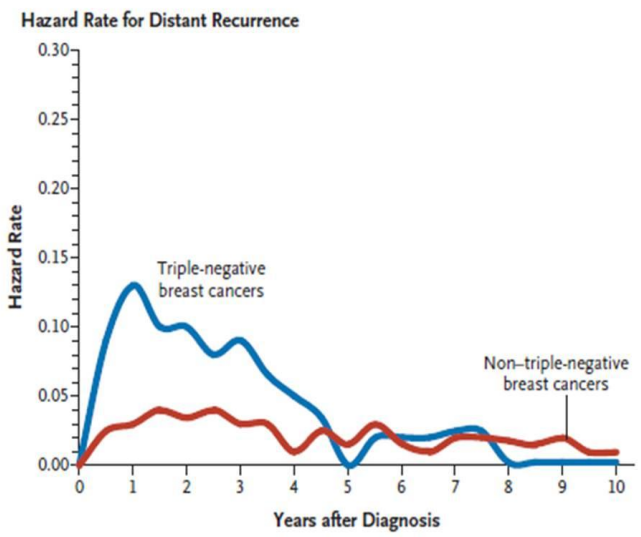

Figure 3. The hazard rates for distant recurrence of triple-negative breast cancer and non–triple-negative breast cancer
Adapted from Foulkes et al., 2010.

TNBCs cause a disproportionate number of breast cancer deaths. This poor prognosis is due to intrinsic aggressiveness and lack of treatment options, especially targeted therapies [Foulkes et al., 2010; Lehmann et al., 2011]. The lack of identified molecular targets in the majority of TNBCs implies that chemotherapy remains the treatment of choice for patients with TNBC. Current best standard of care chemotherapy regimens for early breast cancer are third-generation regimens, which include an anthracycline and taxane-based regimen [O'Toole et al., 2013]. Neoadjuvant studies have shown that TNBC is highly chemotherapy sensitive. Despite this high chemotherapy sensitivity, treatment of TNBC remains challenging, and on recurrence, patients with TNBC have worse survival outcomes than patients with hormone receptor–positive breast cancer subtypes [Carey, 2010; Foulkes et al., 2010]. While there may be some benefit in the adjuvant setting, once metastatic disease develops, chemotherapy responses in TNBC are not sustained with a median survival in patients with metastatic TNBC of only 6 months; consequently there is a pressing need to identify new targets for treating patients with TNBC [O'Toole et al., 2013]. Molecular processes and biological drivers that have been targeted in TNBC include vascular endothelial growth factor, inefficient DNA repair mechanisms (ie, PARP), the epidermal growth factor, mammalian target of rapamycin (mTOR), Src oncogene pathway, histone deacetylase, and androgen receptor. In general, clinical introduction of these molecules is hampered by a lack of predictive biomarkers [Brouckaert et al., 2012].

Anyway, because of the heterogeneity of TNBC tumors, it is unlikely that any single treatment will be efficacious in all TNBC patients. The most effective treatment approach for these patients is likely to be a combination of targeted therapies or combined targeted therapy with cytotoxic agents [Duffy et al., 2012].

2. Metastasis

2.1 The metastatic cascade

The process of metastasis is a highly complex and dynamic event that requires tumor cells to dissociate from the primary tumor mass and moving to localize to distant or secondary sites (figure 4) [Carter et al., 1989]. The metastatic process consists of a series of steps all of which must be successfully completed to give rise to a metastatic tumor. The inability to perform any of the steps in the metastatic process leads to metastatic failure [Fidler et al., 2002].

The first step of the metastatic cascade is represented by epithelial to mesenchymal transition, in which tumor cells originating from the primary tumor acquire fibroblastoid characteristics that increase their motility and allow them to invade epithelial and basal membranes [Kalluri and Weinberg, 2009]. As a primary tumor grows, it needs to develop a blood supply that can support its metabolic needs, a process called angiogenesis. These new blood vessels can also provide an escape route by which cells can leave the tumor and enter into the body's circulatory blood system, known as intravasation [Wyckoff et al., 2000]. Tumor cells might also enter the blood circulatory system indirectly via the lymphatic system [Hunter et al., 2008; Talmadge et al., 2010].

Tumor cells that entered the lymphatic vessels give rise to lymph node metastasis most often occurring in the ipsilateral axilla, whereas hematogenic/systemic spread usually gives rise to metastasis in the bones, lungs, liver, or brain. Tumor cells need to survive in the circulation until they can arrest in a new organ; here, they might extravasate from the circulation into the surrounding tissue. Once in the new site, cells must initiate and maintain growth to form pre-angiogenic micrometastases; this growth must be sustained by the development of new blood vessels in order for a macroscopic tumor to form [Iiizumi et al., 2008].

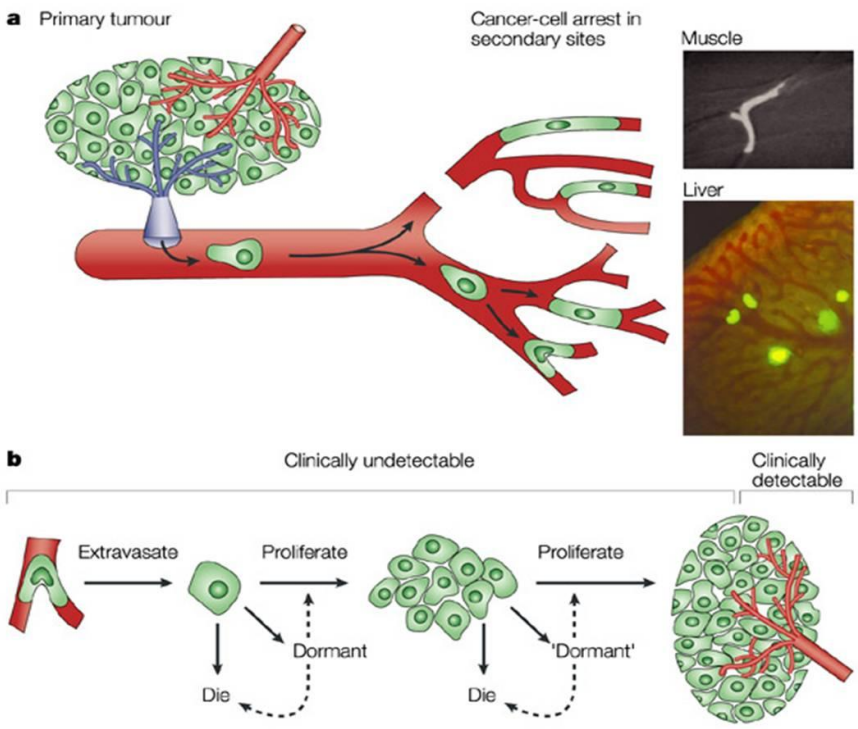

Figure 4. Schematic representation of the hematogenous metastasis
A) Escape of cancer cells from a primary tumor, and arrest in secondary sites. Cells that are able to escape from a primary tumor into the blood circulation are then carried by the flow to secondary sites, where they are arrested by size restriction in small capillaries in the new organ. Examples shown are cells arrested in muscle and in liver.
B) Possible fates of cancer cells in a secondary site, following the arrival of circulating cancer cells in an organ. Cancer cells can exist in a secondary site as solitary cells, small pre-angiogenic metastases or larger vascularized metastases. At each step, only a subset will proceed, and the remainder of cells might either go into a state of dormancy, or die. Only a proportion of vascularized metastases are clinically detectable, and solitary cells and micrometastases are generally clinically undetectable.
Adapted from Carter et al., 1989.

2.2 Inefficiency of metastatic process

Metastasis is an inefficient process; it has been estimated that only about 0.01% of cancer cells that enter the circulation will eventually survive and give rise to micrometastases [Joyce and Pollard, 2009]. Large numbers of cancer cells might be detected in the blood in cancer patients, and yet very few of these develop into overt metastases.

Many *in vivo* studies [Cameron et al., 2000; Chambers et al., 2000, 2001; Luzzi et al., 1998; Varghese et al., 2002] have led to the conclusion that early steps in the hematogenous metastatic process, from the time that cancer cells enter the bloodstream until they extravasate into secondary organs, are completed efficiently. By contrast, subsequent steps in the metastatic process are completed inefficiently, with only a small subset of cancer cells in a secondary site initiating cell division to form micrometastases, and only a small

proportion of these micrometastases persisting to become vascularized and progressively growing macroscopic metastases. Regulation of growth of a subpopulation of cells that arrested in an organ was therefore responsible for the overall metastatic inefficiency. In all cases, the initial arrest of cells was very efficient, but the initiation and persistence of growth was much less efficient. Metastatic inefficiency seems to be due primarily to the regulation of cancer-cell growth in secondary sites.

2.3 Clinical features of breast cancer metastasis

Approximately 10–15% of patients with breast cancer has an aggressive disease and develops distant metastases within 3 years after the detection of the primary tumor. However, the manifestation of metastases at distant sites 10 years or more after the initial diagnosis is also not unusual [Hellman et al., 2000]. Approximately one-third of women with breast tumors that have not spread to the lymph nodes develop distant metastases, and about one-third of patients with breast tumors that have spread to the lymph nodes remain free of distant metastases 10 years after therapy [Hellman, 1994; Rosen et al., 1989]. Patients with breast cancer are therefore at risk of experiencing metastasis for their entire lifetime. The heterogeneous nature of breast cancer metastasis makes it difficult not only to define cure for this disease, but also to assess risk factors for metastasis. Although some of the morphologically distinct, special types of breast tumor, which represent 5–10% of all breast cancers, have certain favorable prognostic features; histological typing in general is only a weak prognostic marker of metastasis risk [Tavassoli et al., 2003]. Traditional pathologic factors, such as histologic type, histologic grade, LVI, tumor size, and axillary lymph node status, together with hormone receptor status and HER2 status, represent the principal means for assessing prognosis and determining the likelihood of therapeutic response in patients with breast cancers [Carter et al., 1989; Elston et al., 1991; Koscielny et al., 1984; Page, 1991; Rosen et al., 1989]. Today, the traditional prognostic markers are able to confidently identify the group of approximately 30% of patients, who are most likely to have either a very favorable or a very poor outcome. For the remaining 70% of patients, of whom approximately 30% will still develop metastases, new prognostic markers are needed to help identify low-risk and high-risk groups, to pinpoint those patients who are most likely to benefit from systemic adjuvant treatment.

3. FOXP3

FOXP3 is a gene involved in immune system responses and it functions as the master regulator in the development and function of regulatory T cells (Tregs) [Fontenot et al., 2003, Hori and Sakaguchi, 2004, Khattri et al., 2001; Sakaguchi et al., 2008]. Treg cells are the main effectors the body uses to fight autoimmunity by suppressing auto-reactive lymphocytes which escape negative selection in the thymus. Tregs were initially defined as immunosuppressive CD4+ T cells expressing constitutively the subunit of the interleukin 2 receptor (CD25) on their surface ($CD4^{+}CD25^{high}$ $FOXP3^{+}$ cells) [Sakaguchi, 2005, Setoguchi et al., 2005]. The nuclear expression of FOXP3 is now considered as the most specific marker for these cells, which is essential for development of immune tolerance [Sakaguchi, 2005, Setoguchi et al., 2005]. However, it should be emphasized that not all FOXP3 positive T cells are necessarily Treg cells and their activity may depend on the level of FOXP3 expression and on FOXP3 isoforms expressed [Liu et al., 2006].

The IL-7 receptor CD127 is another excellent marker of Tregs cells in human peripheral blood. This cell surface marker is expressed at low levels on the majority of Tregs and distinguishes up to 10% of CD4+ T cells as potential Treg cells. FOXP3 expression in this T cell subset controls CD127 expression: the more FOXP3 expression, the less CD127. $CD4+CD25+CD127^{low}$ FOXP3 positive cells are highly suppressive [Liu W et al., 2006].

Alterations in numbers of Tregs expressing FOXP3 have been found in various pathological conditions. Loss of FOXP3 functions in mice and human leads to Tregs deficiency, resulting in lethal autoaggressive lymphoproliferation. On the contrary, the up-regulation of FOXP3 expression on T cells has led to marked reductions in autoimmune disease severity in models of diabetes, multiple sclerosis, asthma, inflammatory bowel disease, thyroiditis and renal disease in animal studies [Xia et al., 2007]. High levels of Tregs have been reported in peripheral blood, lymph nodes, tumor specimens, and ascites of patients with different types of cancer, suggesting a role for these cells in cancer progression. Tumor patients have a local relative excess of FOXP3 positive Tregs, which inhibits the body's self defence [Mizukami et al., 2008].

Several studies have recently described the expression of FOXP3 in non-hematopoietic derived cells, including normal epithelial cells and cancer cells of multiple lineages and of different tissue origins.

3.1 Forkhead proteins

Forkhead proteins are a large family of functionally different transcription factors that have been implicated in a variety of cellular processes [Kaufmann and Knochel, 1996]. All members of the forkhead family contain a characteristic DNA-binding forkhead-box (FKH) domain which acts as both a transcriptional activator and repressor of genes involved in embryonic development, speech and language development, and regulation of the immune system [Coffer and Burgering, 2004]. The FKH domain is highly conserved and consists of approximately 100 amino acids of helix–turn–helix class proteins which produce three alpha helices, beta strands and two loops, or "wings" [Kaestner et al., 2000]. Outside this conserved domain, forkhead proteins are different in sequence, structure, and function.

Nomenclature for the forkhead proteins

In the past twenty years, more than 100 members of the forkhead transcription-factor family have been identified. This led to a revision of the nomenclature used to identify these proteins. The term FOX was used to describe all chordate transcription factors containing the DNA-binding FKH domain. A phylogenetic analysis has resulted in the definition of 15 classes for all known FOX proteins, so these transcription factors are classified in terms of structure and not function. The term FOX is followed by a letter denoting one of the 15 subclasses (A to Q) agreed on by phylogenetic analysis, and finally, a number was used to define each member of the subclass [Kaestner et al., 2000]. Therefore, the actual name of any Fox protein is "Fox, subclass N, member X", or for example, FoxP3. The convention for naming human FKH proteins is that all letters are capitalised (eg. FOXP3) while, in mice, only the first letter is capitalized (Foxp3); the first letter and the subclass is capitalised for all other chordates (FoxP3) [Kaestner et al., 2000; Kim, 2007]. Italic script is used to describe the gene.

3.2 FOXP3 gene

The FOXP3 gene is well conserved in mammals [Ziegler and Buckner, 2006]. It is located on the X chromosome at Xp11.23 and it is submitted to X chromosome inactivation [Bennett et al., 2001; Wang et al., 2009]. The gene contains 11 coding exons (exons 1-11) and 3 non coding exons [Bennett et al., 2001]. The two 5' non coding exons are located significantly up-stream of the coding exons and are spliced into a common non coding exon [Floess et al., 2007; Kaur et al., 2010; Lal and Bromberg, 2009; Smith et al., 2006].

FOXP3 gene mutations

The FOXP3 gene was initially discovered in mice with the X-linked recessive immunodysregulation and severe autoimmunity now known as scurfy [Chatila et al., 2000]. An aminoacid insertion in exon 8 leads to the scurfy phenotype in mice [Bennett et al., 2001a]. FOXP3 was also found to be mutated in the human IPEX (Immunodysregulation Polyendocrinopathy Enteropathy X-linked) syndrome [Bennett et al., 2001a, 2001b; Bennett and Ochs, 2001]. Both scurfy and IPEX are considered fatal, often leading to death by overwhelming autoimmunity by 3 weeks (scurfy) or 2 years (IPEX). Consistent with these findings, FOXP3 has been sequenced in a large cohort of patients with phenotypic features of IPEX and numerous FOXP3 mutations were found [Lopez et al., 2006]. About 60% of IPEX patients have missense mutations in exons 9, 10, and 11 [Bennett et al., 2001a; Harbuz et al., 2010; Owen et al., 2003; Rubio-Cabezas et al., 2009; Torgerson et al., 2007]. In breast cancer patients, a total of 27 somatic mutations in all 11 coding exons and intron-exon boundary regions have been identified in 36% of 65 patients by PCR [Zuo et al., 2007a, 2007b]. Interestingly, the mutations are not randomly distributed in FOXP3 gene and the overwhelming majority of them are either in the functional domains or within intron 11 [Zuo et al., 2007a].

Polymorphisms of the FOXP3 gene, including single nucleotide polymorphisms (SNP) and microsatellite polymorphisms have been reported in patients with autoimmune diseases or cancers [Lin et al., 2011]. Reported polymorphisms include SNPs in the promoter region, in the intron regions, and downstream of the coding regions, and microsatellite polymorphism in the promoter region, and in the intron region [Lan et al., 2010]. While most of the polymorphisms seem to be clinically irrelevant, a few alleles show weak clinical correlation with autoimmune diseases [Inoue et al., 2010; Wang et al., 2010].

3.3 FOXP3 protein

FOXP3 gene encodes FOXP3 protein, which is 431-amino acid long. FOXP3 molecular weight is 47 KDa. It contains four potential functional domains named repressor, zinc finger, leucine zipper and FKH domains (figure 5).

Repressor domain appears to be the main region responsible for the repression of target genes. Many studies showed that mutations in this domain were responsible for a significantly increased transcriptional activity of the protein [Nair et al., 2013].

The zinc finger domain is located centrally in the protein. To date, no role has been clearly defined for this region.

Leucine zipper structures are involved in protein-protein interactions, often leading to homo- and hetero-oligomerization. Many FKH proteins have been found to homo- or hetero-oligomerize, including FOXP3. It has been reported that FOXP3 self-associates and can complex with FOXP1, and that the ability to form these interactions is elemental for normal protein function [Li et al., 2007]. IPEX patients with deletions in this region suffer from severe disease due to the inability of FOXP3 to self-associate, and/or associate with other forkhead family members such as FOXP1 [Li et al., 2007].

The FKH domain, whose structure has been previously described, is critical for both DNA binding and FOXP3 nuclear localization [Ziegler, 2006]. FOXP3 interacts with DNA at consensus sequences found within the promoters of several genes [Schubert et al., 2001]. Reporter and chromatin immunoprecipitation assays indicated that FOXP3 binds such sequences in the promoters of IL-2, CD25, CTLA-4 and CD127 genes [Wu et al., 2006]. The value of the FKH region should not be underestimated, as a protein lacking this region failed to translocate to the nucleus thereby preventing interaction with DNA.

FOXP3 protein has a relatively short half-life of approximately 21 min as it undergoes rapid polyubiquitination and proteosomal degradation [Lee et al., 2008].

FOXP3 protein isoforms

The FOXP3 protein is highly conserved [Lal et al., 2009; Sadlon et al., 2010; Zheng et al., 2010]. To understand FOXP3 function it is critical to realize that, in contrast with mouse Tregs in which FOXP3 is only expressed as a full-length protein, human Treg cells express both full/length protein and three splice variants [Aarts-Riemens et al., 2008; Allan et al., 2005; Kaur et al., 2010; Smith et al., 2006; Ziegler, 2006] (figure 5).

The longest form (wild-type FOXP3, WTFOXP3) resembles the murine full-length FOXP3.

The main deletional isoform (Δ2 isoform, Δ2FOXP3) lacks exon 2 (aa 71–105), which is part of the repressor domain in the FOXP3 protein. It has been proposed that Δ2FOXP3 acts as a dominant negative isoform [Li et al., 2007; Xu et al., 2010].

Both WTFOXP3 and Δ2FOXP3 are expressed in approximately equal proportion in human Tregs [Yagi et al., 2004].

Another splice variant of FOXP3, called Δ7FOXP3, has been identified in *ex vivo* CD4+CD25+ T cells and CD8+ regulatory T cell clones [Kaur et al., 2010]. Δ7FOXP3 lacks the 81 bp region

that encodes exon 7 of FOXP3, which is a part of the leucine zipper domain of the protein. Since this motif is commonly used as structural dimerization element, mutations in exon 7 have been linked to IPEX syndrome, suggested to be caused by impaired dimerization of the FOXP3 protein. The absence of exon 7 abrogates the suppressive function of Tregs [Kaur et al., 2010].

Human Tregs can also express Δ2Δ7FOXP3 isoform that lacks both exon 2 and exon 7 (aa 245–272) [Mailer et al., 2009]. This isoform is expressed at relatively low-copy number in naturally occurring Tregs. Δ2Δ7FOXP3 could play a role in regulating the function of the other FOXP3 isoforms and may be involved in cancer pathogenesis, as it is overexpressed by certain malignant cells [Kaur et al., 2010; Mailer et al., 2009].

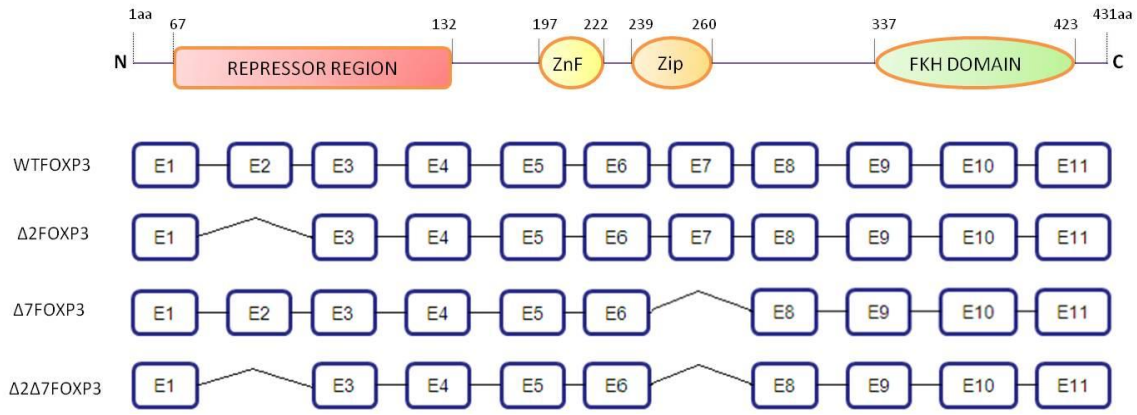

Figure 5. FOXP3 structure and splice variant forms in regulatory T cells
Schematic structure of FOXP3 protein and exon organization in full length (FL) and in FOXP3 splice variants described in Tregs. ZnF, zinc finger domain; Zip, leucine zipper domain.
Adapted from Triulzi et al., 2012.

3.4 FOXP3 subcellular localization

FOXP3 protein is synthesized in the cytoplasm of cells and then actively transported to the nucleus. As a transcription factor, the ability to transport to the nucleus is a pre-requisite for the effective functioning of FOXP3; it has been demonstrated that FOXP3 nuclear localization is required for transcriptional repression [Lopes et al., 2006]. FOXP3 is expressed constitutively within the nucleus of Tregs and of those normal FOXP3-expressing epithelial cells [Sakaguchi et al., 2005]. Despite this, few reports have comprehensively studied factors involved in the transportation of human FOXP3 from the cytoplasm to the nucleus. Due to the size limitation of nuclear pores, proteins above ≈40 KDa of size use an active transport machinery in the presence of specific transport signals to pass the nuclear envelope in either direction [Nigg et al. 1997].

FOXP3 is localized in different subcellular compartments in conventional human CD4+ T cells and Treg cells [Magg et al., 2012]. The nuclear-cytoplasmic shuttling of FOXP3 can be attributed to a nuclear localization signal (NLS) [Lopes et al., 2006] and to two newly identified nuclear export sequences (NESs) [Magg et al., 2012].

A C-terminal fragment of FOXP3 containing the entire DNA binding region FKH with short flanking sequences at each end was found to be both necessary and sufficient for import of FOXP3 to the nucleus. This domain appears to act as the primary targeting sequence [Lopes et al., 2006]. Mutation of two basic amino acids to acidic amino acids (K415/416E) within this NLS (414RKKR417) near the C-terminal end of the FKH domain abrogates nuclear import of FOXP3 [Lopes et al., 2006].

In human FOXP3 two separate leucine-rich regions (NES1 and NES2) have been identified, which match with the consensus export signal, previously reported to be present in many nucleo-cytoplasmic shuttling proteins [Henderson et al., 2000]. The first NES (NES1) lies in the exon 1-2 boundary zone while the second NES (NES2) localizes within the leucine-zipper domain and is encoded by exons 6 and 7. NES1 and NES2 are both affected by alternative splicing of exons 2 and 7, respectively [Smith et al., 2006; Walker et al., 2003]. In theory, both NESs could have a differential impact on subcellular distribution of the alternatively spliced human FOXP3 isoforms. Disruption of both export sequences almost entirely prevented nucleo-cytoplasmic redistribution.

These results show that the alternative FOXP3 isoforms localize differently in primary human T cells according to the presence or absence of the respective nuclear export signals. While WTFOXP3 rapidly shuttled from the nucleus to the cytoplasm, the isoforms Δ2FOXP3 and Δ7FOXP3, lacking either of the two NESs translocated to the cytoplasm with slower kinetics. The isoform Δ2Δ7FOXP3, lacking both export sequences, localized to the nucleus [Magg et al., 2012].

4. FOXP3 and Cancer

4.1 Regulatory T cells and cancer

Regulatory T cells, which represent about 5% of circulating CD4+ T lymphocytes in the human peripheral blood, comprise several functionally distinct cell subsets [Duhen et al., 2012]. Tregs are responsible for maintaining immune responses in balance and preventing excessive and dangerous immune reactivity. Tregs are divided into natural Tregs, which are thymically derived T cells, and inducible Tregs, which upregulate FOXP3 expression and are derived in the periphery from naive CD4+ T-cell precursors under tolerogenic conditions. Tregs are highly enriched in the tumor microenvironment and are well known for their roles in tumor progression. In fact, they are considered to be important for limiting antitumor immune responses resulting in peripheral tolerance of cancer cells. The mechanisms of immune regulation mediated by Tregs are: secretion of soluble or membrane-tethered immunosuppressive molecules, direct cytolytic activity, metabolic disruption, and suppression of dendritic cells. Recently, the role of Tregs beyond immune suppression in tumors has been investigated; Tregs are directly involved in promoting angiogenic reprogramming of the tumor microenvironment [Facciabene et al., 2011], highlighting a multifaceted role for Tregs in promoting cancer through tumor immune escape and angiogenesis.

An increased number of Tregs has been shown in a multitude of cancers, including melanoma, ovarian, breast, colorectal, lung, and pancreatic cancers [Zou, 2006]. Regulatory CD4+CD25+ T cells were increased in tumor sites in non–small cell lung (NSCLC) and ovarian cancers, and these cells secreted large amounts of TGF-b that inhibited CD8+ effector T-cell *in vitro* functions [Woo et al., 2001]. In a study on ovarian cancer patients, Tregs that were isolated from the tumor site, ascites, or peripheral blood were equally able to suppress tumor-antigen–specific immune responses, suggesting that Tregs contribute to the promotion of ovarian cancer, likely due to their enhanced recruitment or local expansion rather than an enhanced suppressive capacity acquired in the tumor microenvironment [Curiel et al., 2004].

In breast cancers the percentage of Treg cells increases in parallel with the disease stage, from normal to DCIS and from DCIS to invasive carcinoma [Bates et al., 2006]. In patients with invasive carcinoma the presence of high numbers of FOXP3 positive T cells predicts worse relapse-free survival and decreased overall patient survival [Bates et al., 2006], and

may indicate that the presence of Treg cells promotes tumour progression by inhibiting immunosuppression.

FOXP3 expression in Treg cells may result in impaired immunological function with maintenance of low-level chronic inflammation. Chronic inflammation may predispose to cancer. Increased and sustained inflammation may provide a mechanism for involvement of FOXP3 in cancer development. Mutation of an X-linked cancer suppressor gene, such as FOXP3, in females may result in its mosaic expression. In Tregs, the mosaic FOXP3 expression produces an elevated expression of inflammatory cytokines, sustaining inflammation [Medema and Burgering, 2007].

Increased numbers of Tregs in tumors have been associated with poor survival in many solid tumors, including breast [Bates et al., 2006], gastric [Sasada et al., 2003], and ovarian cancers [Sato et al., 2005].

As regards breast cancer, it has been observed that poor prognostic factors (negative hormonal receptor status, high tumor grade, and nodal involvement) were associated with a significantly higher number of CD3, CD8, and FOXP3 expressing cells in the infiltrate [Ladoire et al., 2008]. Chemotherapy resulted in a decrease in FOXP3 positive cells in the infiltrate, whereas the level of CD8 and CD3 infiltrate remained unchanged [Ladoire et al., 2008]. Pathologic complete response to chemotherapy was found to be associated with an absence of immunosuppressive FOXP3 cells and the presence of a high number of CD8+ T cells and cytotoxic cells in cancer infiltrate. Since Tregs are potential inhibitors of anti-tumor response, the infiltration by FOXP3+ Tregs may be associated with increased relapse and shorter survival of patients with both *in situ* and invasive breast cancer [Generali et al., 2009].

Recently, a new explanation for the association of CD4+ T-cell and Treg cell markers with a more aggressive behaviour in advanced breast cancers has been proposed, by demonstrating that tumour-infiltrating CD4+CD25+FOXP3+ Treg cells are a major source of RANKL, which stimulates the metastatic progression of RANK-expressing breast carcinoma cells [Tan et al., 2010]. The pro-metastatic function of T cells can be replaced by exogenous RANKL [Tan et al., 2010].

Thus, since Treg cells play an important role in the establishment of aggressive tumor phenotypes, targeting these cells is a promising approach for cancer immunotherapy. Such approaches could include local depletion of Tregs in the tumor mass and attenuation of Tregs function [Sakaguchi et al., 2008].

4.2 FOXP3 expression in normal and malignant cells

The transcription factor FOXP3 is well known marker of CD4+CD25+ regulatory T cells. Recently, FOXP3 expression has been described in normal cells and in non-hematopoietic-derived cancer cells, suggesting that FOXP3 exerts a broader function than that on Tregs. FOXP3 expression has been demonstrated in different histological types of cancer (breast, melanoma, urinary bladder, tongue, gastric, esophageal, pancreas, colorectal, stomach, thyroid, glioma and NSCLC).

Several studies have examined FOXP3 expression in normal and malignant breast epithelial cells: there are opposing reports indicating that its expression in breast cancer decreased [Zuo et al., 2007a, 2007b], increased [Gupta et al., 2007; Merlo et al., 2009; Ohara et al., 2009; Won et al., 2013], or not changed in breast cancer compared to the normal epithelium [Zuo et al., 2007a, 2007b].

Zuo and colleagues [Zuo et al., 2007b] analyzed FOXP3 expression in human breast cancer patient samples and reported that 21% of 275 analyzed samples expressed FOXP3 within tumor cells, whereas FOXP3 expression was found in 80% of the normal cells. In this study only nuclear positivity for FOXP3 was scored as a positive result. Similarly, considering nuclear FOX3 staining in a large series of breast cancers, FOXP3 expression was only found in 16 out of 1547 samples (1%) [Droeser et al., 2013].

Different results were obtained by our group by evaluating FOX3 expression in 397 primary breast cancer specimens from Milan 3 and Milan 1 trials. FOXP3 stained positive in the majority of the breast cancer tissue examined (57% and 73% in the Milan 3 and 1 trials, respectively). Both cytoplasmic and nuclear staining was scored as a positive result. Always considering both cytoplasmic and nuclear staining, positive tumoral FOXP3 expression has been observed in 38.6% (105/272) of breast carcinomas, while normal breast ducts and acini were negative for FOXP3 expression [Won et al., 2013].

Quantitation of FOXP3 transcripts has been performed in breast carcinoma samples and in their adjacent normal tissue. According to FOXP3 protein expression in breast cancer specimens, the relative FOXP3 mRNA amount in breast carcinoma was significantly upregulated when compared to normal breast tissue expression [Gupta et al., 2007; Ohara et al., 2009].

Studies performed on other tissues have produced data showing a distribution of FOXP3 expression restricted only to cancer cells. For example, there was no detectable expression of FOXP3 in normal duct cells of the pancreas whereas FOXP3 was readily detectable in

pancreatic cancer cells [Hinz et al., 2007]. FOXP3 is widely expressed in human melanoma cells and cell lines but not in normal human melanocytes [Ebert et al., 2008]. FOXP3 over-expression has been also demonstrated in esophageal cancer cells, while normal esophageal mucosal cells did not express FOXP3 [Wang et al., 2012]. No FOXP3 expression was observed in the cells of normal brain tissue, whereas FOXP3 protein expression has been detected in 40 human glioma samples [Wang et al., 2013]. The positive rate of FOXP3 expression in non-small cell lung cancer (54.7%) was significantly higher than that of normal lung tissue [Fu et al., 2013].

In contrast to these findings, Wang and colleagues found that FOXP3 was expressed in 100% of epithelial cells in healthy prostate samples, while 68.5% of prostate cancer samples failed to express detectable levels of FOXP3. Furthermore, in samples with clearly identifiable prostate epithelial neoplasia, there was a significant reduction in FOXP3 mRNA levels [Wang et al., 2009]. Similar findings have been reported in samples of ovarian cancer where FOXP3 protein was clearly identifiable in normal ovarian epithelial samples, but weak or no expression was found in malignant samples [Zhang and Sun, 2010].

4.3 FOXP3 protein isoforms in cancer cells

Besides full-length FOXP3 expression, some types of cancers predominantly express FOXP3 splice variants in addition to those occurring in non-transformed cells. In cutaneous melanomas, in some breast and ovarian cancers, and malignant T cells of Sezary syndrome, specific splice variants of the FOXP3, such as Δ2FOXP3, Δ2Δ3FOXP3, Δ2Δ7FOXP3 and Δ8, were reported to be preferably expressed [Kaur et al., 2010; Smith et al., 2006]. The Δ2Δ3FOXP3 splice variant results in a truncated FOXP3 with a premature stop codon, and therefore might contribute to the malignant progression of cells [Wang et al., 2009].

4.4 Genes involved in carcinogenesis regulated by FOXP3

In vitro studies indicate an important role of FOXP3 in controlling oncogenic factors in epithelial cells by regulating the expression of a number of genes implicated in cancer, including both tumor suppressor and oncogenes. In particular, FOXP3 was reported to inhibit breast tumor growth through directly repressing the transcription activity of HER2, SKP2, and c-MYC while inducing the transcription activity of LATS2 and p21 tumor suppressor genes (figure 6).

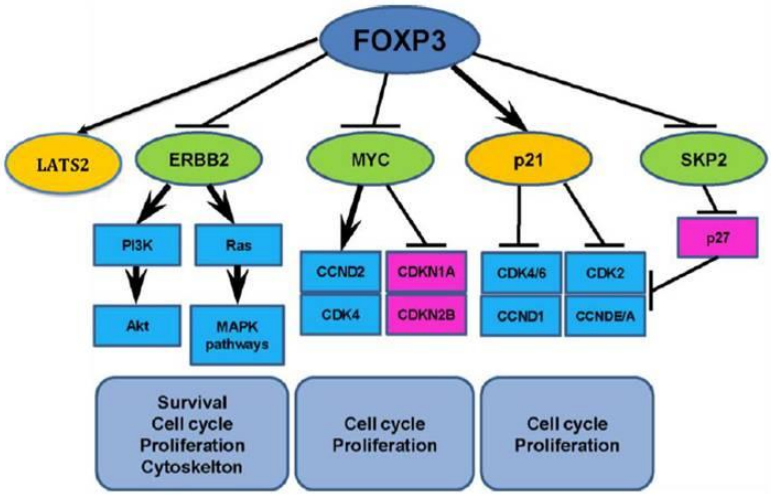

Figure 6. Signaling pathways of FOXP3 in epithelial cells
FOXP3 suppresses breast cancer and prostate cancer growth by inducing tumor-suppressor genes and repressing oncogenes. The genes are direct targets for FOXP3 and their regulation is essential for growth inhibition by FOXP3.
Adapted from Katoh et al., 2010.

- **ErbB2/HER2**

FOXP3 can repress HER2 transcription in human breast cancers by directly binding to the ErbB2 gene promoter [Zuo et al., 2007b]. Loss of nuclear FOXP3 contributes to HER2 overexpression in breast cancer [Mahmoud et al., 2010; Zuo et al., 2007b]. Analysis of malignant breast cancer cell lines with significantly reduced levels of FOXP3 revealed an overexpression of HER2 transcripts in breast cancer cells compared to normal epithelial cells. Finally, when WTFOXP3 was silenced using siRNA in normal mammary epithelial cells, a 7-fold increase was observed in ErbB2 mRNA and cell-surface expression of HER2. These data suggest that FOXP3 can influence mammary carcinogenesis [Zuo et al., 2007b].

- **SKP2**

S-phase kinase-associated protein 2 (SKP2) has been reported in a wide variety of cancers [Nakayama and Nakayama, 2006] and it is overexpressed in nearly 50 % of breast cancers [Sonoda et al., 2006]. Such cancers have a poorer prognosis than those not overexpressing SKP2 [Radke et al., 2005; Signoretti et al., 2002]. SKP2 is expressed during S and G2 phases of the cell cycle and regulates p27 degradation, thus facilitating cell cycle progression. The expression of SKP2 and FOXP3 has been investigated in malignant human breast tissues. It was found that 56% of FOXP3 negative samples overexpressed SKP2. These findings suggest that FOXP3 is able to reduce SKP2 expression. FOXP3 directly represses SKP2 expression by

binding to specific regions within the SKP2 gene, and a deletion in either binding site results in an increased level of SKP2 expression [Zuo et al., 2007a].

- **C-Myc**

C-Myc is a sequence-specific transcription factor and an important player in various cellular processes including cell cycle and apoptosis. It is overexpressed in more than 30% of all human cancers and in 80% of prostate cancers [Grandori et al., 2000]. Overexpression of c-Myc contributes to more aggressive and poorly differentiated cancer phenotypes. FOXP3 is able to repress the expression of c-Myc [Wang et al., 2009]. A clear correlation between FOXP3 and c-Myc was demonstrated by knocking down FOXP3 expression in human prostate cells leading to an increase in both c-Myc transcripts and protein and, consequently, an increased rate of proliferation. Corresponding to this, when FOXP3 was transfected into human prostate cancer cell lines, the expression of c-Myc was almost completely abrogated [Wang et al., 2009].

- **LATS2**

LATS2 is an enzyme of the Hippo-pathway [Li et al., 2011]. This pathway largely contributes to regulating cell cycle, cell proliferation and apoptosis by suppressing the expression of oncogenic YAP. LATS2 is significantly downregulated in breast [Takahashi et al., 2005], prostate [Powzaniuk et al., 2004], and brain [Jiang et al., 2006] cancers. FOXP3 is a direct transcriptional activator of LATS2 in epithelial cells of the prostate and breast where mutations in FOXP3 often result in decreased levels of LATS2 [Li et al., 2011]. When WTFOXP3 is transfected into MCF-7 breast cancer cells, there was a significant increase in LATS2 expression.

- **p21**

p21 is a protein encoded by the CDKN1A gene. p21 causes cell cycle arrest in the G1 phase and, therefore, is important in cell cycle progression [Xiong et al., 1993]. The expression of p21 has been implicated in many forms of cancer, particularly breast cancers [Pinto et al., 2005]. FOXP3 can upregulate p21 expression [Liu et al., 2009]. A specific FOXP3 binding site in the intron 1 is essential for p21 induction by FOXP3. FOXP3 binding to intron 1 of CDKN1A gene increases H3 histone acetylation. This acetylation reduces histone deacetylases HDAC2- and HDAC4-binding affinity increasing the expression of CDKN1A gene [Liu et al., 2009]. When FOXP3 was knocked down in MCF-7 breast cancer cells and in human mammary epithelial cells, there was a decrease in the expression of p21 transcripts and protein [Liu et

al., 2009]. Furthermore, when human breast cancer samples were assessed for FOXP3 and p21 expression, only 30% of FOXP3 negative cases expressed p21 [Liu et al., 2009].

Together, all these data from *in vitro* studies increasingly point to the critical role of FOXP3 as a tumor suppressor in at least breast and prostate carcinoma cellular models (table 1) [Triulzi et al., 2013].

Cancer cell model	**Histotype**	**FOXP3 target**	**FOXP3-mediated biological effect**	**Ref**
Mm-TSA_FOXP3 Hs-MCF7_FOXP3 Hs-SKBR3_FOXP3	Breast	*HER2 repression	Growth inhibition	Zuo et al (2007b)
Mm-TSA_FOXP3 Hs-MCF7_FOXP3	Breast	*SKP2 repression	Growth inhibition	Zuo et al (2007a)
Hs-MCF7_FOXP3	Breast	*p21 induction	Growth inhibition	Liu et al (2009)
Mm-TSA_FOXP3 Hs-MCF7_FOXP3	Breast	*LATS2 induction	Growth inhibition	Li et al (2011)
Hs-BT459_FOXP3 Hs-MDA-MB-231_FOXP3	Breast	*SATB1 repression	N.A.	McInnes et al (2011)
Hs-PC3_FOXP3 Hs-DU145_FOXP3	Prostate	*c-MYC repression	Growth inhibition	Wang et al (2009)
Hs-SKOV3_FOXP3	Ovary	MMP2 repression uPA repression	Growth, migration and invasion inhibition	Zhang et al (2010)

Table 1. FOXP3 ectopic expression *in vitro* models and biological effects
*Direct regulation demonstrated by CHIP or luciferase analysis. N.A.: Not Available; CFA: colony formation assay; WH: wound healing; MTA: Matrigel Transwell assay.

4.5 FOXP3 in mouse models

In order to analyze FOXP3 expression in different tissues in the absence of regulatory T cells, FOXP3 expression has been investigated in $Rag2^{-/-}$ mice, which lack T lymphocytes [Chen et al., 2008]. FOXP3 mRNA and protein were detected in mammary, bronchial, and prostate epithelial cells, but not in intestine, kidney, liver, or heart. No evidence of FOXP3 was found in epithelial cells of $Rag2^{-/-}$ scurfy ($FOXP3^{sf/sf}$) mice, used as negative control, which carry a naturally occurring mutation in FOXP3 that results in an early stop codon and missing FOXP3 mRNA and protein expression (table 2).

A role for FOXP3 as a tumor suppressor gene in human cancers has been suggested in a study of female mice heterozygous for the scurfy mutation of FOXP3 (BALB/c $FOXP3^{sf/+}$) and showing a high rate of cancer development [Zuo et al., 2007b]. About 60% of the tumors were mammary carcinomas. Normal epithelial cells transcribed mostly the wild-type FOXP3 allele, while the cancer cells transcribed the mutant allele, resulting in FOXP3 expression only in normal mammary epithelium (table 2).

In another mouse model, Wang et al. (2009) showed that mice carrying prostate-specific ablations of FOXP3 ($FOXP3^{flox/y}$; PB-$Cre4^{+}$) developed prostatic hyperplasia and prostatic intraepithelial neoplasms that are putative precancerous prostate lesions, suggesting a direct link between the lack of FOXP3 and malignant transformation (table 2).

These results suggest that loss of FOXP3 in mammary and prostatic epithelial tissues leads to tumor formation.

Mouse model	FOXP3-mediated features	Ref
BALB/c, $Rag2^{-/-}$ and $Rag2^{-/-}$ $FOXP3^{sf/sf}$	Specific FOXP3 expression in mammary, bronchial and prostate epithelial cells in $Rag2^{-/-}$ mice.	Chen et al (2008)
BALB/c $FOXP3^{sf/+}$	Development of mammary carcinomas, lymphomas, hepatomas and sarcomas. FOXP3 expression only in normal epithelial cells.	Zuo et al (2007b)
$FOXP3^{fl/y}$; PB-$Cre4^{+}$ (FOXP3 prostatic specific ablation)	Development of prostatic hyperplasia and intraepithelial neoplasm.	Wang et al (2009)

Table 2. FOXP3 expression and correlation with tumor onset in three mouse models
sf: scurfy; fl: flox

4.6 FOXP3 expression correlates with prognosis in human cancer

In sharp contrast to a putative onco-suppressor role for FOXP3, many recent studies on FOXP3 expression in different histological types of cancer have correlated FOXP3 expression in tumor cell with poor prognosis (table 3).

Merlo and colleagues demonstrated for the first time a significantly increased expression of FOXP3 by breast cancer cells. Immunohistochemical (IHC) and statistical analyses of archival material from two series of breast cancer patients (Milano 1 and Milano 3 trials) indicated that the expression of FOXP3 in tumors was inversely associated with patient survival and the risk increased with increasing FOXP3 staining intensity (log-rank $p<0.0001$; figure 7A).

Comparison of overall survival of Milan 3 trial patients whose tumors showed positive immunoreactivity with anti-FOXP3 antibody (weak and strong; n=105) versus patients with tumors showing no FOXP3 staining (n=78) revealed a striking univariate association between these parameters ($p<0.0001$; figure 7B). FOXP3 was also a strong prognostic factor for distant metastasis-free survival ($p=0.0001$; figure 7C), but not for local recurrence incidence risk (figure 7D).

Moreover, multivariate analysis revealed a similar hazard ratio for FOXP3 expression and lymph node positivity: the 10-year survival probability of node-negative/FOXP3-negative patients was 100%; node-negative/FOXP3-positive patients had survival rates similar to those of node-positive/FOXP3-negative patients (82%); and when both markers were positive, the survival probability was around 40% [Merlo et al., 2009]. To note, in most breast carcinomas, FOXP3 staining was localized predominantly in the cytoplasm, although both cytoplasmic and nuclear staining was present in some specimens and a few showed only nuclear staining.

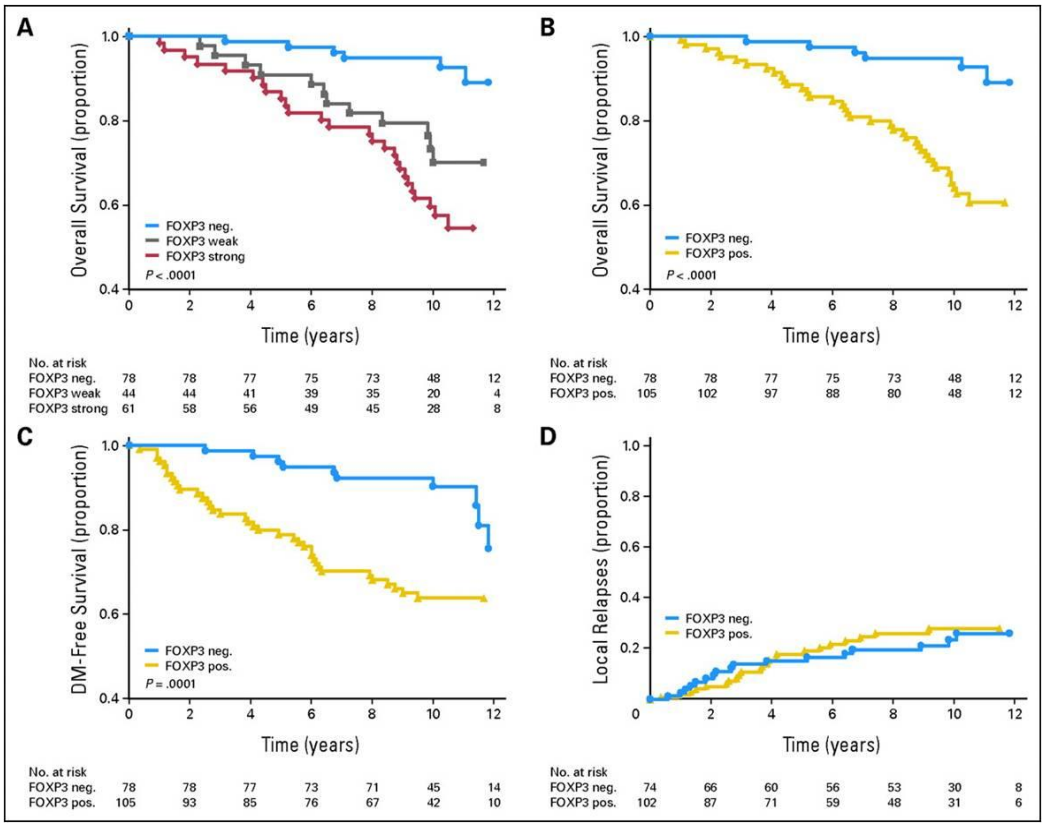

Figure 7. FOXP3 prognostic significance in breast cancer Milan 3 trial
(A) Association between FOXP3 strong, weak and negative staining intensity with overall survival. Association between FOXP3 positive and negative staining (B) with overall survival, (C) distant metastasis (DM)-free survival, (D) and local recurrence incidence. *P* values were calculated with use of the log-rank test.

Recently, high mRNA FOXP3 levels were demonstrated to significantly associate with higher risk of recurrence in TNBC and in ER-positive/HER2-negative breast cancer subgroups, whereas no differences were found in HER2-positive tumors expressing or not FOXP3 [Nair et al., 2013].

In another recent study FOXP3 expression has been also evaluated in 183 breast cancer patients using IHC assay of tissue microarray. Out of all breast cancer samples, 132 tumors were scored as FOXP3 negative, 33 as weak positive, and 18 as strong positive. FOXP3 staining was localized in the cytoplasm only or both the cytoplasm and nucleus in all tumor specimens. Both FOXP3-weak-positive and strong-positive patients were associated with significantly higher nuclear grade, higher histologic grade and more negative ER status compared to FOXP3-negative patients. The proportion of TNBC is higher in FOXP3 positive patients than in FOXP3 negative patients. FOXP3-strong-positive patients had slightly poorer disease-free survival (DFS) and disease-specific survival (DSS) compared to FOXP3-negative or FOXP3-weak-positive patients, though the differences were not statistically significant.

However, in the node-positive subgroup, patients with strong FOXP3 expression have significantly shorter DFS and DSS, whereas FOXP3-negative and FOXP3-weak-positive patients showed similar favorable prognoses [Kim et al., 2013].

In another study positive tumoral FOXP3 expression was observed in 38% of breast carcinomas. FOXP3 positivity was significantly related to a higher histological grade, positive p53 expression, and higher Ki67 expression [Won et al., 2013].

FOXP3 expression has been associated to poorer survival when it was expressed in urinary bladder tumor cells. No significant differences were observed between the cytoplasmic, nuclear, and cytoplasmic/nuclear staining groups in terms of patient survival [Winerdal et al., 2011].

To determine whether FOXP3 expression in tongue squamous cell carcinoma (TSCC) cells could correlate with tumor progression, IHC analysis was done in TSCC samples. FOXP3 positivity in tumor cells was detected in 59% of cases, with heterogeneous subcellular staining ranging from cytoplasmic to nuclear. FOXP3 expression in TSCC cells was associated with pathologic differentiation ($p=0.04$) and T stage ($p=0.000$), while it was inversely associated with patient survival. Notably, it was not associated with local recurrence, confirming that FOXP3 might drive metastasis [Liang et al., 2011].

A study on non-small-cell lung cancer (NSCLC) patients revealed tumor cell FOXP3 expression in 31% of analyzed specimens [Tao et al., 2012]. While the mainly cytoplasmic expression of FOXP3 in lung cancer cells per se was not associated with either lymph node positivity or with survival in this study, another IHC analysis of NSCLC tissue [Dimitrakopoulos et al., 2011], in which the consistently nuclear FOXP3 staining was stronger in tumor cells than in adjacent normal bronchial epithelium, did find a correlation between FOXP3 positivity in cancer cells and lymph node metastases.

Recently, also Fu et al. (2013) detected FOXP3 immunostaining in NSCLC cells, which primarily exhibited diffuse staining in the cytoplasm alone, in both the nucleus and the cytoplasm, or the nucleus alone [Fu et al., 2013]. FOXP3 expression in patients with lymph node metastasis was significantly higher than in patients without lymph node metastasis, and FOXP3 percent positive rate rose with an increase in TNM staging. These results suggest that FOXP3 expression correlates with NSCLC metastasis and poor prognosis.

IHC staining of FOXP3 was performed to examine the association of FOXP3 expression with clinic-pathological features of 194 patients with gastric cancer who underwent surgical resection. FOXP3 expression was localized often (79.3%) in the nuclei of signet ring cell

carcinoma tissues [Yoshii et al., 2012]. Few FOXP3-positive tumor cells were detected in poorly differentiated adenocarcinomas, and none were detected in well and moderately differentiated adenocarcinomas. FOXP3 expression was significantly associated with node involvement and TNM stage IV. The 3-year survival rate of patients with positive expression of FOXP3 in tumor tissue was 74.3% whereas in patients whose tumors did not express FOXP3 was 86.5%, indicating a potential association of FOXP3 with poor prognosis. In this study it was demonstrated that signet ring cell carcinoma cells might have a Treg-like activity, which would allow them to escape from immune surveillance, thereby resulting in cancer progression such as lymph node metastasis [Yoshii et al., 2012].

The frequency of FOXP3 positive cancer cells in primary tumors correlated with the incidence of lymph node metastases in gastric tumor specimens [Wang et al., 2010]. In these samples FOXP3 staining was detected in the nucleus of peritumoral epithelial cells and in the nucleus/cytoplasm of some gastric cancer cells.

A correlation between FOXP3 expression and lymph node metastases incidence was also reported for esophageal squamous carcinoma [Xue et al., 2010], where FOXP3 mRNA and protein expression was not only higher in tumors than in normal mucosa, but also higher in advanced stages than in early stages. FOXP3 expression was found in 48% of esophageal cancer tissues. This overexpression had a significant correlation between tumor staging and lymph node metastasis. The FOXP3 negative group showed significantly better overall survival than the overexpressing group (32.3% vs. 13.8%, $p=0.001$). Cox regression analysis showed that tumor stage and FOXP3 protein expression were independent prognostic risk factors [Wang et al., 2012].

IHC detection of FOXP3 expression in human melanoma cells [Quaglino et al., 2011] revealed that FOXP3 expression was significantly associated with visceral spread after treatment; in fact, samples from five of seven patients with visceral progression showed positive FOXP3 staining, while none of eight patients with lower or negative FOXP3 staining developed visceral metastases.

FOXP3 expression has been correlated with prognosis even in colorectal cancer (CRC) patients. Kim et al. (2013) provided for the first time evidence of a significantly increased tumor-related expression of FOXP3 in CRC cells. FOXP3 positive cancer cells were detected in 60 out of 65 CRC patients by IHC analysis. Among all patients, those with high FOXP3 expression levels in cancer cells had a poorer prognosis than those with low FOXP3 expression levels ($p<0.001$) [Kim et al., 2013].

In vitro, FOXP3 expression by tumor cells has been correlated with the inhibition of T-cell proliferation, indicating that cancer cells may share growth-suppressive effects with Tregs and that mimicking Tregs functions may represent a novel mechanism of immune evasion [Grimmig et al., 2013].

FOXP3 expression has been detected also in glioma cancer cells. IHC analysis detected FOXP3 in 35 out of the 40 glioma patients and high levels of FOXP3 were observed in 26 out of the 27 high-grade glioma samples. Statistical analysis suggested that the up-regulation of FOXP3 was significantly correlated with the histologic grade of glioma and that patients with high expression of FOXP3 protein exhibited a poorer prognosis than those with low FOXP3 expression [Wang et al., 2012].

All these data point to the association between FOXP3 expression in tumor cells and poor patient prognosis. Notably, FOXP3 has not been associated with local recurrence but only with a possible role in driving metastatic spread.

In contrast with these findings, Ladoire and colleague detected cytoplasmic FOXP3-expressing tumor cells by immunohistochemistry in 103 patients with primary invasive HER2-overexpressing breast treated with neo-adjuvant chemotherapy, with or without Trastuzumab. FOXP3 expression was associated with better relapse-free and overall survival and was independent of other clinic-pathological variables. This study raises the question of whether the putative suppressive role of FOXP3 in cancer cells depends on the oncogenic pathways involved in breast tumor cell growth; indeed, in the subgroup of Trastuzumab-treated patients, FOXP3 expression did not correlate with prognosis [Ladoire et al., 2011].

Cancer histotype	N° FOXP3 pos /Tot (%)	FOXP3 localization	Prognosis	Ref
Prostate	29/92 (31)	N	N.A.	Wang et al (2009)
Ovary	0/27 (0)	-	N.A.	Zhang et al (2010)
Pancreas	24/39 (61)	C and N	N.A.	Hinz et al (2007)
Breast	261/397 (66)	C and N	Poor (DM-FS)	Merlo et al (2009)
Breast, HER2+	59/113 (52)	C	Good (DFS and OS)	Ladoire et al (2011)
Breast	405/1097 (37)	C	Good (OS and LN)	Ladoire et al (2012)
Breast, TN	70/285 (25)	RNA	Poor (DFS)	Nair et al (2013)
HER2- ER+	267/832 (33)			
Breast (LN+)	51/183 (28)	C and N	Poor (DFS)	Kim et al (2013)
Breast	105/272 (39)	C and N	N.A.	Won et al (2013)
Urinary bladder	17/37 (46)	C and N	Poor (OS)	Winerdal et al (2011)
Tongue	48/81 (59)	C and N	Poor (OS)	Liang et al (2011)
NSCLC	22/44 (50)	N	Poor (LN metastases)	Dimitrakopoulos et al (2011)
NSCLC	27/87 (31)	C	No prognostic	Tao et al (2012)
NSCLC	29/53 (55)	C and N	Poor (LN)	Fu et al (2013)
Gastric	71/122 (58)	C and N	Poor (LN metastases)	Wang et al (2010)
Stomach	49/92 (60)	N	Poor (LN)	Yoshii et al (2012)
Esophageal	80/112 (71)	C and N	Poor (LN metastases)	Xue et al (2010)
Esophageal	29/60 (48)	-	Poor (OS and LN)	Wang et al (2012)
Melanoma	5/15 (33)	C	Poor (visceral metastases)	Quaglino et al (2011)
Colorectal	60/65 (92)	C	Poor (OS and LN)	Kim et al (2013)
Cervical	32/40 (80)	N	No prognostic (LN)	Zeng et al (2012)
Thyroid	244/266(90%)	C	N.A	Cunha et al (2012)
Glioma	35/40 (87)	N	Poor (OS)	Wang et al (2013)

Table 3. FOXP3 expression, subcellular localization, and prognosis in different human cancer histotypes.
C: cytoplasm; N: nucleus; N.A.: not available; DM-FS: distant metastasis-free survival; DSF: disease-free survival; OS: overall survival; LN: lymph node.

4.7 FOXP3 supports metastatic process: GSEA analysis

The lack of experimental evidences demonstrating a loss of tumor suppressor function due to FOXP3 mutated forms and their correlation with prognosis, leaves open the hypothesis of a possible pro-metastatic role of FOXP3. Consistent with this hypothesis GeneSet Enrichment Analysis (GSEA) performed at the National Cancer Institute of Milan [Triulzi et al., 2013] evidenced that the expression of several genes implicated in cell migration and metastasis was induced by FOXP3. In particular, an enrichment of molecules involved in the pathway of TGFβ, in epithelial to mesenchymal transition (EMT), and in focal adhesions was found. Moreover, an enrichment in cell cycle pathway-related molecules was found in FOXP3-negative cells, consistent with already published results which suggested a role of FOXP3 in inhibiting cancer cell proliferation.

The apparently discrepant role of FOXP3 in breast tumors might reflect the fact that metastatic potential and high proliferation rates are two different necessary, but not always coexistent, aspects of tumor progression, at least during the acquisition of invasive and migratory properties possibly driven by an EMT program leading to low proliferative potential. Thus, FOXP3, like other onco-suppressor proteins [Evdokimova et al., 2009], may play a positive role in tumor progression by reducing cell proliferation but thereby support the program of epithelial-mesenchymal transition and survival spread of disseminated metastatic cells [Triulzi et al., 2013].

Materials and Methods

1. Triple negative breast cancer specimens and patients

Tumor samples from 81 women with confirmed triple negative breast carcinomas were obtained in the form of formalin-fixed paraffin embedded (FFPE) blocks. All tumor specimens have been previously scored for breast cancer markers (ER, PR and HER2 status) and other characteristics.

All patients have received adiuvant chemotherapy.

Patients had agreed with the use of samples from their tumors for investigations.

2. Cell culture

Human breast cancer cell lines MDA-MB-231, HCC1937, MCF7, SKBr3, MDA-MB-468, BT474, MDA-MB-361 were purchased from ATCC (Rockville, MD). MDA-MB-231, MCF7 and SKBr3 cell lines were maintained in RPMI 1640 medium (Invitrogen). MDA-MB-468, BT474 and MDA-MB-361 cell lines were maintained in DMEM (Dulbecco's modified Eagle's medium); HCC1937 cell line was maintained in RPMI 1640 medium with 1 mM sodium pyruvate, 1% non essential amino acids and 10 mM Hepes. Each medium was supplemented with 10% fetal bovine serum (FBS) and 2 mM glutamine (both from Sigma-Aldrich). Cells were maintained at 37°C in a 5% CO_2 in air atmosphere.

Human Tet-Off MDA-MB-231 recipient cells were grown in RPMI 1640 medium containing 10% Tet system-approved FBS (Clontech) and 500 μg/ml G418 (Clontech). Human Tet-Off MDA-MB-231-WTFOXP3 and Tet-Off MDA-MB-231-Δ2FOXP3 cells were grown in RPMI 1640 medium containing 10% Tet system-approved FBS (Clontech), 300 μg/ml G418 (Clontech), 200 μg/ml hygromycin and 100 ng/ml doxycycline (Clontech).

3. Vectors

3.1 Generation of WTFOXP3-pcDNA3 and Δ2FOXP3-pcDNA3 vectors

The human full-length FOXP3 and Δ2FOXP3 cDNA was cloned into the expression vector pcDNA3.1 (Invitrogen Corp., Carlsbad, CA, USA) according to standard methodology. WTFOXP3 and Δ2FOXP3 cdna was amplified from two pCMV6-XL4-FOXP3 vectors (Origene, Rockville, MD) by PCR. For PCR amplification specific primers were used: FOXP3 5'-AAGGATCCATGGACTACAAGGACGACGACGACAAGCCCAACCCCAGGCCTGGC-3' (forward primer) and FOXP3 5'-AAGATATCTCAGGGGCCAGGTGTAGGG -3' (reverse primer).

Subsequently, PCR products and pcDNA3.1 empty vector were digested with EcoRV and BamHI enzymes (New England Biolabs, Ipswich, MA), and cDNA was ligated into pcDNA3.1 to produce the WTFOXP3-pcDNA3 and Δ2FOXP3-pcDNA3 expression vectors.

3.2 Tet-Off system

The first critical component of the Tet System is the regulatory protein, based on Tetracyclin repressor protein (TetR). In the Tet-Off System, this 37-kDa protein is a fusion of amino acids 1–207 of TetR and the C-terminal 127 amino acids of the Herpes simplex virus VP16 activation domain. Addition of the VP16 domain converts the TetR from a transcriptional repressor to a transcriptional activator, and the resulting hybrid protein is known as the tetracycline-controlled transactivator (tTA). tTA is encoded by the pTet-Off regulator plasmid, which also includes a neomycin-resistance gene to permit selection of stably transfected cells.

The second critical component is the response plasmid which expresses the gene of interest under control of the tetracycline-response element (TRE). TRE located just upstream of the CMV promoter. This CMV promoter lacks the strong enhancer elements normally associated with the CMV immediate early promoter. Because these enhancer elements are missing, there is extremely low background expression of the gene of interest from the TRE in the absence of binding by the TetR domain of tTA.

The ultimate goal in setting up a functional Tet System is creating a double stable Tet cell line which contains both the regulatory and response plasmids. When cells contain both the regulatory (pTet-Off) and the response (pTRE-Gene of interest) vectors, the gene of interest is only expressed upon binding of the tTA protein to the TRE. tTA binds the TRE and activates transcription in the absence of tetracycline (Tc) or doxycycline (DOXI). With the Tet-Off system, it is necessary to keep Tc or DOXI in the medium to maintain the native (off) state. Because Tc and DOXI have relatively short half-lives, Tc or DOXI was added to the medium at least every 48 hours to suppress the expression of the interest gene.

3.3 Generation of Tet-Off MDA-MB-231 recipient cell clones

MDA-MB-231 cells were stably transfected with pTet-Off regulator plasmid and selected in the presence of 500 μg/ml G418 in the culture medium. The resistant clones were assayed for luciferase activity.

3.4 Luciferase activity assay

To verify inducible system functionality Tet-Off MDA-MB-231 recipient cells were seeded on 48-well plates. When a cell confluence of 90% was achieved cells were transiently co-transfected with pTRE2hyg-Luc (0.2µg/well) plasmid (Clontech), which contains the *Firefly Luciferase* encoding gene, and with pRL-TK (Promega) (0.07µg/well) plasmid, which contains *Renilla Luciferase* gene, using Lipofectamine 2000 (Invitrogen). Cells were maintained in culture medium with or without doxycycline for 48 hours and then collected to performe luciferase activity assay. Firefly and Renilla luciferase activities were measured with Dual-Glo Luciferase Assay System Kit (Promega), according to the manufacturer's instructions. Relative luciferase activity was calculated as ratio between Firefly luciferase and Renilla luciferase activity. The Tet-Off MDA-MB-231 #23 clone with the lowest background and the highest induction of luciferase in response to doxycycline was selected.

3.5 Generation of inducible WTFOXP3-pTRE2hyg and Δ2FOXP3-pTRE2hyg vectors

The human full-length FOXP3 and Δ2FOXP3 cDNA was cloned into the expression vector pTRE2hyg (Clontech, Mountain View, CA) according to standard methodology. WTFOXP3 and Δ2FOXP3 cdna was amplified from two pCMV6-XL4-FOXP3 vectors (OriGene, Rockville, MD) by PCR. For PCR amplification specific primers were used: FOXP3 5'-GCTAGCATGGACTACAAGGACGACGACGACAAGCCCAACCCCAGGCCTGGC-3' (forward primer) and FOXP3 5'-AAGATATCTCAGGGGCCAGGTGTAGGG -3' (reverse primer).

Subsequently, PCR products and pTRE2hyg empty vector were digested with EcoRV and NheI enzymes (New England Biolabs, Ipswich, MA), and cDNA was ligated into pTRE2hyg to produce the expression vectors WTFOXP3-pTRE2hyg and Δ2FOXP3- pTRE2hyg.

3.6 Generation of inducible bulk population and single clones

For stable transfection Tet-Off MDA-MB-231 #23 cells were seeded on a 100mm dish. When cell confluence of approximately 90% was achieved cells were transfected with WTFOXP3-pTRE2hyg or Δ2FOXP3-pTRE2hyg plasmid using Lipofectamine 2000 (Invitrogen) according to modified manufacturer's instructions. To obtain bulk populations, 48 hours post-transfection either 300 µg/ml G418 and 200 µg/ml hygromycin (Sigma Aldrich) was added to the medium in the presence of 100 ng/ml doxycycline. After 16 days all antibiotic-resistant clones were pooled and analyzed for FOXP3 expression induction.

To generate single stable clones, 48 hours post-transfection cells were washed, trypsinized and divided onto three 10mm plates. Either 300 µg/ml G418 and 200 µg/ml hygromycin (Sigma Aldrich) was added to the medium in the presence of 100 ng/ml doxycycline. After 15 days, antibiotic-resistant single clones were selected and FOXP3 inducible expression was analyzed.

3.7 Analysis of inducible FOXP3 expression

In order to verify the modulation of FOXP3 expression by doxycycline, cells were grown in the selective medium containing or not 100 ng/ml doxycycline. After 48 hours cells were trypsinized and collected for Western blot analysis.

4. Immunohistochemistry

4.1 FOXP3 staining

FFPE tissue samples were deparaffinized and rehydrated. The endogenous peroxydase activity was blocked with 3% hydrogen peroxide in methanol for 30 minutes. Antigen retrieval was carried out by heating slides for 6 minutes at 95°C in 5 mM citrate acid buffer (pH 6.0). Non-specific binding was blocked with 1% human albumin for 30 minutes at room temperature. The slides were subsequently incubated overnight at 4°C with rat anti-human FOXP3 antibody, clone PCH101 (dilution 1:250; eBioscience, San Diego, CA). Subsequently, sections were washed in two changes of Phosphate Buffered Saline (PBS) solution for 5 minutes per wash, then covered in biotinylated rabbit anti-rat secondary antibody (dilution 1:200, DAKO) for 30 minutes and washed as before. Sections were covered with streptavidin (diluition 1:300; DAKO) for 30 minutes and washed. Color was developed by 5 minutes incubation with 3,3'-diaminobenzidine and sections were washed for 5 minutes in running tap water. Slides were counterstained with Mayer's hematoxylin for 1 minute, then dehydrated through a series of alcohol concentrations, cleared in xylene and mounted.

Expression of FOXP3 was evaluated independently by two pathologists both blinded to the clinic-pathologic data. Discrepancies between the two observers were reviewed jointly to reach consensus.

4.2 Vimentin staining

Vimentin staining on FFPE sections of lungs was performed using Vector Mouse on Mouse (M.O.M.^{TM}) Kits according to the manufacturer's instructions. Slides were incubated with

mouse anti-human vimentin antibody clone V9 (dilution 1:400; DAKO) for 1 hour at room temperature. Color was developed by 5 minutes incubation with 3,3'-diaminobenzidine and sections were washed for 5 minutes in running tap water. Slides were counterstained with Mayer's hematoxylin for 1 minute, then dehydrated through a series of alcohol concentrations, cleared in xylene and mounted.

5. PCR

5.1 RNA extraction

Total RNA was isolated from breast cancer cells using Trizol Reagent (Invitrogen) and from FFPE sections of human breast carcinomas using RNeasy Mini Kit (Qiagen Inc., Hilden, Germany) according to the manufacturer's instructions.

RNA was quantified by nanodrop (Biophotometer, Eppendorf, Hamburg, Germany) at 260 nm and 280 nm. The 260/280 ratio provides an estimate of nucleic acid purity. Pure preparations of RNA have OD 260/280 values of 1.8 and 2.0 respectively.

5.2 Reverse Transcription

First strand cDNA synthesis was performed using Superscript III RNase H Reverse Transcriptase. 5µg of total RNA was incubated at 65°C for 5 minutes with 1µl Oligo$(dT)_{20}$ (50µM) (Invitrogen Life Technologies, MD, USA), 1µl of 10 mM deoxynucleotide tri-phosphate Mix (Invitrogen Life Technologies, MD, USA), and distilled water was added to the volume of 13 µl. This mixture was then incubated on ice for at least 1 minute. Then 4µl of 5X First Strand Buffer, 1µl of 0.1M DTT, 1µl RNAseOUT (Recombinant RNAse Inhibitor 40U/µl) and 1 µl of Superscript III (200U/µl, Script™ III Reverse Transcriptase, Invitrogen Life Technologies, MD, USA) were added and mixed by pipetting. The mixture was reverse transcribed at 50-55°C for 50 minutes, before inactivating at 70°C for 15 minutes, prior to cooling.

5.3 Standard PCR

Standard PCR on cDNA from human breast cancer specimens was carried out using FOXP3 5'-GCCCTTGGACAAGGACCCGATG-3' (forward primer) and FOXP3 5'-CATTTGCCAGCAGTGGGTAGGA-3' (reverse primer). PCR involved 30 cycles of 95°C for 20 minutes, 62°C for 30 seconds, 72°C for 1 minute, followed by 10 minutes at 72°C prior to cooling.

A standard 20 µl reaction for a PCR was performed: 1 µl deoxynucleoside triphosphate solution, 2 µl of 10x PCR buffer concentrate, 0.5 µl of Taq polymerase enzyme, 1 µl (10 pmol), forward primer solution, 1 µl (10 pmol), reverse primer solution, 1 µl (50 mM) MgCl2, 2 µl (1 ng) template DNA, dH2O to make up to 20 µl. PCR products were resolved using 1% agarose gel electrophoresis.

5.4 Quantitative Real-Time PCR

Quantitative Real-Time PCR for FOXP3 was performed using ABI Prism 7900 Real-Time PCR instrument (Applied Biosystems). For amplification, 4.5 µL of cDNA were incubated with 10 µL of SYBR Green PCR Master Mix (Applied Biosystem), and with sense and reverse primers at a final concentration of 10 pmol/µL. The reaction mixture was brought up to a final volume of 20 µL with RNase-free distilled water. The human housekeeping gene GAPDH was used as endogenous control. Thermocycling conditions were: 50°C for 2 minutes; 95°C for 10 minutes; 95°C for 15 seconds and 60°C for 1 minute for 40 cycles.

Reaction was carried out using FOXP3 (forward primer) 5'-GCCCTTGGACAAGGACCCGATG-3' and FOXP3 (reverse primer) 5'-CATTTGCCAGCAGTGGGTAGGA-3', GAPDH (forward primer) 5'-CATGGCCTCCAAGGAGTAAG-3' and GAPDH (reverse primer) 5'-GACTGAGTGTGGCAGGGAC-3'. The amplification was carried out and analyzed using an ABI Prism 7900HT Sequence Detection System Software (Applied Biosystem). To rule out contamination from buffers and tubes, a negative control with water instead of the cDNA template was used on every plate. We used a relative quantification method (ΔΔCt method) to calculate the gene expression values as described (User Bulletin No. 2, Applied Biosystems). In brief, the amplification plot is the plot of fluorescence versus PCR number. The threshold cycle value (Ct) is the fractional PCR cycle number at which the fluorescent signal reached the detection threshold. Therefore, cDNA copy number and Ct are inversely related. Data were analyzed with the Sequence Detector System (SDS) software version 2.1 (ABI) and Ct value was automatically converted to fold change RQ value. The Fold change was calculated by applying the equation (RQ) = $2^{-(\Delta\Delta CT)}$. For each sample, ΔCT represents the difference between CT of each target gene and the internal control gene (GAPDH). For each specific gene ΔΔCT represents the difference between ΔCT of the different samples and the control. For each control sample ΔΔCT=0 and 2^0=1, by definition. Using this method, data are presented as differences in gene expression (relative gene expression) normalized to an endogenous reference gene and relative to a control group.

6. Western blot

Proteins were extracted from breast cancer cells by sonication in a lysis buffer (Tris HCl 0.1 M + SDS 4%). Mechanical microdissection with the same lysis buffer was performed on frozen primary tumors from SCID mice and on human breast tumor specimens. Lysates were centrifuged at 12,000 rpm for 10 minutes to collect the supernatants. Protein concentrations were quantified using BCA Protein Assay Kit (Thermo scientific). Whole-protein extracts are separated by electrophoresis on pre-casted polyacrylamide gels (Life Technologies Italia, Monza, Italy) and then transferred to PVDF membranes (Millipore, Billerica, MA). Non-specific binding was blocked with 5% fat-free milk for 1 hour at room temperature. The membrane was then incubated with rat anti-human FOXP3 antibody clone PCH101 (dilution 1:250; eBioscience, San Diego, CA) or mouse anti-human vinculin antibody (dilution 1:10000; Sigma-Aldrich, MO, USA) overnight at 4°C. After three washes with PBS+ buffer, the membranes were incubated with the horseradish peroxidase-conjugated secondary antibodies (dilution 1:5000; Amersham Pharmacia Biotech, Piscataway, NJ) for 1 hour at room temperature. After 3 washes with PBS buffer, proteins are detected using chemiluminescence ECL Western blot (Amersham Pharmacia Biotech, Piscataway, NJ) according to the manufacturer's instructions. Blots were then analyzed by Quantity One® software (Bio-Rad, CA, USA) following the User's Guide. Western blot for vinculin expression was used as loading control.

7. Immunofluorescence

To determine FOXP3 localization and induction in breast cancer cells, MDA-MB-231 cells grown *in vitro* were fixed and permeabilized. Afterwards cells were stained with rat anti-human FOXP3 antibody clone PCH101 (dilution 1:100; eBioscience, San Diego, CA) for 1 hour at room temperature and then incubated with anti rat 555 Alexafluor-conjugated secondary antibody (dilution 1:1000; Invitrogen). Nuclei were visualized by DRAQ5. Coverslips were mounted on glass slides using Prolong (Calbiochem, San Diego, CA) and examined with confocal microscope (Microradiance 2000, BioRad) equipped with Argon (488 nm), Green HeNe (543 nm) and Red diode (633 nm) lasers. Images were obtained using a X60 oil immersion lens (512x512 pixels) and analyzed using Image-Pro Plus v. 7.0.1 (MediaCybenetics) software.

8. *In vitro* assays

8.1 Transient transfection

MDA-MB-231 cells were plated into 6-well plates and allowed to adhere for 24 hours. The transfection of WTFOXP3-pcDNA3, Δ2FOXP3-pcDNA3 or empty vectors (as negative control) was performed using Lipofectamine-2000 (Invitrogen) according to the manufacturer's recommendation. After 5 hours of transfection the culture medium with serum was added. Migration and invasion assays were carried out 48 hours post-transfection.

8.2 Migration assay

A transwell (8μm pore size; Costar, Corning, USA) assay was used to analyze cell migration. 1×10^5 cells/well were placed in the upper chambers in serum-free RPMI-1640 medium, and the lower chambers were filled with RPMI-1640 supplemented with 10% FBS. Following incubation for 24 hours at 37°C, non-migrating cells on the top surface of the membrane were removed with a cotton swab. The membranes were fixed with absolute ethanol for 20 minutes at -20°C, stained with sulforhodamine b for 30 minutes and then washed with acetic acid 1% in water. Images of migrated cells were captured using an optical microscope and quantified with ImageJ program.

8.3 Invasion assay

Cell invasion assay was performed using a transwell plate (8 μm pore size; Costar, Corning, USA) coated with Matrigel (Becton Dickinson, NJ, USA). Briefly, Matrigel was diluted to a concentration of 2mg/ml, and 50μl of this solution were placed into a polycarbonate filter and air-dried. Filters were placed into wells and 700μl of RPMI-1640 culture medium supplemented with 10% FBS were added into the lower chamber. Cells were resuspended in serum-free RPMI-1640 medium and 1×10^5 cells in 0.2ml defined medium were plated into the upper chamber. Following incubation for 48 hours at 37°C, cells that did not penetrate the pores of the membrane were removed with a cotton swab. The membranes were fixed with absolute ethanol for 20 minutes at -20°C, stained with sulforhodamine b for 30 minutes and then washed with acetic acid 1% in water. Images of invaded cells were captured using an optical microscope and quantified with ImageJ program.

8.4 Stable transfection and proliferation assay

MDA-MB-231 cells were seeded in 100mm dishes, and transfected with 6µg of WTFOXP3-pcDNA3, Δ2FOXP3-pcDNA3 or empty vector (as control) using Lipofectamine-2000 (Invitrogen, Carlsbad, CA, USA) for 48 hours without antibiotic selection. Transfected cells were plated into 6-well plates at a density of 200 cells/well and cultured in medium containing 800 µg/ml G418 (Sigma, St. Louis, MO) until all the cells in the non-transfected control culture were killed. The antibiotic-resistant cells were maintained in the medium containing G418. Three weeks after the onset of drug selection, cells were fixed and stained with Toluidine Blue. Colonies were taken imaged and quantified using ImageJ program.

9*. In vivo* assays

9.1 Mice

All experiments were carried out using 8- to 10-week-old Several Combined Immuno Deficiency female (SCID) mice (Charles River, Calco, Italy). Mice were maintained in laminar flow rooms at constant temperature and humidity, with food and water given ad libitum. Experiments were approved by the Ethics Committee for Animal Experimentation of the Fondazione IRCCS Istituto Nazionale Tumori of Milan according to institutional guidelines.

9.2 Metastatic assays

For spontaneous metastatic assay, tumor cells in exponential growth phase were harvested using trypsin then washed and resuspended in Ca^{2+}- and Mg^{2+} -free PBS, to give a dose of 5×10^6 cells in 100µl. 100µl of matrigel were added to these cells immediately before injection into the mammary fat pad of mice. Mice were then monitored for overall health and total body weight. Tumor-bearing mice are given water containing doxycycline. When tumors reach a volume of ~150 mm^3, FOXP3 was induced in half of injected mice by removal of doxycycline from water. Tumor volume was measured twice weekly using a caliper, applying the formula 0.5 x $d1^2$ x d2, where d1 and d2 are the smaller and larger diameters, respectively. For experimental metastatic assay mice were injected with 1×10^6 cells in 100µl Ca^{2+}- and Mg^{2+} -free PBS through lateral tail vein injection. The injected cells were induced or non-induced to express FOXP3 in mice given water with or without doxycycline. At the end of the experiments mice were euthanized and autopsied. To evaluate the regulation of FOXP3 expression through doxycycline in spontaneous metastatic assay primary tumors were frozen in liquid nitrogen and stored at -80°C for Western blot analysis.

For the evaluation of spontaneous and experimental metastases lungs were removed, fixed in 10% buffered formalin and embedded in paraffin. 5µm-thick sections stained with hematoxylin/eosin were examined by IHC using anti-human vimentin antibody.

10. Statistical analyses

Disease-free survival (DFS) was defined as the time elapsed from date of diagnosis to the date of first recurrence, loco-regional or systemic. Survival functions were assessed using the Kaplan-Meier estimator, while log-rank test was used to compare survival distributions. All the analyses on triple negative breast cancer cohort were conducted using SAS software (SAS Institute Inc, Cary, NC).

Data obtained in *in vitro* and *in vivo* experiments were analyzed with Graph Pad Prism (GraphPad Software, Inc., San Diego) and evaluated using Student's t-test.

Two-sided *p* values lower than .05 were considered statistically significant.

Results

1. FOXP3 expression in triple-negative breast cancer correlates with poor prognosis

Data obtained in our previous retrospective study [Merlo et al., 2009] were related to old trials performed in 1973-1980 (Milano 1) and 1987-1989 (Milano 3). In these trials only lymph node-positive patients have been treated with chemotherapy after surgical resection of tumor, whereas node-negative patients (≈60% of trial patients) did not receive any chemotherapic treatment until relapse. Since data from a cohort of triple negative breast cancer (TNBC) cases were available in our laboratory, we evaluated FOXP3 expression in 81 paraffin-embedded primary TNBC specimens in order to confirm Milano 1 and Milano 3 trial data in a cohort of chemotherapy-treated breast cancer patients.

TNBC subtype accounts for 15% to 20% of newly diagnosed breast cancer cases. This breast cancer subtype tends to relapse with distant metastases rather than local recurrences [Lin et al., 2008]. TNBC patients have an increased risk of distant recurrence following diagnosis compared to other breast cancer subtypes [Dent et al., 2007] and show a peak of recurrence 1–3 years after the initial diagnosis with a quick drop thereafter [Foulkes et al., 2010].

The clinic-pathological characteristics of all triple negative breast cancer patients are summarized in table 4. Expression of FOXP3 was evaluated independently by two pathologists both blinded to the clinic-pathologic data. Discrepancies between the two observers were reviewed jointly to reach consensus. Out of all tumor specimens immunostained for FOXP3, 34 were scored as negative, 25 as weak positive, and 22 as strong positive. In most breast carcinomas, FOXP3 staining was localized predominantly in the cytoplasm, whereas the other specimens showed both cytoplasmic and nuclear staining (figure 8).

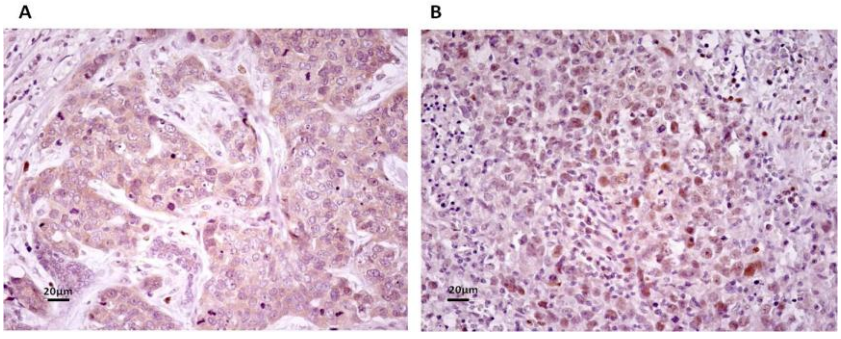

Figure 8. FOXP3 subcellular localization in triple negative breast cancer specimens.
Immunohistochemical staining of paraffin-embedded triple negative breast cancer tissue revealed FOXP3 expression in triple negative breast cancer cells. FOXP3 positive staining was localized predominantly in the cytoplasm (A) or both in cytoplasm and nucleus (B).

FOXP3 expression in triple negative breast tumors was associated with worse prognosis. Figure 9 shows the Kaplan-Meier curve for the disease-free survival of patients according to FOXP3 expression status. FOXP3 positive patients (weak and strong FOXP3 positivity; n=47) had poorer disease-free survival compared to FOXP3-negative patients (n=34) (p=0.014 by Cox proportional hazards model; HR 2.619, 95% CI 1.175-5.842).

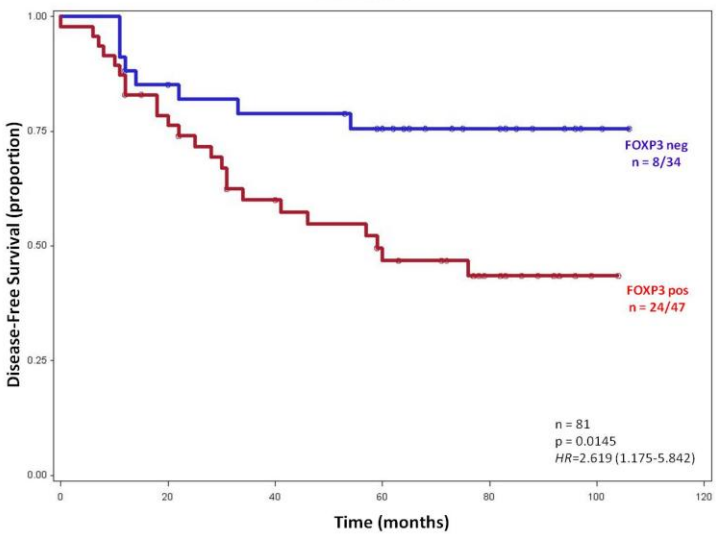

Figure 9. Disease-free survival according to FOXP3 expression in triple negative breast cancer patients
Survival analysis according to FOXP3 expression. Kaplan-Meier curve for disease-free survival according to FOXP3 expression status in all patients. * p value was calculated using the log-rank test comparing FOXP3-weak/strong-positive patients vs. FOXP3-negative patients.

To determine whether FOXP3 expression was associated with the clinical characteristics of triple negative breast cancer, we correlated its expression with age, lymph node positivity, tumor size and grade, necrosis, calcification, multifocality, DCIS and cytokeratines 5/6 positivity. The frequency of clinic-pathological characteristics and FOXP3 expression, grouped according to the presence (weak and strong) or absence of FOXP3 immunostaining are listed in table 5. Positive FOXP3 expression was significantly correlated with lymph node positivity (*p*=0.0269), while no association was found with age or other pathologic parameters.

Characteristic	Number/Total	%
Age >50 years	57/94	60
Lymph node positivity	34/81	42
Tumor size >2.0 cm	37/94	39
Tumor grade III	81/93	87
Necrosis	72/89	81
Calcification	26/82	32
Multifocality	20/86	23
DCIS	31/87	36
Cytokeratines 5/6 positivity	57/90	63

Table 4. Clinic-pathologic characteristics of triple negative breast cancer patients

Characteristic	FOXP3 neg Number/Total (%)	FOXP3 pos Number/Total (%)	p*
Age > 50 years	20/34 (61)	28/47 (60)	ns
Lymph nodes positivity	9/34 (27)	24/47 (52)	0.0269
Tumor size >2.0 cm	13/34 (39)	25/47 (40)	ns
Tumor grade III	29/34 (86)	41/47 (87)	ns
Necrosis	25/34 (74)	40/47 (85)	ns
Calcification	9/34 (28)	16/47 (34)	ns
Multifocality	8/34 (23)	12/47 (24)	ns
DCIS	12/34 (37)	16/47 (35)	ns
Cytokeratines 5/6 positivity	19/34 (57)	31/47 (67)	ns

Table 5. Correlation of FOXP3 expression with clinic-pathologic characteristics of 81 triple negative breast cancer patients.

In agreement with the results obtained in the previous analyses of FOXP3 expression in breast cancer patients (Milano 3 and Milano 1 trials), our results indicated that FOXP3 expression correlated with a higher risk of relapse in triple negative breast cancer patients treated with current oncological therapies.

2. FOXP3 expression in human breast cancer and in breast cancer cell lines

In the light of the results previously obtained we set out to further investigate the role of FOXP3 in breast cancer through *in vitro* and *in vivo* experiments.

To investigate the feasibility of using breast carcinoma cell lines as *in vivo* and *in vitro* models, first of all we assessed FOXP3 expression in a panel of breast carcinoma cell lines with different molecular characteristics and in human IHC FOXP3-positive primary breast carcinoma samples.

Whole-cell lysates were obtained from MDA-MB-231, MDA-MB-468, HCC1937 triple negative cell lines, MCF-7 (ER+/PR+) cells, BT474 and MDA-MB-361 (ER+/PR+/HER2+) cells and HER2-overexpressing SKBr3 cell line and from frozen human breast cancer specimens. Western blot analysis showed FOXP3 expression in breast cancer cell lines (figure 10A) and human breast cancer samples (figure 10B). As shown in figure 10C quantitative analysis of Western blot showed that FOXP3 levels both in cancer cell lines and in carcinoma samples were drastically lower than in Treg cells used as positive control. Human breast cancer samples had a 33-fold reduction in FOXP3 protein levels in comparison to Tregs, whereas a 5000-fold reduction in FOXP3 levels with respect to Tregs was found in tumor cell lines. Thus, FOXP3 expression in human breast cancer samples was significantly higher (300-fold increase) than in breast cancer cell lines.

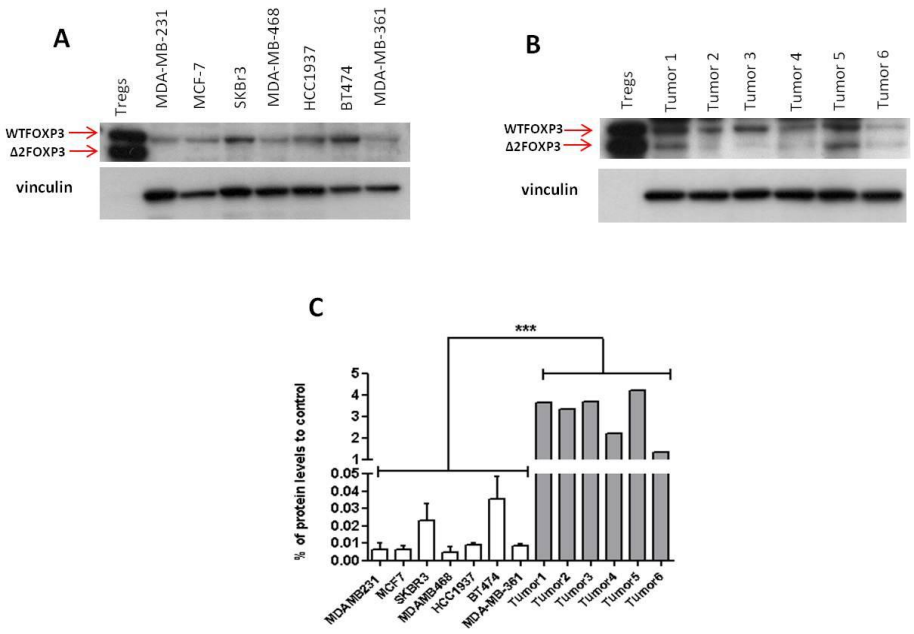

Figure 10. FOXP3 expression in human breast cancer and in breast cancer cell lines
Western blot analyses of FOXP3 expression in breast carcinoma cell lines (A) and in human primary breast carcinoma samples (B), using vinculin as loading control. C) Quantitative analysis of Western blot results, with FOXP3 levels expressed as percentage with respect to positive control (Tregs). For breast cancer cell lines, data are mean±SD of 2 independent experiments. ***p<0.0001 by unpaired t-test.

FOXP3 expression in breast cancer cells has been examined also at the mRNA level. RNA was isolated from MDA-MB-231, MCF-7, SKBr3, MDA-MB-468, HCC1937 and BT474 breast cancer cell lines. Following cDNA synthesis, cell lines were subjected to quantitative real-time PCR. The housekeeping gene GAPDH was used as control. FOXP3 mRNA was revealed in all tumor cell lines analyzed, although at very low levels, and the amount of FOXP3 mRNA was drastically lower in cancer cell lines (1000-fold reduction) than in Treg cells (figure 11). These data were consistent with FOXP3 protein expression analysis data.

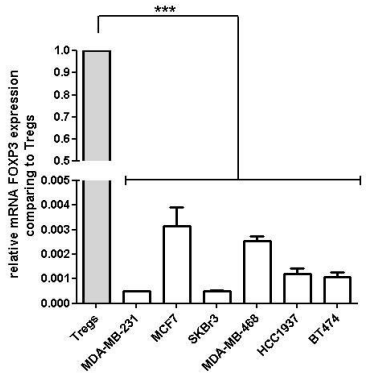

Figure 11. FOXP3 mRNA expression in breast cancer cell lines
FOXP3 mRNA expression in a panel of breast cancer cell lines quantified by real-time PCR. Results were normalized to GAPDH. Graph bars represent FOXP3 mRNA amount expressed as relative levels with respect to Tregs (Tregs=1). Results are representative of three independent experiments. Error bars represent SD; ***p<0.0001 by unpaired t-test.

A detailed analysis of Western blot results (figures 10A e 10B) revealed that the protein ran as a closely spaced doublet in Tregs and human breast cancer lysates, whereas a single band was detected in the lysates of all breast cancer cell lines. The upper isoform represented the full lenght FOXP3 (WTFOXP3), whereas the lower isoform represented the main deletional isoform Δ2FOXP3 lacking exon 2 (amino acids 71–105) described in naïve human CD4+ T cells and CD4+CD25+ regulatory T cells [Allan et al., 2005; Li et al., 2007; Xu et al., 2010]. Deletion of exon 2 is predicted to result in an approximately 4-kDa decrease in the molecular weight of FOXP3, which corresponds to the lower band detected in lysates from human breast cancer specimens [Allan et al., 2005]. Moreover, the molecular weight of the Δ2FOXP3 isoform appears identical to that of the lower band in Tregs, further supporting our hypothesis regarding its identity. RT-PCR was performed to confirm the presence of both WTFOXP3 and Δ2FOXP3 isoforms in human breast cancer samples. RNA was isolated from human breast cancer samples previously analyzed for FOXP3 expression by Western blot analysis. Following cDNA synthesis, samples were subjected to standard PCR. Tregs cDNA was used as positive control. As shown in figure 12, both WTFOXP3 and Δ2FOXP3 transcripts were identified in all human breast cancer specimens analyzed.

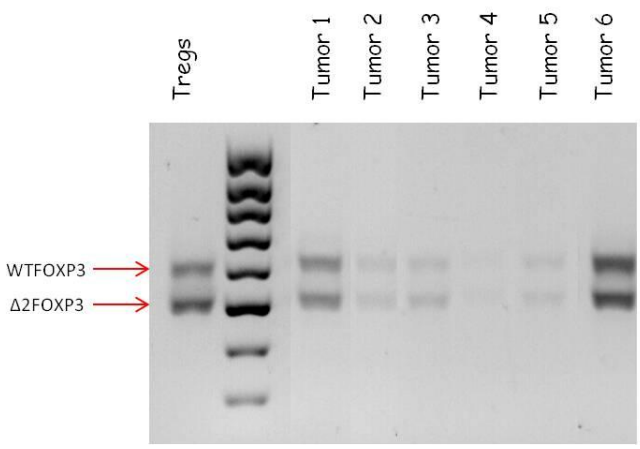

Figure 12. FOXP3 mRNA expression in human breast cancer

Human breast cancer samples were tested for FOXP3 mRNA expression. The reaction performed using specific FOXP3 forward and reverse primers revealed both WTFOXP3 and Δ2FOXP3 mRNA expression in all human breast cancer samples studied. The reaction with Tregs cDNA (positive control) confirmed the product size of ≈600bp and ≈500bp for WTFOXP3 and Δ2FOXP3, respectively.

The biological role of Δ2FOXP3 isoform has been investigated in CD4+CD25- T cells. Data suggest that Δ2FOXP3 possesses transcriptional repressor activity toward the IL-2 promoter in CD4+ T cells [Allan et al., 2005]. Moreover, FOXP3-transduced T cells were tested for their ability to proliferate in response to immobilized anti-CD3 mAbs. The antiproliferative effect was significantly lower in Δ2FOXP3-transfected cells than in T cells overexpressing WTFOXP3. Finally, Δ2FOXP3 overexpression showed a moderate decrease in IL-2 production in comparison to WTFOXP3-transduced T cells which showed a significantly reduced capacity to produce IL-2 upon activation. Together these results indicate that the two isoforms may have distinct functions *in vivo* [Allan et al., 2005].

Δ2FOXP3 isoform is overexpressed by certain malignant cells; for example, in an aggressive variant of cutaneous T cell lymphoma (Sezary Syndrome) the transformed T cells express Δ2FOXP3 isoform. To our knowledge, in current literature no data exist on the biological role of this splice variant in cancer cells.

Given these preliminary results we reasoned as following:

i) Since we demonstrated that many breast cancer cell lines, representing different breast cancer subtypes, expressed significantly lower levels of FOXP3 than primary human breast cancer samples, we assumed that the overexpression of FOXP3 in breast cancer cell lines could be a chance to mimic *in vivo* conditions;

ii) Since WTFOXP3 was detected in all human breast cancer and breast cancer cell lines analyzed, whereas the Δ2FOXP3 isoform was visible solely in human breast tumors, we asked whether FOXP3 role in human breast cancer may depend on Δ2FOXP3 isoform and in particular if this isoform can have a different biological role than full length FOXP3 in breast cancer progression.

3. Triple-negative breast cancer cell line as *in vitro* and *in vivo* tumor model

In order to investigate FOXP3 function in breast cancer and its involvement in promoting metastasis, we attempted to stably overexpress FOXP3 in breast cancer cells. Among several available mammalian inducible expression systems, the Tet-off system has been successfully used in different cell lines [Gossen et al., 1995; Weng et al., 1998]. For this reason our initial efforts were to use the Tet-Off system to generate stable FOXP3 expressing breast cancer cells. Among breast cancer cell lines, we choose the human breast cancer cell line MDA-MB-231 for its biological characteristics.

The MDA-MB-231 breast cancer cell line was obtained from a patient in 1973 at M.D. Anderson Cancer Center. With epithelial-like morphology, the MDA-MB-231 breast cancer cells appear phenotypically as spindle shaped cells. *In vitro*, the MDA-MB-231 cell line has an invasive phenotype and it has abundant activity in the Boyden chamber invasion assay. The MDA-MB-231 cell line is also able to grow on agarose, an indicator of transformation and tumorigenicity, and displays a relatively high colony forming efficiency. Moreover, this established breast adenocarcinoma cell line is widely used by the scientific community for studying *in vivo* metastasis based on its ability to grow orthotopic tumors in athymic mice able to spontaneously metastasize to other organs. Few established breast cancer cell lines metastasize in mice, and among them most only in experimental settings, for example via tail vein bypassing the crucial and physiologically relevant steps of migration and invasion inside the primary tumor.

Moreover, we selected this breast cancer cell line as cellular model since, as previously shown, a significant association between FOXP3 expression and decreased disease-free survival ($p=0.014$) was found in triple negative breast cancer specimens.

4. Role of FOXP3 in breast cancer metastasis: *in vitro* assays

As active migration and invasion of tumor cells are pre-requisites for tumor-cell metastasis, we sought to identify the effects of FOXP3 overexpression on MDA-MB-231 cell migration and invasion capability. Furthermore, we also investigated FOXP3 overexpression effect on MDA-MB-231 cell growth.

Prior to establishing Tet-Off bulk cultures with an inducible FOXP3 expression, we investigated the effect of FOXP3 overexpression in transiently transfected MDA-MB-231 cells. To this purpose, MDA-MB-231 cells were transfected with the pcDNA3 plasmid encoding WTFOXP3 (WTFOXP3-pcDNA3 cells), Δ2FOXP3 (Δ2FOXP3-pcDNA3 cells), or the corresponding empty vector (mock cells). Western blot analysis indicated that FOXP3 protein expression was increased in both WTFOXP3-pcDNA3 and Δ2FOXP3-pcDNA3 transfected cells (figure 13).

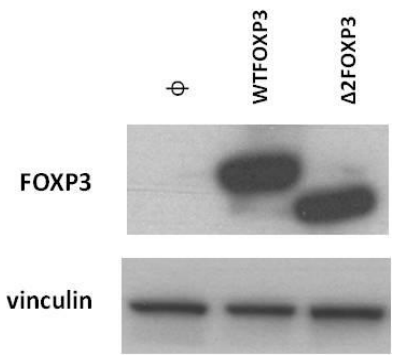

Figure 13. Western blot analysis of FOXP3 expression in MDA-MB-231 FOXP3-transfected cells
Western blot analysis of FOXP3 expression in WTFOXP3-, Δ2FOXP3- and empty vector (ϕ)-transfected MDA-MB-231 cells. Vinculin served as a loading control.

4.1 FOXP3 enhances MDA-MB-231 cancer cell migration capability

The migration assay was carried out using a transwell membrane. WTFOXP3-pcDNA3 cells, Δ2FOXP3-pcDNA3 cells and mock cells were detached from the tissue culture plates, resuspended in serum-free RPMI 1640 medium (1×10^5 cells/well), and then loaded to the upper side of the chamber. Serum containing RPMI 1640 medium was added to the lower chamber. After 24 hours of incubation, filter inserts were removed from the wells. Cells on the upper surface of the filter were removed using cotton swabs. Those on the lower surface were fixed with absolute ethanol in PBS and stained with sulforhodamine B (SRB). Images of

migrated cells were captured using an optical microscope and quantified with ImageJ program.

Overexpression of WTFOXP3 and Δ2FOXP3 in MDA-MB-231 cells induced a 2.3-fold and 1.8-fold increase, respectively, in the migration rate compared to matching mock cells (figure 14). The increase was statistically significant (p=0.01 and p=0.03 for WTFOXP3- and Δ2FOXP3-transfected cells, respectively).

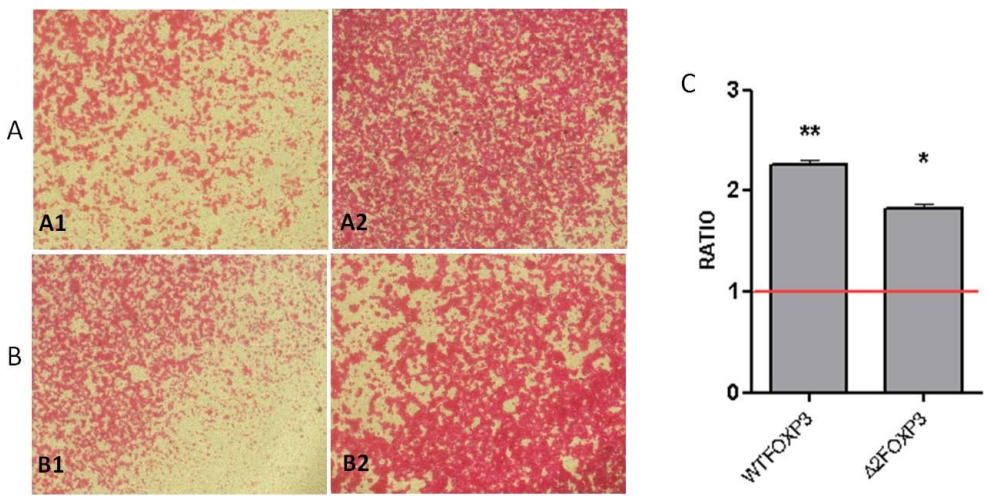

Figure 14. Promotion of MDA-MB-231 cell migration by FOXP3 overexpression

Representative images of migrated cells in mock cells (A1 and B1), WTFOXP3- (A2) and Δ2FOXP3- (B2) transfected cells using the transwell migration assay. C) A diagram of the migrated cells, as determined by using an optical microscope and the ImageJ program for quantification of migrated cells. Data are mean±SD of the ratio between the number of migrated WTFOXP3-/Δ2FOXP3- and empty vector-transfected cells (Ratio=1, red line). Results are representative of 3 independent experiments. * $p<0.05$; ** $p<0.01$ by unpaired t-test vs empty vector-transfected cells.

4.2 FOXP3 enhances MDA-MB-231 cancer cell invasion capability

The cell invasion assay was performed using a transwell chamber coated with matrigel. WTFOXP3-pcDNA3, Δ2FOXP3-pcDNA3 and mock cells were plated into the upper chamber at a density of 1×10^5 cells/well with serum-free medium, while the lower chamber contained medium with 10% FBS. When the cells were allowed to invade the matrigel for approximately 48 hours, the invasive cells were fixed with absolute ethanol and stained using SRB, while the non-invasive cells were scraped with cotton tips. Finally, the invasive cells were taken imaged and quantified using ImageJ program. WTFOXP3-pcDNA3 and Δ2FOXP3-pcDNA3 cells showed a 2.6-fold (p=0.03) and 3.4-fold (p=0.02) penetration rate through the matrigel-coated membrane compared to mock cells (figure 15), indicating that FOXP3 significantly increased the invasion ability of MDA-MB-231 cells.

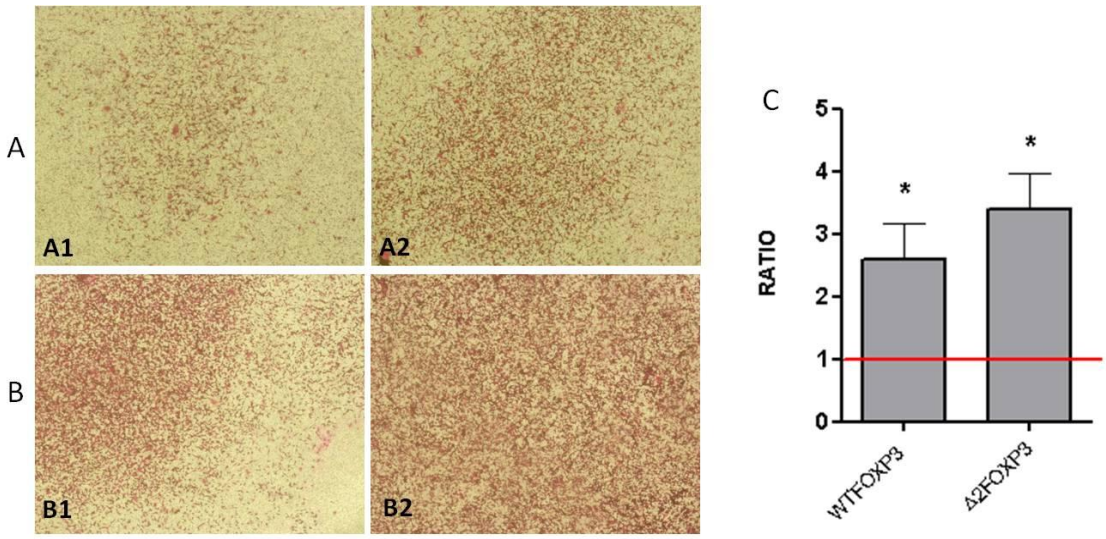

Figure 15. Promotion of MDA-MB-231 cell invasion by FOXP3 overexpression
Representative images of invasive cells in mock cells (A1 and B1), WTFOXP3- (A2) and Δ2FOXP3- (B2) transfected cells using the matrigel invasion assay. C) A diagram of the invasive cells, as determined by using an optical microscope and the ImageJ program for quantification of invaded cells. Data are mean±SD of the ratio between the number of invaded WTFOXP3- or Δ2FOXP3- and empty vector-transfected cells (Ratio=1, red line). Results are representative of 3 independent experiments. * p<0.05 by unpaired t-test vs empty vector-transfected cells.

4.3 FOXP3 inhibits MDA-MB-231 cell growth

To assess whether FOXP3 had a functional effect on MDA-MB-231 breast cancer cell growth, colony forming assay on WTFOXP3-pcDNA3 cells, Δ2FOXP3-pcDNA3 cells and mock cells was performed. 48 hours after transfection cells were plated into 6-well plates at a density of 200 cells/well and maintained in a selective medium containing antibiotic. Only those cells which have integrated the plasmid survived, since they contained the drug resistant gene. The medium was refreshed every three days. After 3 weeks of cell culture under antibiotic selection, culture dishes were stained with Toluidine Blue and the number of colonies was evaluated. As shown in figure 16, WT*FOXP3* and Δ2FOXP3 overexpression consistently decreased the ability of breast cancer cells to form colonies. WTFOXP3-pcDNA3 and Δ2FOXP3-pcDNA3 cells had a 1.5- and 2-fold reduction in colony number, respectively, compared to mock cells (p<0.01 by unpaired t-test) (figure 16).

In agreement with already published data supporting the role for FOXP3 as an onco-suppressor gene in human cancer [Liu et al., 2009; Zuo et al., 2007a; Zuo et al., 2007b], our results demonstrated a significant growth-inhibitory activity of FOXP3 on breast cancer cells.

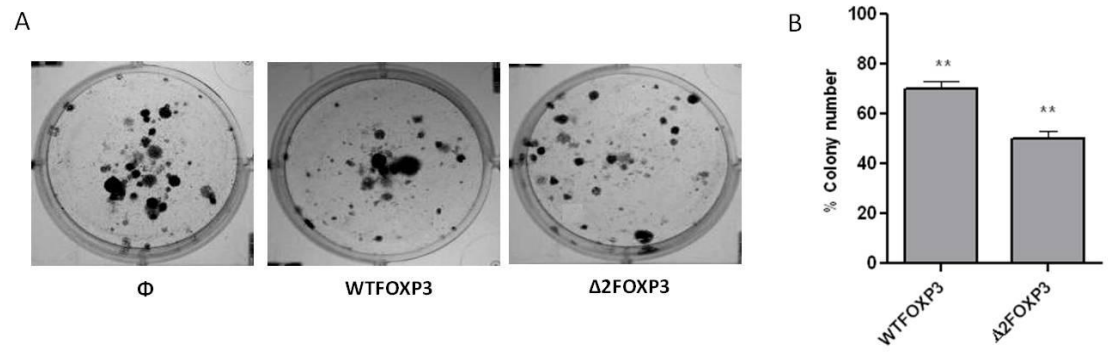

Figure 16. Inhibition of MDA-MB-231 cell colony number by FOXP3 overexpression
A) Representative images of colony assay showing the inhibition of colony formation in MDA-MB-231 cells after transfection with a vector encoding WTFOXP3, Δ2FOXP3 or with the empty vector. B) A diagram of colony number reduction, as determined by using the ImageJ program for quantification of the number of colonies. The number of colonies for each cell clone is expressed as percentage compared with matching empty vector transfected cells (100%). Results are representative of 3 independent experiments. Error bars = standard deviation. ** p<0.01 by unpaired t-test vs mock cells.

Our *in vitro* results demonstrated that both WTFOXP3 and Δ2FOXP3 isoforms significantly increased migration and invasion capability of breast cancer cells. Taking advantage of the correlation between FOXP3 and poor prognosis in triple negative breast cancer patients, and considering the pro-migratory and pro-invasive effect of transient FOXP3 overexpression in triple negative breast cancer cells, to further investigate *in vivo* role of FOXP3 in breast cancer metastasis, we planned to build an inducible Tet-Off construct that stably expressed FOXP3. The inducible system provides tight and inducible gene expression so that it is possible to induce high expression of FOXP3 gene *in vivo*. Moreover, for our *in vivo* experiments we generated a mixed cell population stably expressing FOXP3 to ensure that the *in vivo* effect of FOXP3 expression was not related to the intrinsic characteristic of a single selected clone.

5. Generation of a vector for inducible FOXP3 expression in triple negative breast cancer cells

We generated two constructs (pTRE2hyg-WTFOXP3 and pTRE2hyg-Δ2FOXP3) that stably expressed our gene of interest in an inducible Tet-Off system so as to be able to regulate FOXP3 transcription through the use of tetracyclines. The entire cDNA of WTFOXP3 or Δ2FOXP3 was cloned into the pTRE2hyg plasmid within the multiple cloning site (MCS) sequence. The plasmid contains the Tet response element (TRE) sequence which makes it responsive to the tTA protein of the Tet-Off system. In the absence of tetracycline tTA protein binds to the promoter and activates transcription. The TRE sequence also contains a

part of the Citomegalovirus promoter able to ensure high expression of the gene of interest cloned downstream.

To verify the functionality of the inducible system in MDA-MB-231 recipient cell clone (Tet-Off MDA-MB-231 #23 clone), previously transfected with the pTet-Off Advanced plasmid, the plasmid containing the reporter gene pTRE2hyg *Firefly Luciferase* (pTRE2hyg-Luc) and the control plasmid pRL-SV40 containing the sequence of the *Renilla Luciferase* reporter gene were co-transfected into Tet-Off MDA-MB-231 #23 cells. 48 hours after transfection, the cells maintained in culture in the presence or absence of doxycycline (DOXI) (100ng/ml) were detached with trypsin and collected for assessment of luciferase activity. In the Tet-Off system, doxycycline represses the expression of the gene placed under control of the TRE sequence. Luciferase assay indicates that cells grown in the absence of doxycycline (DOXI-) exhibit greater luciferase activity than those grown in the presence of doxycycline (DOXI+) (figure 17). So, we concluded that in the Tet-Off MDA-MB-231 cells the inducible system functioned and that these cells can be used to create a model with inducible expression of FOXP3.

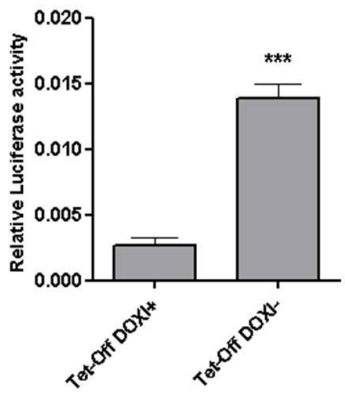

Figure 17. Functionality of Tet-Off system in recipient MDA-MB-231 cells

MDA-MB-231 cells were transfected with pTRE2hyg-Luc vector and maintained in culture in the presence or absence of doxycycline (100ng/ml). Cells maintained in the presence of doxycycline (DOXI+) showed a luciferase activity significantly lower than that detected in cells grown in the absence of doxycycline (DOXI-). Bars indicate standard deviation calculated on three replicates ***: $p < 0.001$.

5.1 Generation of stable cell populations with inducible expression of FOXP3

The pTRE2hyg-WTFOXP3 and pTRE2hyg-Δ2FOXP3 plasmids containing the coding sequence of WTFOXP3 and Δ2FOXP3, respectively, were transfected into recipient Tet-Off MDA-MB-231 cells. Stably transfected cells (Tet-Off MDA-MB-231-WTFOXP3 and Tet-Off MDA-MB-231-Δ2FOXP3) were maintained in the selective medium. The result was a stable cell mixed population (bulk culture). In order to verify the modulation of FOXP3 expression by doxycycline, these cells were grown in the selective medium containing or not doxycycline. After 48 hours cells were trypsinized and lysed. FOXP3 expression in total lysates was

analyzed by Western blot (figure 18). Data showed that a 4-fold and 7-fold increased expression of FOXP3 in Tet-Off MDA-MB-231-WTFOXP3 and Tet-Off MDA-MB-231-Δ2FOXP3 bulk cultures, respectively, was induced after doxycycline removal from the culture medium. These results demonstrated an efficient doxycycline-mediated induction of both WTFOXP3 and Δ2FOXP3 isoform expression in stably transfected MDA-MB-231 breast cancer cells. Also immunofluorescence analysis showed a strong induction of FOXP3 expression in both WTFOXP3- and Δ2FOXP3-transfected cells. In almost all tumor cells a nuclear localization of FOXP3 protein was observed (figure 19).

So, this inducible model has been employed in our further investigation on the involvement of FOXP3 in breast cancer metastatic process.

Figure 18. Western blot analysis of FOXP3 expression induction in MDA-MB-231 cells
Western blot analysis of FOXP3 expression in WTFOXP3- (A) or Δ2FOXP3-(B) stably transfected MDA-MB-231 cells with (DOXI+) or without (DOXI-) doxycycline induction. Vinculin served as a loading control.

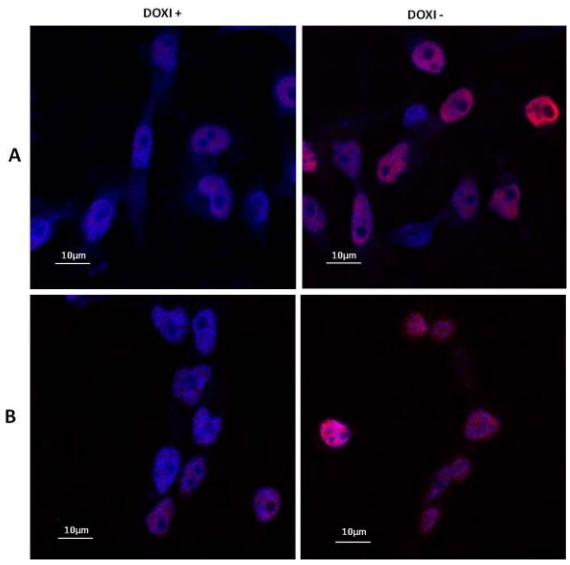

Figure 19. Immunofluorescence analysis of FOXP3 expression induction in MDA-MB-231 cells
Immunofluorescence analysis of FOXP3 expression in WTFOXP3- (A) or Δ2FOXP3- (B) stably transfected MDA-MB-231 cells with (DOXI+) or without (DOXI-) doxycycline induction. Merged images: DRAQ5; FOXP3.

6. Role of FOXP3 in breast cancer metastasis: *in vivo* assays

To demonstrate FOXP3 involvement in breast cancer metastasis, we aimed to correlate FOXP3 expression with the ability of FOXP3-overexpressing breast cancer cells to form spontaneous lung metastases.

6.1 Effect of WTFOXP3 over-expression in MDA-MB-231 breast cancer cells on spontaneous lung metastases

To assess the role of WTFOXP3 on spontaneous lung metastases $5x10^6$ Tet-Off MDA-MB-231-WTFOXP3 cells, grown in the presence of doxycycline in the culture medium were resuspended in Matrigel, which promotes the rooting and growth of tumor cells *in vivo*, and injected into the mammary fat pad of 16 female Severe Combined Immunodeficient (SCID) mice. Since we previously observed that FOXP3 inhibits tumor cell proliferation *in vitro*, we decided to suppress FOXP3 expression until the appearance of a palpable tumor. To this aim all mice received doxycycline (1mg/ml) in their drinking water, until tumor volume reached a mean size of 150 mm^3. Then mice were randomized into two groups: one was provided with drinking water (DOXI-) and the other was provided with doxycycline (1mg/ml) (DOXI+) in drinking water *ad libitum* for the duration of the experiment. Tumor volume was measured twice weekly using a caliper, applying the formula $0.5 \times d1^2 \times d2$, where d1 and d2 are the smaller and larger diameters, respectively. Doxycycline treatment had no effect on the animal weight, and 30 days after tumor cell injection, primary tumors showed comparable volume in the two mice groups (figure 20B). At this time primary mammary tumors and lungs were surgically removed.

Western blots of tissue extracts from primary tumors showed that FOXP3 expression was higher in tumors from DOXI- mice compared with tumors in DOXI+ mice, indicating that regulation of FOXP3 expression through doxycycline was maintained *in vivo* (figure 20A). Lung metastases were analyzed on formalin-fixed paraffin-embedded lung sections immunostained with anti-vimentin antibody. An average number of metastatic lesions of 12.5 ± 27.4 (mean±SD) was observed in mice not doxycycline-treated (DOXI-) and an average number of 35.8 ± 24.6 (mean±SD) metastases was observed in doxycycline-treated mice (DOXI+) (figure 20C). The difference in the number of lung metastases between DOXI+ and DOXI- groups did not reach significance (p=0.09).

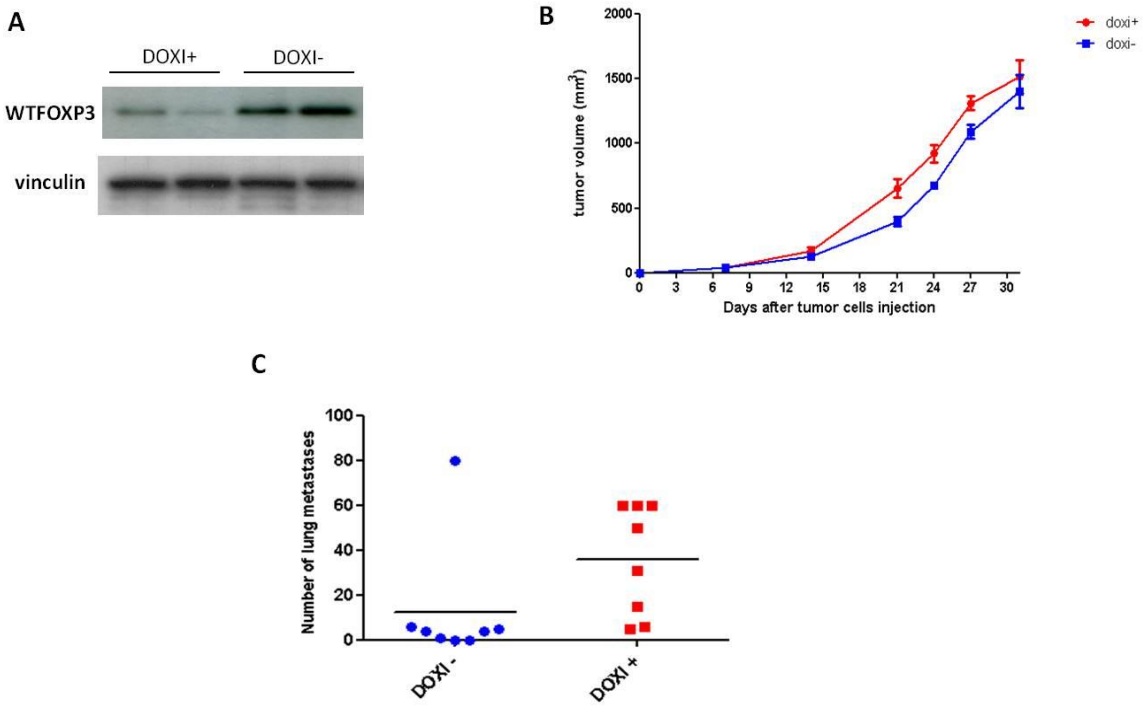

Figure 20. Effect of WTFOXP3 expression in MDA-MB-231 breast cancer cells on spontaneous lung metastases

A) Western blot analysis of FOXP3 expression in tumors grown in mice injected with Tet-Off MDA-MB-231-WTFOXP3 cells. Results are representative of 16 injected-mice, treated (n=8) or not (n=8) with doxycycline.
B) Tumor volume against time after injection of Tet-Off MDA-MB-231-WTFOXP3 cells. Doxycycline treatment did not affect tumor growth. Each point represents the mean ± standard deviation of the mean.
C) Number of lung metastases at 30 days after injection of Tet-Off MDA-MB-231-WTFOXP3 cells into the mammary fat pad. For each mouse, lung metastases were evaluated as the mean number in 3 microscopic fields (3.0 X 3.0 mm^2) randomly selected in each histological section stained with α-human vimentin antibody to detect breast carcinoma cells.

6.2 Effect of Δ2FOXP3 overexpression in MDA-MB-231 breast cancer cells on spontaneous lung metastases

At the same time, to evaluate Δ2FOXP3 isoform potential to contribute to breast cancer metastasis, we performed the same experimental protocol used to investigate WTFOXP3 isoform role in spontaneous metastasis assay. $5x10^6$ Tet-Off MDA-MB-231-Δ2FOXP3 bulk culture cells grown in the presence of doxycycline in the medium were resuspended in matrigel and injected into the mammary fat pad of 16 female SCID mice. All mice received doxycycline (1mg/ml) in their drinking water, until tumor volume reached a mean size of 150 mm^3. Then mice were randomized into two groups (8 mice per group): one was provided with drinking water (DOXI-) and the other was provided with doxycycline (1mg/ml) (DOXI+) in drinking water *ad libitum* for the duration of the experiment. Doxycycline treatment had no effect on the animal weight, and 30 days after tumor cell injection, primary tumors

showed comparable volume in the two mice groups (figure 21B). At this time primary mammary tumors and lungs were surgically removed.

Compared to doxycycline-treated mice, primary tumors grown in mice watered without doxycycline displayed a significant increase in FOXP3 expression, indicating that regulation of FOXP3 expression through doxycycline was maintained *in vivo* (figure 21A). Lung colonization was measured at the assay endpoint by IHC analysis of formalin-fixed paraffin-embedded lung sections. The average number of metastatic tumors was 33.9±32.7 (mean±SD) in doxycycline-treated mice and 10.3±18.1 (mean±SD) in not doxycycline-treated mice (figure 21C). The difference in the number of lung metastases between DOXI+ and DOXI- groups did not reach significance (p=0.12).

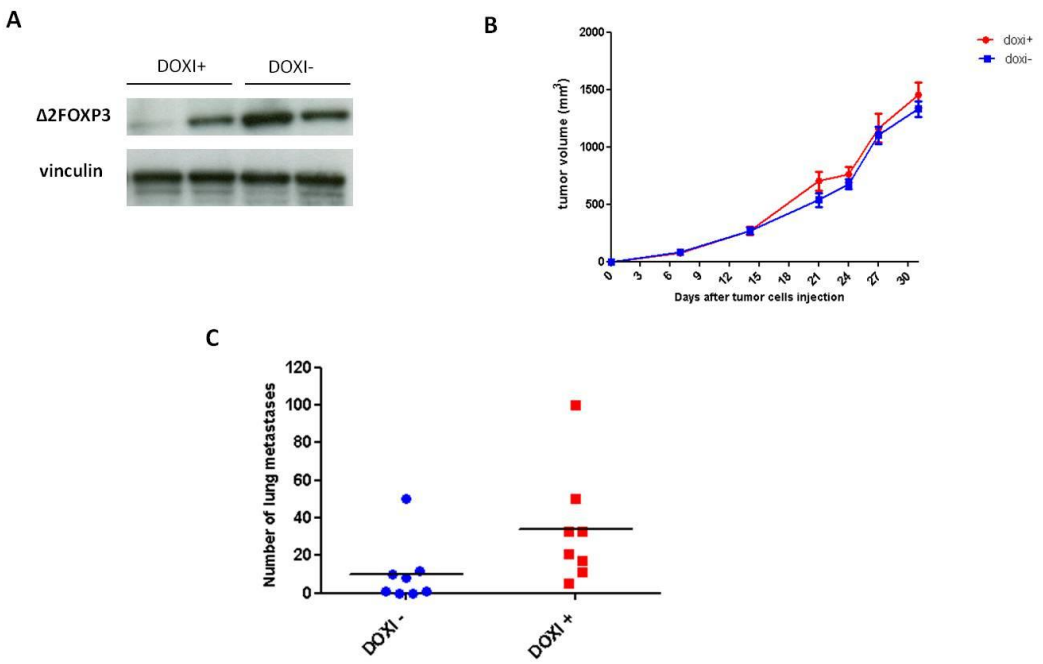

Figure 21. Δ2FOXP3 expression in MDA-MB-231 breast cancer cells enhances spontaneous lung metastases

A) Western blot analysis of FOXP3 expression in tumors grown in mice injected with Tet-Off MDA-MB-231-Δ2FOXP3 cells. Results are representative of *16* injected-mice, treated (n=8) or not (n=8) with doxycycline.
B) Tumor volume against time after injection of Tet-Off MDA-MB-231-Δ2FOXP3 cells. Doxycycline treatment did not affect tumor growth. Each point represents the mean ± standard deviation of the mean.
C) Number of lung metastases at 30 days after injection of Tet-Off MDA-MB-231-Δ2FOXP3 cells the mammary fat pad. For each mouse, lung metastases were evaluated as the mean number in 3 microscopic fields (3.0 X 3.0 mm^2) randomly selected in each histological section stained with α-human vimentin antibody to detect breast carcinoma cells.

These findings do not support a role of Δ2FOXP3 isoform in promoting breast cancer metastasis, suggesting that the hypothesis of Δ2FOXP3 role in metastatic process does not look right.

Moreover, *in vivo* results suggested that WTFOXP3 and Δ2FOXP3 isoforms have a similar biological effect when expressed in a triple-negative breast cancer cell line in terms of spontaneous metastasis induction since the mean number of spontaneous lung metastases was superimposable in WTFOXP3- and Δ2FOXP3-overexpressing tumor bearing mice (12.5 and 10.3 for WTFOXP3- and Δ2FOXP3-MDA-MB-231 injected mice, respectively). The decrease, although not statistically significant, in the number of spontaneous lung metastases with both FOXP3 isoforms, is in contrast with the pro-metastatic role of FOXP3 previously emerged from our immunohistochemical analyses of breast cancer patient specimens.

7. FOXP3 subcellular localization in cancer cells and its biological role

The nuclear expression of FOXP3 in human benign breast tissue is well documented [Chen et al., 2008]. However FOXP3 localization in cancerous epithelia is less definitive.

Zuo et al. (2007b) assessed the subcellular distribution of FOXP3 in human breast cancer patient samples and reported that 21% of 275 breast cancer samples expressed only nuclear FOXP3 within the epithelial cells of the tumor, whereas 80% of the non-malignant cells expressed nuclear FOXP3.

Conversely, other reports, including ours, described a cytoplasmic FOXP3 staining of tumor cells in several types of cancer [Hinz et al., 2007; Karanikas et al., 2008; Ladoire et al., 2011; Merlo et al., 2009; Winerdal et al., 2011]. Our IHC analysis of breast carcinoma specimens (Milano 1 and Milano 3 trials) showed that FOXP3 positive staining was localized predominantly in the cytoplasm, although both cytoplasmic and nuclear staining was present in some specimens and a few specimens showed only nuclear staining. A similar heterogeneous FOXP3 localization in breast cancer cells has emerged in our recent analysis of TNBC cohort. Out of 47 breast cancer specimens scored as positive for FOXP3, 28 showed FOXP3 staining predominantly in the cytoplasm, while in 19 specimens both cytoplasmic and nuclear staining was present (figure 8).

While our investigation on FOXP3 role proceeded, many studies on different cancer types provided evidences for a strong correlation of elevated FOXP3 expression in cancer cells with

poor prognosis, particularly with metastasis [Fu et al., 2013; Kim et al., 2013; Liang et al., 2011; Quaglino et al., 2011; Xue et al., 2010; Wang et al., 2010; Winerdal et al., 2011]. Similarly to what observed in our IHC analyses on human breast cancer specimens, even in all these studies FOXP3 localization in tumor cells was reported to range from predominantly cytoplasmic to both cytoplasmic and nuclear.

It's currently difficult to interpret the significance of FOXP3 cytoplasmic localization; however, since the role of FOXP3 is transcription regulation, which mainly occurs in the nucleus, cytoplasmic FOXP3 localization could affect its biological role (see review Triulzi et al., 2013). Thus, we hypothesized that a cytoplasmic ''non functional'' localization of FOXP3 protein could explain the discrepancy between the onco-suppressive role of FOXP3 in our *in vivo* experiments, performed with transfected cells showing FOXP3 nuclear localization (figure 19), and the worse prognosis of patients with FOXP3-positive tumors.

In the light of these considerations we decided to investigate whether FOXP3 in tumor cells may have distinct biological activities and prognostic values according to its subcellular localization.

8. Selection of stable Tet-Off MDA-MB-231 WTFOXP3 clones with cytoplasmic localization of FOXP3 protein

Since the immunofluorescence staining previously performed on Tet-Off MDA-MB 231 WT FOXP3 bulk culture showed the presence of some, although few, cells expressing FOXP3 in the cytoplasm, in order to investigate whether FOXP3 overexpression could play a different role on breast cancer metastasis depending on its subcellular localization, the construct expressing WTFOXP3 under the control of the Tet-Off promoter was newly transfected into Tet-Off MDA-MB-231 recipient cells. FOXP3-transfected cells were diluted in a 96-well plate to seed only one cell per well, and grown in a selective culture medium. Several stable clones were obtained and screened for FOXP3 expression by Western blot in order to verify the modulation of FOXP3 expression by doxycycline. Depending on the individual clone an increased expression of FOXP3, ranging from 1- to 7-fold, was observed after doxycycline removal from the culture medium (data not shown). Among the clones showing a high induction of FOXP3 levels, we searched for clones with a predominantly cytoplasmic localization of the protein. To this aim immunofluorescence assay was perfomed on cells in which FOXP3 expression was regulated by doxycycline. Tet-Off MDA-MB-231-WTFOXP3#1

clone which showed a predominantly cytoplasmic FOXP3 localization was selected. The metastatic capability of Tet-Off MDA-MB-231-WTFOXP3#1 clone was evaluated and compared to that of Tet-Off MDA-MB-231-WTFOXP3#4 clone, which showed a predominantly nuclear FOXP3 localization, and selected for a FOXP3 induction similar to Tet-Off MDA-MB-231-WTFOXP3#1 clone (figure 22).

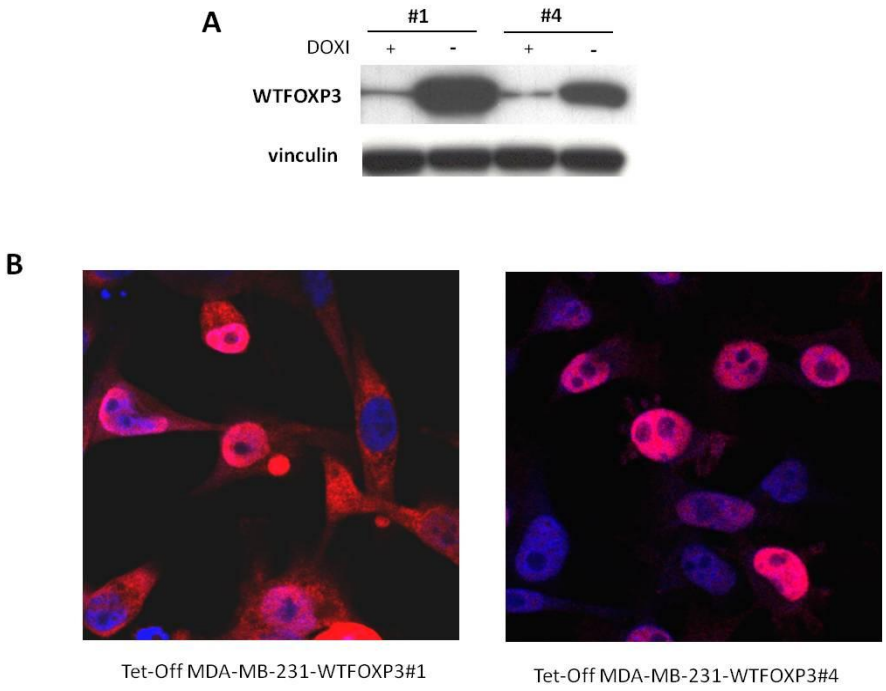

Figure 22. FOXP3 expression and subcellular localization in MDA-MB-231 stable clones
A) Western blot analysis showed the induction of FOXP3 expression in Tet-Off MDA-MB-231-WTFOXP3#1 clone and Tet-Off MDA-MB-231-WTFOXP3#4 clone. FOXP3 expression levels were compared after 48 hours of culture in the presence (DOXI+) or absence (DOXI−) of doxycycline. Vinculin was used as loading control.
B) Immunofluorescence analysis of FOXP3 expression in Tet-Off MDA-MB-231-WTFOXP3#1 clone and Tet-Off MDA-MB-231-WTFOXP3#4 clone cells, in which FOXP3 expression was induced upon doxycycline removal from the culture medium. Merged images: DRAQ5; FOXP3.

8.1 Correlation between FOXP3 subcellular localization in stable MDA-MB-231 clones and metastatic capability *in vivo*

We evaluated the *in vivo* effect of FOXP3 overexpression in Tet-Off WTFOXP3#1 and Tet-Off WTFOXP3#4 cells by performing both spontaneous and experimental metastasis assays, using the same conditions as described above.

In spontaneous metastasis assay cells were injected into the mammary fat pad of 20 female SCID mice. FOXP3 expression was induced in primary tumors of not doxycycline-treated mice (figures 23A and 24A). Tumor size was monitored for 30 days after tumor cell injection, then tumors were harvested and lungs were resected for analyses. As shown in figures 23B and 24B tumor growth was not affected by doxycycline treatment.

FOXP3 overexpression in Tet-Off WTFOXP3#4 cell-injected mice (DOXI-) led to a significantly reduced number of spontaneous lung metastases after a 30-day period (p=0.01) (figure 23C). The average number of metastatic lesions decreased from 4.6±4.4 (mean±SD) in doxycycline-watered mice to 0.7±1.5 (mean±SD) in not doxycycline-treated mice.

On the contrary, a significant increase (p=0.04) in the number of lung metastases was observed in Tet-Off WTFOXP3#1 cell-injected mice bearing FOXP3-overexpressing tumors (DOXI-) versus controls. The mean number of metastases was 4.8±6.4 and 16.4±15.9 (mean±SD) in doxycycline-treated and not doxycycline-treated mice groups respectively (figure 24C).

Similar results were obtained by performing experimental metastasis assay.

FOXP3 overexpression in Tet-Off WTFOXP3#4 cell-injected mice (DOXI-) led to a significant reduction in the number of experimental lung metastases (p=0.01) (figure 23D). The average number of metastatic lesions decreased from 55±7.1 (mean±SD) in doxycycline-watered mice to 29.3±19 (mean±SD) in not doxycycline-treated mice.

On the contrary, a significant increase (p=0.02) in the number of experimental lung metastases was observed in Tet-Off WTFOXP3#1 cell-injected mice bearing FOXP3-overexpressing tumors (DOXI-) versus controls. The mean number of metastases was 0.4±0.8 and 9.8±9.7 (mean±SD) in doxycycline-treated and not doxycycline-treated mice groups, respectively (figure 24D).

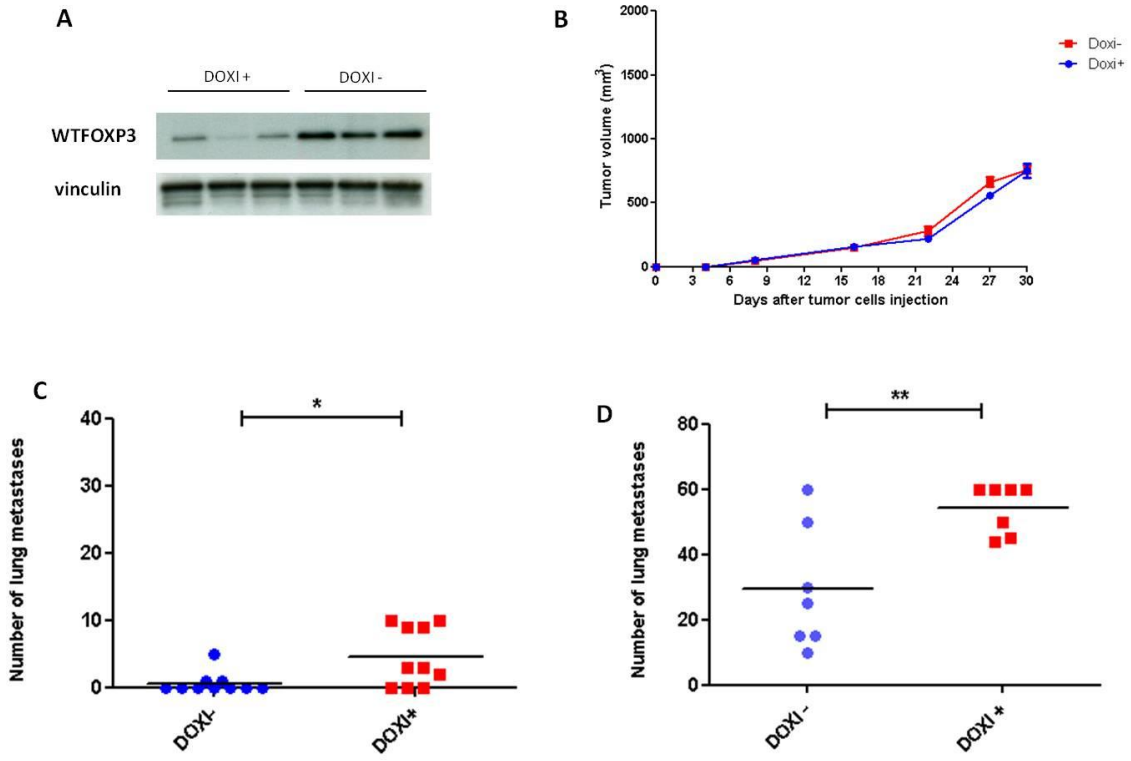

Figure 23. Predominantly nuclear FOXP3 expression in MDA-MB-231-WTFOXP3#4 clone inhibits both spontaneous and experimental lung metastases

A) Western blot analysis of FOXP3 expression in tumors grown in mice injected with Tet-Off MDA-MB-231-WTFOXP3#4 cells into the mammary fat pad. Results are representative of 20 injected-mice, treated (n=10) or not (n=10) with doxycycline. B) Tumor volume against time after injection of Tet-Off MDA-MB-231-WTFOXP3#4 cells. Doxycycline treatment did not affect tumor growth. Each point represents the mean ± standard deviation.

C) Number of lung metastases at 30 days after injection of Tet-Off MDA-MB-231-WTFOXP3#4 cells into the mammary fat pad. D) Number of lung metastases at 21 days after i.v. injection of Tet-Off MDA-MB-231-WTFOXP3#4 cells into the mice lateral tail vein. For each mouse, lung metastases were evaluated as the mean number in 3 microscopic fields (3.0 X 3.0 mm^2) randomly selected in each histological section stained with α-human vimentin antibody to detect breast carcinoma cells. * $p<0.05$; ** $p<0.01$ by unpaired t-test.

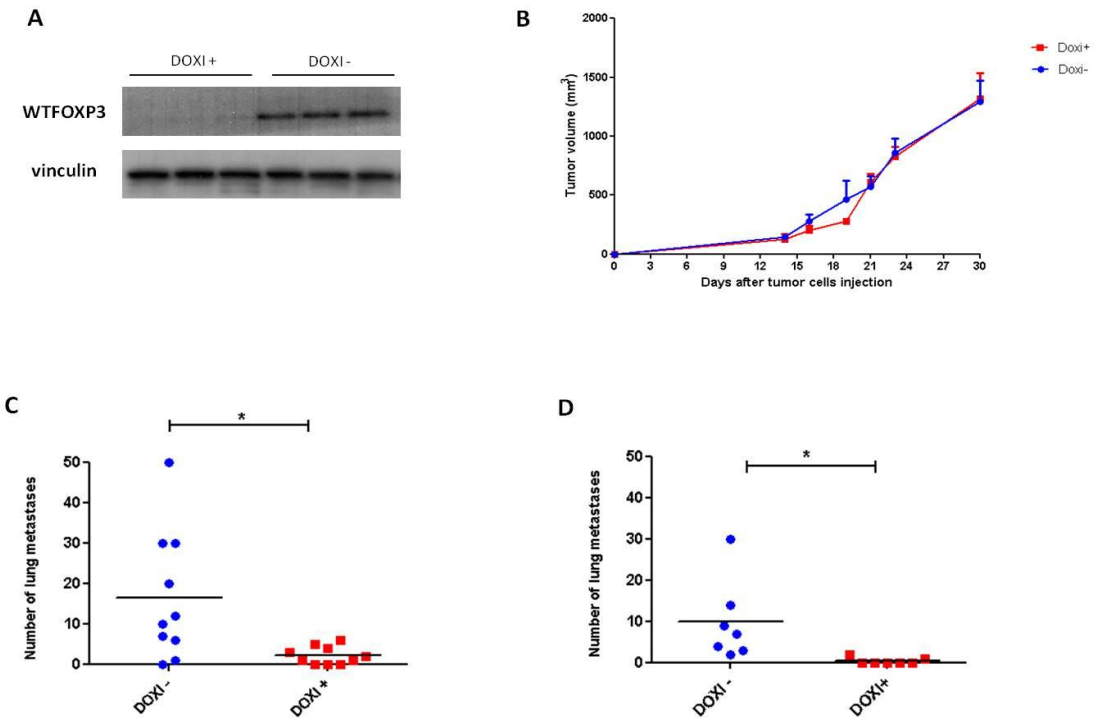

Figure 24. Predominantly cytoplasmic FOXP3 expression in MDA-MB-231-WTFOXP3#1 clone enhances both spontaneous and experimental lung metastases

Western blot analysis of FOXP3 expression in tumors grown in mice injected with Tet-Off MDA-MB-231-WTFOXP3#1 cells into the mammary fat pad. Results are representative of 20 injected-mice, treated (n=10) or not (n=10) with doxycycline. B) Tumor volume against time after injection of Tet-Off MDA-MB-231-WTFOXP3#1 cells. Doxycycline treatment did not affect tumor growth. Each point represents the mean ± standard deviation. C) Number of lung metastases at 30 days after injection of Tet-Off MDA-MB-231-WTFOXP3#1 cells into the mammary fat pad. D) Number of lung metastases at 21 days after i.v. injection of Tet-Off MDA-MB-231-WTFOXP3#1 cells into the mice lateral tail vein. For each mouse, lung metastases were evaluated as the mean number in 3 microscopic fields (3.0 X 3.0 mm^2) randomly selected in each histological section stained with α-human vimentin antibody to detect breast carcinoma cells. * $p<0.05$ by unpaired t-test.

The evaluation of metastatic capability of two clones with different FOXP3 subcellular localization led to opposite results. A significant decreased number of both spontaneous and experimental lung metastases was observed in mice injected with Tet-Off WTFOXP3#4 cells which showed a predominantly nuclear FOXP3 localization. On the other hand, when mice were injected with Tet-Off WTFOXP3#1 cells which showed a predominantly cytoplasmic FOXP3 localization a significant increased number of both spontaneous and experimental lung metastatic tumors was observed.

These results support our hypothesis that FOXP3 discrepant role in breast cancer metastasis may be due to its different subcellular localization. When FOXP3 localized in the nucleus it can play its transcriptional activity, resulting in an onco-suppressive effect, while its cytoplasmic localization unabled this transcription factor to perform its biological functions, leading to an opposite *in vivo* effect.

FUTURE PERSPECTIVES

Our findings indicate that FOXP3 subcellular localization in breast tumor cells is an important determinant of prognosis, supporting the involvement of this transcription factor in breast cancer metastasis. To further investigate the biological significance of FOXP3 cytoplasmic localization we generated a stable FOXP3-transfected MDA-MB-231 cell bulk population with a forced localization of FOXP3 to the cytoplasm.

FOXP3 nuclear localization was found to be dependent on the presence of the FKH domain. In particular, a C-terminal fragment of FOXP3 containing the entire FKH domain with short flanking sequences at each end was found to be both necessary and sufficient for import of FOXP3 to the nucleus [Lopes et al., 2006]. Mutation of two amino acids within this nuclear localization signal (NLS) domain abrogates nuclear import of FOXP3 [Lopes et al., 2006].

By targeted mutation at specific sites within NLS region we created a single mutant construct (mutWTFOXP3-pTre2hyg). This vector expresses FOXP3 full-length protein carrying a specific amino acid mutation in the FKH domain that impairs its nuclear localization. MutWTFOXP3-pTre2hyg vector was then stably expressed in Tet-Off-MDA-MB-231 breast cancer cells, so as to be able to regulate FOXP3 transcription through the use of doxycycline. Preliminary *in vitro* investigations on mutWTFOXP3-MDA-MB-231 cells in which FOXP3 localized in the cytoplasm are ongoing. We aimed to evaluate both spontaneous and experimental metastatic capability of mutWTFOXP3 MDA-MB-231 cells to confirm whether FOXP3 cytoplasmic localization enhances breast cancer metastasis.

DISCUSSION

The transcription factor FOXP3 is an X-linked gene well known for its crucial importance in the generation of CD4+CD25+ regulatory T cells (Tregs). Although expression of this transcriptional factor specifically characterizes naturally occurring Tregs, it is now clear that FOXP3 is also expressed by many tumor cells including breast cancer cells. Despite increasing knowledge about the biology of FOXP3, the significance of its expression in human cancer cells is not clearly understood.

FOXP3 is reported to repress the transcription of HER2 oncogene in human breast cancer by directly binding to the ErbB2 gene promoter; moreover, FOXP3 is able to bind to specific regions within the SKP2 gene directly repressing its expression [Zuo et al., 2007a; Zuo et al., 2007b]. FOXP3 is also been described to be a transcriptional activator of LATS2 and p21 tumor suppressor genes in breast epithelial cells [Liu et al., 2009; Li et al., 2011].

It has been also reported that FOXP3 expression in cancer cells may be a predictive biomarker of anthracycline efficacy, since FOXP3 expression in breast cancer cells was associated with a better overall survival in patients treated with anthracycline-based chemotherapy but not in those treated with sequential anthracycline-taxane therapy [Ladoire et al., 2011, 2012].

In sharp contrast to a putative onco-suppressor role for FOXP3, several studies had correlated FOXP3 expression in different histological types of cancer with poor prognosis, and particularly with metastasis [Bates at al., 2006; Gobert et al., 2009; Jaberipour et al., 2010; Mansfield et al., 2009; Merlo et al., 2009]. Our previous retrospective study conducted on human primary breast carcinoma specimens from Milano 1 and Milano 3 trials demonstrated for the first time a significantly inverse association between FOXP3 expression in breast cancer cells and patient survival. FOXP3 was also a strong prognostic factor for distant metastasis-free survival, but was not a significant predictor of local recurrence incidence risk [Merlo et al., 2009]. Since Milano 1 (1973-1980) and Milano 3 (1987-1989) trial lymph node-positive patients (≈ 40%) received adjuvant chemotherapy or hormone therapy after surgery, whereas node-negative patients have been treated at the time of relapse, we aimed to confirm the impact of FOXP3 on breast cancer survival in a cohort of breast cancer patients (2002-2006) all treated with post-surgical chemotherapy according to current oncological treatment guidelines. The choice to focus our analyses on triple negative breast cancers (TNBC) was based on the fact that this highly aggressive cancer subtype, with a particularly poor prognosis [Dent et al., 2009], usually presents a short

disease-free interval after surgery and adjuvant treatment and tends to relapse with distant metastases rather than local recurrences [Lin et al., 2008].

FOXP3 expression was evaluated in primary TNBC specimens from adiuvantly-treated patients and subsequently correlated to patient clinical characteristics and survival. FOXP3 positive patients had poorer disease-free survival compared to FOXP3-negative patients, confirming the significant impact of FOXP3 on the outcome of breast cancer patients even in this particular breast carcinoma subtype.

This data has been recently confirmed by Nair and colleagues [Nair et al., 2013]. In this study the correlation between FOXP3 gene expression and patient disease-free survival was determined in three different breast cancer subtypes (TNBC, ER+/HER2- and HER2+ breast cancers). FOXP3 expression was found to significantly associate with higher risk of recurrence in TNBC and ER+/HER2-negative breast cancer subgroups, whereas no differences were found in HER2+ tumors expressing or not FOXP3.

In the light of all these results which confirmed the association between FOXP3 expression and poor prognosis, we decided to further investigate the role of FOXP3 in breast cancer through *in vitro* and *in vivo* experiments. FOXP3 expression was assessed in a panel of different breast carcinoma cell lines, including triple negative breast cancer cell lines, and in human IHC FOXP3-positive primary breast carcinoma specimens. Quantitative analysis of Western blot showed that FOXP3 expression level in all human breast cancer cell lines was significantly lower than in human breast cancer specimens. Thus, we assumed that the overexpression of FOXP3 in breast cancer cell lines could be a chance to mimic *in vivo* conditions. Since our previous results showed an association between FOXP3 expression and poor outcome in triple negative breast cancer patients we decided to keep on our investigations on FOXP3 role in this specific breast cancer subtype. MDA-MB-231 cell line, which showed very low level of FOXP3 expression, has been selected as cellular model in our *in vitro* and *in vivo* experiments, and stably transfected to overexpress FOXP3.

Western blot analysis revealed that all breast cancer cell lines expressed only full-length FOXP3, whereas human breast cancers expressed both the full-length FOXP3 and the main deletional isoform Δ2FOXP3. The biological role of Δ2FOXP3 splice variant that completely lacks the second coding exon has been investigated in CD4+CD25- T cells. Since human $CD4^{+}CD25^{+}$ Tregs, which constitutively expressed high levels of both WTFOXP3 and Δ2FOXP3 isoforms do not produce detectable amounts of most cytokines, including IL-2, Allan and colleagues investigated whether ectopic expression of WTFOXP3 and/or Δ2FOXP3 in

CD4+CD25- T cells resulted in a Treg-like phenotype. Δ2FOXP3-transfected T cells only moderately reduced IL-2 production in comparison to WTFOXP3-transduced T cells that showed a significantly reduced capacity to produce IL-2 upon activation. Further analyses in Δ2FOXP3-transfected Jurkat T cells indicated that Δ2FOXP3 possesses a transcriptional repressor activity toward the human IL-2 promoter. Finally, transduced T cells were tested for their ability to proliferate in response to immobilized anti-CD3 mAbs, based on incorporation of tritiated thymidine. The antiproliferative effect was significantly lower in Δ2FOXP3-transfected cells than in T cells overexpressing WTFOXP3 (proliferation rate 30%±11% and 64%±41% in WTFOXP3- and Δ2FOXP-transfected cells, respectively). Together these finding suggested that the two FOXP3 isoforms may have distinct functions *in vivo* [Allan et al., 2005].

In current literature Δ2FOXP3 isoform expression has been reported in cancer cells, e.g melanoma cells and malignant T cells of Sezary syndrome; however, to the best of our knowledge, no data exist on the biological role of this splice variant in cancer cells. We hypothesized that Δ2FOXP3 isoform could have a distinct role from that of the full-length protein in breast cancer progression.

Conflicting results were obtained by investigating the effects of WTFOXP3 and Δ2FOXP3 overexpression in triple negative breast cancer cells on proliferative, migratory and invasive cell capabilities *in vitro*. On the one hand a significant growth-inhibitory activity of both WTFOXP3 and Δ2FOXP3 on breast cancer cells was observed in agreement with already published data supporting the role for FOXP3 as a tumor-suppressor gene in human cancer [Zuo et al., 2007b; Zuo et al., 2007a; Liu et al., 2009]; on the other side WTFOXP3 and Δ2FOXP3 isoform expression was found to significantly increase migration and invasion capability of breast cancer cells.

Considering the pro-migratory and pro-invasive effect of transient FOXP3 overexpression in triple negative breast cancer cells, we further investigated the *in vivo* role of each FOXP3 isoform in breast cancer metastasis exploiting a stable cell mixed population (bulk culture) with an inducible expression of WTFOXP3 or Δ2FOXP3. To correlate FOXP3 expression with the ability of FOXP3-overexpressing breast cancer cells to form spontaneous lung metastases, mice were injected with bulk culture cells and FOXP3 expression in tumor-bearing mice was regulated by doxycycline removal or administration in drinking water.

In mice injected with WTFOXP3-transfected cells the average number of lung metastases decreased from 35.8±24.6 (mean±SD) in doxycycline-watered mice to 12.5±27.4 in not

doxycycline-treated mice. Similarly, the number of lung spontaneous metastases in Δ2FOXP3-transfected mice was 33.9±32.7 and 10.3±18.1 in doxycycline-treated and not-treated mice, respectively.

The number of spontaneous lung metastases was very similar in WTFOXP3- and Δ2FOXP3-overexpressing tumor-bearing mice. These findings do not support a role of Δ2FOXP3 isoform in promoting breast cancer metastasis.

Further, the induction of both WTFOXP3 and Δ2FOXP3 isoforms led to a decrease in the number of spontaneous lung metastases. This inhibition of metastatic capability is in contrast with the pro-metastatic role of FOXP3 previously emerged from our immunohistochemical analyses of breast cancer patient specimens.

While our investigation on FOXP3 role proceeded, many studies on different cancer types provided evidences that patients with FOXP3 positive tumors have a significantly shorter progression free survival and/or overall survival compared to patients with FOXP3 negative tumors [Fu et al., 2013; Liang et al., 2011; Nair et al., 2013; Quaglino et al., 2011; Xue et al., 2010; Wang et al., 2010; Winerdal et al., 2011]. As regards breast cancer, FOXP3 expression has been recently analyzed in 183 patients by Kim and colleagues (2013). FOXP3-strong-positive patients showed a significantly shorter disease-free survival than FOXP3-negative and weak-positive patients, which had similar favorable prognoses, indicating that strong FOXP3 expression is an important prognostic factor for recurrence and poor survival [Kim et al., 2013]. Consistent with our IHC analyses of breast carcinoma specimens from Milano 1 and Milano 3 trials [Merlo et al., 2009] and from a cohort of triple negative breast cancer patients, in all these studies subcellular staining of FOXP3 was found to be heterogeneous, ranging from cytoplasmic to both cytoplasmic and nuclear and, in few cases, only nuclear.

The mechanism underlying FOXP3 cytoplasmic localization is still under investigation.

Chen et al. demonstrated that in Treg cells, TCR-mediated post-translational modifications could mediate the regulation function, and influence the subcellular distribution of FOXP3; they revealed a change in the subcellular localization of FOXP3 from a more cytoplasmic/perinuclear to a nuclear expression pattern in Tregs activated with anti-CD3/anti-CD28 antibodies [Chen et al., 2006]. FOXP3 contains at least three distinct functional domains, including forkhead (FKH) domain, a leucine zipper, and a zinc finger. The FKH domain is critical for nuclear localization. FOXP3 with mutations at the carboxyl end of the FKH domain (two lysine residues (K415 and K416) to glutamic acid), when expressed in T cell lines, is localized to the cytoplasm [Lopes et al., 2006].

The fact that many tumors display cytoplasmic staining may be a result of defects in the nuclear localization signals of FOXP3, possibly due to acquired mutations. Mutations have been found in breast cancer, with numerous single base-pair changes detected in 23 out of 65 human breast carcinoma samples [Zuo et al., 2007b]. Frequent FOXP3 gene mutations and deletions, together with post-translational modifications and splice variations may result in cytoplasmic localization of FOXP3 protein in breast cancer cells, and the cytoplasmic function may differ from nuclear function [Wang et al., 2009; Hancock et al. 2009].

Since the role of FOXP3 is transcription regulation, which mainly occurs in the nucleus, a cytoplasmic FOXP3 localization could affect its biological role. The concept that FOXP3 cytoplasmic localization unables this transcription factor to perform its onco-suppressive functions has been suggested by two very recent studies.

The subcellular localization of FOXP3 within tumor infiltrating CD4+ T cells has been found to be predictive of recurrence in a cohort of oral squamous cell carcinoma patients [Weed et al., 2013]. CD4+ T cells showed a mutually exclusive FOXP3 expression in both cellular compartments, suggesting that two well defined subsets of FOXP3+ CD4+ T cells infiltrated the tumor. CD4+ T cells expressing FOXP3 in the cytoplasm were indicative of a favorable prognosis (no recurrence within three years) whereas a high concentration of CD4+ T cells showing nuclear FOXP3 localization was strongly associated with recurrence [Weed et al., 2013].

Moreover, Takenaka et al. (2013) reported a heterogeneous subcellular localization of FOXP3 in breast cancer cells, similarly to what we have reported in our IHC analyses. Cytoplasmic FOXP3 expression in tumor cells was significantly associated with larger tumor size and the presence of metastatic lymph nodes. The prognostic value of tumor-cell FOXP3 expression was determined according to FOXP3 subcellular localization. Nuclear FOXP3 expression was significantly associated with an improved overall survival in breast cancer patients, whereas cytoplasmic FOXP3 expression in tumor cells was found to significantly associate with poor overall survival [Takenaka et al., 2013].

Our evaluation of the metastatic capability of two FOXP3-overexpressing clones with different FOXP3 subcellular localization supported the hypothesis that failure of FOXP3 localization in the nucleus of cancer cells may contribute to tumorigenesis by inactivating its tumor-suppressive function. In fact, a significant decrease in the number of both spontaneous and experimental lung metastases was observed in mice injected with breast cancer cells showing a predominantly nuclear FOXP3 localization. Contrarily, when mice

were injected with breast cancer cells which showed a predominantly cytoplasmic FOXP3 localization a significant increase in the number of both spontaneous and experimental lung metastatic tumors was observed.

Taken together, these results suggested that FOXP3 intracellular localization is an important factor to be considered when assessing FOXP3 prognostic significance, confirming our hypothesis.

In conclusion, results of this thesis indicate that FOXP3 expression in breast cancer cells has crucial function in the development of metastases and suggest that its role depends on subcellular localization. Studies are ongoing to confirm the importance of FOXP3 subcellular localization in breast cancer.

Moreover, further investigations are required to identify the underlying mechanism(s) by which FOXP3 expression in breast cancer cells affects prognosis. The discovery of genes, molecules and/or cellular functions regulated by FOXP3 in tumor cells would afford the rational explanation of its role. The proof of FOXP3 pro-metastatic function would support FOXP3-targeted therapeutic strategies for breast cancer. However, since FOXP3 expression in Tregs has a crucial role of in regulating autoimmunity, the identification of molecules that are expressed/functional in tumor cells but not in Tregs would provide an avenue to develop potential therapeutic targets other than FOXP3 itself. The identification of other potential targets of FOXP3-dependent pathways may provide additional candidates for intervention in breast cancer.

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