Progression of Hormone-Dependent Mammary Tumors After Dormancy: Role of Wnt Pathway

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Abstract

The cancer stem cell theory suggests the existence of cells within breast cancers that possess the ability to self-renew and differentiate, albeit in a deregulated manner, which sustains tumor progression. Therefore, latent breast tumors and/or their metastasis may eventually resume growth thorough signals impacting on cancer stem cells and their niche. Since it has been determined that the Wingless Related Protein (Wnt) signaling is a likely niche factor and regulator of Mammary Stem Cells dynamics, it is conceivable that this pathway play a significant role in the "awakening" of dormant tumors. We have previously shown that in virgin females, MMTV-induced pregnancy-dependent (ER+PR+) tumor transplants were able to remain dormant for up to 300 days, but were able to resume growth after hormone stimulation. In a subsequent transplant generation, all these tumors became ER−PR− and grew in virgin females, indicating that cancer dormancy facilitated progression to hormone-independence. Our data also showed that mutations altering expression of genes involved in the Wnt pathway were prone to be selected during progression. To gain more insight into the mechanisms underlying these observations, we compared the gene expression profile of tumors that either underwent or not dormancy before progressing to hormone-independency. Confirming our

previously reported data, we found that the most significant up-regulated gene in hormone-independent tumors that progressed after dormancy was *Wnt1*. In addition, in this group we have determined a systematic down-modulation of previously described mediators of normal pubertal mammary gland development. Using a hierarchical clustering analysis to classify breast cancer patients, we have also identified a specific group of breast carcinomas with significant modulation of genes also deregulated in the MMTV-induced tumors that resumed growth after dormancy. Interestingly, that group of human samples was mainly composed by patients with basal-like breast carcinomas, which also showed down-regulation of genes associated to pubertal mammary development. Therefore, we believe that the cluster of co-regulated genes in basal human breast cancer and mouse mammary tumors resuming growth after dormancy might be mechanistically associated to the activation of Wnt pathway, which might induce proliferation from mammary progenitor basal cells.

Introduction

Breast Cancer

Cancer diseases result from the accumulation of mutations, chromosomal instabilities and epigenetic changes that together facilitate an increased rate of cellular evolution and damage, which progressively impairs the detailed and complex system of regulation of cell growth and cell death. Changes in gene activities are further influenced by the microenvironment within and in the vicinity of tumor cells as well as by exogenous factors. When one combines all of these aspects with inborn genetic variations among individuals, there is every kind of reason to expect tumors to display prodigiously diverse phenotypes. Breast tumors, like most solid cancers, are heterogeneous and consist of several pathological subtypes with different histological appearances of the malignant cells and different clinical presentations and

outcomes, and the patients show a diverse range of responses to a given treatment. Furthermore, breast tumor tissue shows heterogeneity with respect to its microenvironment, including the types and numbers of infiltrating lymphocytes, adipocytes, stromal and endothelial cells. The cellular composition of tumors is a central determinant of both the biological and clinical features of an individual's disease (Sorlie 2007).

The management of breast cancer has been dramatically changed with the advent of widespread screening programs and the systematic use of adjuvant hormonal therapy and chemotherapy. Recent data have shown that these changes are having a major impact in outcome, and despite increasing incidence breast cancer mortality is decreasing in most of the Western world. The overview of randomized adjuvant therapy trials has confirmed that systemic therapies (hormone therapy and chemotherapy) are producing cures and has also shown that tamoxifen is of benefit in only patients with estrogen positive (ER+) disease, effectively representing a form of targeted therapy. On the other hand trastuzumab therapy, either concurrent or sequential with adjuvant chemotherapy, is helping patients with HER2-positive tumors. These examples give credence to the old idea that breast cancers are a heterogeneous group of diseases. This has been further confirmed by molecular profiling of breast cancers using array technology showing the biologic and clinical heterogeneity of breast cancer is explained by differences in the genetic composition of the primary tumors (Brenton et al. 2005).

A straightforward interpretation of the recurrent appearance of several different patterns of gene expression among tumors of similar anatomical origin is to regard each as representing a different biological entity. One possible basis for the consistent differences in these patterns between tumor subtypes might be that they originate from different cell types. In fact, it has been found breast tumor subtypes with patterns of gene expression similar to those of luminal epithelial cells (the cells that line the duct and give rise to the majority of breast cancers) and patterns of at least one other subtype (termed basal) that

resembles the pattern found in basal epithelial cells of the normal mammary gland characterized by expression of cytokeratins 5, 6 and 17 (Roarty and Rosen 2010). Advances in microarray technology and pathology have led to improved techniques of sub-classifying breast tumors. Global gene expression profiling enables a subdivision into five individual subclasses (known as the Sorlie–Perou subtypes) found to convey a distinct prognostic and biological message in breast cancer above and beyond established clinical markers (Eriksson et al. 2012). The five groups are the Luminal A, Luminal B, Basal-like, ErbB2, and the Normal breast-like subtypes. Luminal A tumors are mostly ER+, have a low proliferation rate, and are of low grade, whereas Luminal B tumors are also mostly ER+, but may express low levels of hormone receptors, and are usually of high grade and have a higher proliferation rate than Luminal A. The Basal-like subtype, on the other hand, is often characterized by triple-negative tumors (ER-, PR-, and HER2-negative) and a certain cytokeratin pattern, and the ERBB2þ subtype shows amplification and high expression of the ErbB2 gene (also known as HER2 or HER2-neu). Lastly, there is the Normal breast-like subtype, which 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. It is, however, unclear whether the latter subtype is a distinct group or represents poorly sampled tissue (Sorlie 2007). Since breast cancers are a heterogeneous group of diseases, the signaling pathways involved in their progression may be different and unique for each type. In this chapter we will show that activation of the wnt pathway would be particularly relevant for ER+ PR+ mammary tumors to resume growth and progress after long dormancy periods.

Breast Cancer Dormancy and Hormone-Dependency

Clinical cancer dormancy is defined as an unusually long time between removal of the primary tumor and subsequent relapse in a patient who has been clinically disease-free. The condition is frequently observed in certain carcinomas (e.g. breast cancer), B-cell lymphoma, and melanoma, with relapse occurring 5–25 years later. Clinical data suggest that a majority of breast cancer survivors have cancer cells for decades but can remain clinically cancer-free for their lifetime. "A long time" has not been precisely defined, but is meant to exceed the time when recurrence is at a lower rate. In breast cancer, 20% of clinically disease-free patients relapse 7–25 years after mastectomy and, from 10 to 20 years, the rate of relapse is relatively steady at about 1.5%/year (Uhr and Pantel 2011).

A dormancy score based on gene signatures developed by combining dormancy expression profiles from a variety of cancer types was recently generated. Although neither recurrence information nor ER status was used to select these genes or to refine the scores, it was found that luminal, ER+ breast cancers were more likely to have a high dormancy score. Then, it was applied to both breast cancer cell line expression data as well as four published clinical studies of primary breast cancers and it was determined that ER+ breast cell lines and primary tumors had significantly higher dormancy signature scores (P<0.0000001) than ER- cell lines and tumors. Interestingly, positive dormancy genes were more synchronously up-regulated in patient tumors than in cell lines grown *in vitro*, demonstrating the importance of the tumor microenvironment on dormancy properties (Kim et al. 2012). In addition, these authors noticed that the rate of recurrence was significantly reduced for patients whose tumors were ER+ and had a high dormancy score. These results were consistent with the observed clinical outcome that ER+ tumors tend to recur later than ER- tumors (EBCTCG 2005). It has been reported that women taking estrogen and progesterone for menopausal symptoms (hormone replace treatment: HRT) showed an increased risk of breast cancer development, and it has been argued such an effect might be due to the growth of dormant ER+PR+ tumors. Therefore, these women would already had breast cancer at the start of HRT but were unaware of it. In fact, breast autopsy studies of women

who had died of non-breast cancer-related causes and who had exhibited no evidence of breast disease during life found that, on average, 8.9% had undiagnosed ductal carcinoma *in situ* and 1.3% had undiagnosed invasive breast cancer. Remarkably, it was estimated that 82% of these micro-tumors would have been mammographically undetectable and these studies were restricted to women over 40, when they became candidates for HRT. Therefore, these undiagnosed microdiseases, if they fall into the ER+, PR+ luminal subtype, are susceptible to cancer stem cell reactivation and expansion by exposure to hormones. The hypothesis proposed by Horwitz and Sartorius (2008) would also explain why the increased breast cancer risk observed with E+P treatment is restricted to ER+PR+ disease. These ideas also have direct impact on the use of HRT in breast cancer survivors, because any residual disease would be subject to the same hormonal effects. In fact, when HRT safety was analyzed in 447 breast cancer survivors, after 5 years, there were 39 recurrences among 221 women on HRT, compared with 17 recurrences among 221 no-hormone controls (Horwitz and Sartorius 2008).

Stem Cells and Breast Cancer Dormancy

Traditionally, the mechanisms proposed to account for tumor dormancy have hinged on interactions between cancer cells and host cells within the tumor microenvironment. For example, dormancy has been suggested to arise from a requirement to either switch off host immune surveillance or switch on angiogenesis at sites of latent disease. However, there is little or no evidence that immune suppression of the host, as occurs in organ transplant recipients, triggers reactivation of minimal residual disease and breast cancer relapse Likewise, in vivo evidence for an angiogenic switch regulating reactivation of dormant breast cancer is lacking. Alternatively, the tumor dormancy phenomenon might be explained by an emerging concept that places cancer stem cells at the root of solid tumors. Particularly, in breast cancer it is conceivable that mechanisms governing survival and quiescence in normal mammary stem cells may likewise govern survival and quiescence within dormant tumors. This supports the notion that dormant mammary cancers may harbor transformed mammary progenitor cells (Gestl et al. 2007).

It has been postulated that the tumor cells in G0/G1 may be the precursors of the population of tumor cells underlying clinical cancer dormancy. Initially, this appeared to be a reasonable assumption, particularly because dormant tumor cells are the ones that are resistant to conventional therapy and persist. However, studies on circulating tumor cells (CTCs) in breast cancer survivors 7–22 years after mastectomy and clinically disease-free challenge this notion. The short half-life of these CTCs (1–2 h) indicates that there must be a replicating population of tumor cells at secondary sites that replenishes the CTCs and keeps them at the same low level for many years (Meng et al. 2004). Similarly, our experimental data from MMTV-induced pregnancy dependant mammary tumors show that in the absence of hormone stimulus ER+ PR+ tumors remain dormant, but this status does not result from cells remaining in G0, but cells dividing slowly and dying at similar rates (Gattelli et al. 2004).

Mammary stem cells (MaSCs) are the key drivers of self-renewal and differentiation throughout development, particularly in active growth phases, but these cells are also essential for the maintenance of tissue homeostasis. The niche, hypothesized as the local tissue microenvironment, is essential to maintain and regulate stem cells within the mammary gland. The existence of MaSCs was demonstrated by reconstitution of the mammary gland after transplantation of a mammary epithelial fragment into the cleared fat pad (Kordon and Smith 1998). More recent studies have identified functional MaSCs by surface marker expression followed by mammary reconstitution assays, leading to a preliminary understanding of the hierarchical organization of epithelial subtypes that comprise the mammary ductal tree. This has facilitated the investigation of the molecular signaling pathways regulating MaSC self-renewal

and lineage commitment. It has been determined that the Wingless Related Protein (Wnt) signaling is a likely niche factor and regulator of MaSC dynamics, yet how these signals integrate with systemic hormones and local growth factors remains unclear. Similar to normal tissues, the cancer stem cell theory suggests the existence of cells within breast cancers that possess "stem-like" qualities in their ability to self-renew and differentiate, albeit in a deregulated manner, ultimately sustaining tumor progression and driving tumor heterogeneity. The fact that tumorigenesis, in many ways, may follow the hierarchical nature of an adult tissue, suggests that similar or related pathways are involved in both MaSC and cancer stem cell (CSC) dynamics. Specifically, Wnt signaling pathways play important roles in multiple aspects of both MaSC and CSC biology (Roarty and Rosen 2010).

Somatic stem cells maintain tissue homeostasis in the face of wear and injury. To perform this function over the lifetime of the organism, somatic stem cells possess both a strong intrinsic cell survival program and a capability to enter into, and emerge from, extended quiescence. Robust survival and reversible quiescence are not only biological hallmarks of putative mammary stem cells; they are also clinical hallmarks of minimal residual disease (MRD) as encountered in breast cancer patients. Therefore, mechanisms governing survival and quiescence in normal mammary stem cells may likewise govern survival and quiescence within dormant MRD. Viewed this way, the failure of adjuvant therapy to cure the majority of breast cancer patients at risk for relapse might be blamed on a population of malignant progenitor cells resident within MRD that persists despite treatment and ultimately reconstitutes the malignancy (Gestl et al. 2007). The hierarchical model of mammary gland development proposes the existence of stem and progenitor cells, which are under tight control of both cell intrinsic and extrinsic cues, and give rise to the mature mammary epithelium of either the luminal or basal/myoepithelial lineage by a series of lineage-restricted events. Currently, significant challenges exist in understanding the complex interactions between MaSCs, their more committed progeny, and differentiated epithelial cells, all of which are required to maintain MaSC activity and the functional integrity of the mammary gland. To date, only a few of the paracrine mediators of hormonal action have been identified, including Amphiregulin, receptor Activator for Nuclear Factor κB Ligand (RANKL) and Wnt4. Other Wnt proteins likely represent important mediators of hormone action, although their specific functional roles have yet to be defined (Roarty and Rosen 2010).

The Wnt Signaling Pathway

Activation of Wnt signaling is initiated by the binding of Wnt ligands to Frizzled (FZD) 7 transmembrane receptors in combination with Lrp5/6 or Ror1/2 co-receptors. The binding of Wnt ligands to these receptor complexes is regulated by a number of proteins that either bind to the receptor component (such as DKKs, SOST, or Wise/SOSTDC1) or to the Wnt ligand itself, such SFRPs. Multiple downstream signaling pathways are activated following Wnt ligand binding. The best characterized is the *canonical Wnt pathway*. In the absence of Wnt ligand, the cytoplasmic β-catenin protein is degraded through the action of a multi-protein complex, centered on the axin protein, which phosphorylates β-catenin aminoterminal residues, marking it for ubiquitin-dependent degradation. Instead, following Wnt binding to the FZD-Lrp5/6 receptor complex, β-catenin turnover is inhibited leading to its accumulation and subsequent translocation into the nucleus where it forms a complex with members of the T-cell factor/lymphoid enhancing factor (Tcf/Lef) transcription factor family, inducing Wnt target gene expression, such cyclin D1, axin2 and c-myc (Saito-Diaz et al. 2013).

In the *non-canonical Wnt pathways*, the Wnt signal is transduced in a FZD-dependent but Lrp5/6-independent manner, and activates two downstream signaling branches. Wnt induces cytoskeletal changes via activation of the small GTPases RHOA and RAC1 regulating cell polarity (*WNT/PCP pathway*). Wnt also modulates cell adhesion, motility and gene transcription by NFAT

via activation of the heterotrimeric G-proteins, calcium calmodulin dependent kinase II, and protein kinase C (*WNT/Ca++ pathway*).

The Wnt Signalling Pathway in the Mammary Gland

Wnt signals regulate a variety of key processes, to bring about tissue-level effects including cell proliferation, differentiation, polarity and migration. Given the importance of the pathway in so many tissues and its links to the regulation of cell proliferation, it is not surprising that the mutations which induce canonical Wnt signalling drive carcinogenesis, whilst inactivation of the Wnt pathway leads to loss-of-tissue architecture. This highly complex pathway is tightly regulated by positive and negative feedback signals and through tissue-specific interactions with other signal transduction pathways.

The prototype member of the Wnt family, Wnt-1, was originally identified as a site of integration by the mouse mammary tumor virus (MMTV). Although MMTV expression of Wnt-1 drives the formation of mouse mammary tumors it is not expressed at detectable levels in the normal mammary gland. However, other members of the Wnt family are naturally expressed in the breast (Wnt-2, Wnt-4, Wnt-5a, Wnt-5b, Wnt-6 and Wnt-7), which are differentially regulated during the phases of mouse mammary gland development including puberty, pregnancy, lactation and involution (Jarde and Dale 2012).

Following embryonic establishment of a mammary placode, later embryonic stages lead to the invasion of mammary epithelium into the underlying fatty stroma. These minimal epithelial ducts then remain quiescent until the initiation of postnatal ductal development that follows the rise of estrogen levels immediately prior to and during puberty. Postnatal development of the mammary gland is classified into two different steps: ductal development followed by lobular development, which primarily occurs during pregnancy. During sexual maturation, the end of each duct forms a terminal end bud (TEB), a highly proliferative epithelial structure enriched in mammary stem cells.

The end bud elongates and ramifies to form the mammary ductal architecture. Definitive evidence for a role for Wnt signaling in mammary ductal elongation came from loss-of-function studies of the Wnt co-receptors Lrp5 and Lrp6 (Lindvall et al. 2006, 2009). In Lrp5−/- and Lrp6+/, the number of TEBs per gland and the branching complexity were reduced, whilst over-expression of Lrp6 from the MMTV promoter induced increased ductal branching in the young virgin animal's mammary glands (Zhang et al. 2009). These results suggest that the absence of Wnt-responsive mammary cells delays the normal development of the gland and that active Wnt signals and Wnt co-receptor drive the formation of a mature ductal tree. During pregnancy, rapid growth and differentiation of lobulo-alveolar structures fills the stroma with a dense network of epithelial ducts and alveoli (Richert et al. 2000). In the mouse, the Wnt reporter Tcf/Lef1-βgal was prominently expressed during mid-pregnancy within the epithelial cells of the developing alveoli (Boras-Granic et al. 2006) suggesting a role for the Wnt pathway during lobulo-alveolar development.

To demonstrate the requirement for Wnt signaling during mammary gland lobular-alveolar development, studies were conducted using suppressor components of the pathway. The lactating mammary gland from mice over-expressing axin from the MMTV promoter showed severe hypoplasia of the lobulo-alveolar structures, associated with reduced β-catenin and cyclin D1 expression and increased apoptosis (Hsu et al. 2001). Similarly, the over-expression of a dominant negative β-catenin-engrailed fusion protein that lacks the C-terminal β-catenin signaling domain revealed a severe inhibition of lobulo-alveolar development in pregnant mice, associated with reduced proliferation and increased apoptosis (Tepera et al. 2003). These results suggest that the Wnt pathway play a major role during pregnancy and that Wnt signals may provide survival and proliferative signals.

The Wnt pathway may also be involved in the remodeling of the mammary gland during involution. Some Wnt genes were re-expressed during involution, including Wnt-2, Wnt-5a,

Wnt-5b and Wnt-7b. The activation of Wnt signaling in the absence of survival factors may drive involution-induced apoptosis, as previously observed with myc expression in the absence of other growth factors (Jarde and Dale 2012). Taken together, these studies have demonstrated the involvement of the Wnt canonical signaling pathway in the mammary gland development. On the other hand, although crucial for embryo development (Seifert and Mlodzik 2007), the involvement of non-canonical Wnt signaling pathways in normal mammary development is not well understood.

Mammary Stem Cells, Target of the Wnt Signals?

Several lines of evidence suggest that Wnt signaling is involved in the maintenance of the stem/progenitor pool in the mammary gland. It has been demonstrated that the stem/progenitor fraction was increased in the hyperplastic mammary glands of MMTV-Wnt-1 and MMTV-stabilized β-catenin transgenic mice as well as in primary cultures of mammary epithelial cells treated with Wnt-3a (Li et al. 2003; Liu et al. 2004; Shackleton et al. 2006). It has also been demonstrated that MMTV-Wnt-1 and MMTV-stabilized b-catenin induced Wnt signaling in distinct progenitor cell populations (Teissedre et al. 2009).

Combinations of cell surface markers, namely Lin⁻, CD24⁺, CD29^{high} or Lin⁻, CD24⁺, CD49f^{high}, have been described that allow the isolation of cell populations that are highly enriched for mouse mammary stem cells. The majority of stem/progenitor cells in the virgin mouse mammary epithelium were localized in the basal compartment. Interestingly, histological analysis of Wnt-reporter transgenic lines indicated that Wnt-responsive cells were also located in the basal compartment. Isolation of Axin2-lacZ positive cells stained with a live-cell fluorogenic β-galactosidase substrate allowed the characterization and isolation of Wnt-responsive cells in combination with stem cell surface markers. Zeng and Nusse (2010) demonstrated that Wnt-responsive cells generated mammary outgrowths

more efficiently than Axin2-lacZ- negative cells. They also noted that Axin2-lacZ+, Lin-, CD24+, CD29high cells expressed high levels of Lrp5 and Lrp6, in contrast to Axin2-lacZ + equivalent cells. Accordingly, Lrp5−/− mammary epithelial cells exhibited little to no stem cell activity in limiting dilution transplants, whilst Lrp5-high mammary cells were enriched for stem cell activity. In addition, by using s-SHIP promoter-GFP transgenic model that selectively identifies mammary stem cells it has been shown that Wnt-1 increased the number of SMA+; s-SHIP-GFP+, providing more support for the idea that Wnt signaling stimulates the size of this population (Bai and Rohrschneider 2010). Interestingly, although Wnt ligands are expressed by luminal and basal cells, only the basal/myoepithelial cells express Frizzled receptor genes (Kendrick et al. 2008), indicating a directionality of Wnt signaling. This has been supported by the strong induction of Wnt-4 mRNA by progesterone in the luminal population, suggesting that Wnt signals would be paracrine effectors of progesterone-induced mammary stem cell expansion both during pregnancy and at the diestrum phase of the estrus cycle (Jarde and Dale 2012).

Recently, it has been demonstrated an up-regulation of differentiation genes and a marked decrease in the Wnt/Notch signaling ratio in basal stem/progenitor cells of parous mice. This was associated with down-regulation of potentially carcinogenic pathways and a reduction in the proliferation potential of this cell subpopulation both in vitro and in vivo. These observations may identify why early pregnancy has a strong protective effect against breast cancer in humans and rodents, and suggest that inhibitors of the Wnt pathway may be used to mimic the parity-induced protective effect against breast cancer (Meier-Abt et al. 2013).

Wnt signaling would be relevant not only for normal mammary stem cells, but also for survival and expansion of breast cancer stem cells and may contribute to tumor invasion at distant organs. It has been recently reported that periostin (POSTN), a component of the extracellular matrix, is expressed by fibroblasts in the normal tissue and in the stroma of the primary tumors.

Infiltrating tumor cells need to induce stromal POSTN expression in the secondary target organ (*e.g.* the lung) to initiate colonization. The expression of this protein is required to allow cancer stem cell maintenance, and blocking its function prevents development of metastasis. Interestingly, it has been postulated that POSTN exerts this effect recruiting Wnt ligands and thereby increasing Wnt signalling in cancer stem cells (Malanchi et al. 2011).

Association Between Hormone-Dependency and Wnt Activation in Dormant Breast Cancer

To understand the biology of dormant breast cancer *in vivo*, diverse mouse models have been employed (Aguirre-Ghiso 2007; Brackstone et al. 2007) being the Wnt signaling pathway pointed out as relevant in at least some of them (Gestl et al. 2007), particularly in hormone-dependent mammary tumors (Gattelli et al. 2004). We have previously shown that in virgin females, MMTV induced pregnancy-dependent (PD) tumor transplants were able to remain dormant for up to 300 days. During that period, these tumors synthesized DNA, expressed high levels of estrogen and progesterone receptors (ER+ PR+) and were able to resume growth after hormone stimulation. Surprisingly, in a subsequent transplant generation, all these tumors were fully able to grow in virgin females, they expressed very low levels of ER and PR (ER− PR−) and had a monoclonal origin; then, they behaved as hormone-independent (pregnancy-independent: PI) tumors. Therefore, our data indicated that although hormone stimulation was required to rescue PD tumors from dormancy, PI clones prevailed once tumor growth was re-established after long dormancy periods.

In the MMTV as in other hormone-dependent breast cancer animal models, it has not been possible to foresee when and how tumor growth would be released from hormone control, making it very difficult to pinpoint the pathways involved. Interestingly, leaving virgin females carrying latent pregnancy-dependent tumor cells without hormone treatment for more than 4 months allowed us to predict hormone independent behavior in the following passage.

We determined that PI tumor passages arising after a dormant phase usually displayed a lower level of glandular differentiation together with epithelial cell trans-differentiation (squamous metaplasias were frequently detected in those samples). Interestingly, it has been shown that squamous differentiation is the most characteristic histological pattern of mammary tumors of genetically engineered mice with activated Wnt/b-catenin signaling components. Our morphological data showing a higher tendency to form solid cords together with trans-differentiated foci and a very low incidence of pure acinar structures in tumors arising from long dormancy periods suggested to us that mutations altering expression of genes involved in the Wnt pathway were prone to be selected in the progression of PD dormant tumors towards hormone-independence (Gattelli et al. 2004).

Genes Affected by Dormancy Period in Mouse Mammary Tumors: Rationale

In order to gain more insight into the effects triggered by dormancy in hormone-dependent mammary tumors, we compared the gene expression profile of PD and PI MMTV-induced tumors that either underwent (D) or not (ND) long periods of latency. To this purpose, RNA samples were obtained from ER+ PR+ tumors growing in pregnant females, which had been either impregnated immediately (PD/ND) or more than 4 months after tumor transplantation (PD/D) and from ER− PR− transplants that came from either PD/ND (called PI/ND) or PD/D (called PI/D) tumors. To clarify our strategy, Fig. 6.1a, b shows an example of each pattern of tumor growth.

Microarray Data Processing, Statistical and Data Mining Analyses

Total RNA from mammary tumors was obtained using the RNeasy Mini Kit (Qiagen) and hybridized to Affymetrix GeneChip Mouse Gene 1.0 ST
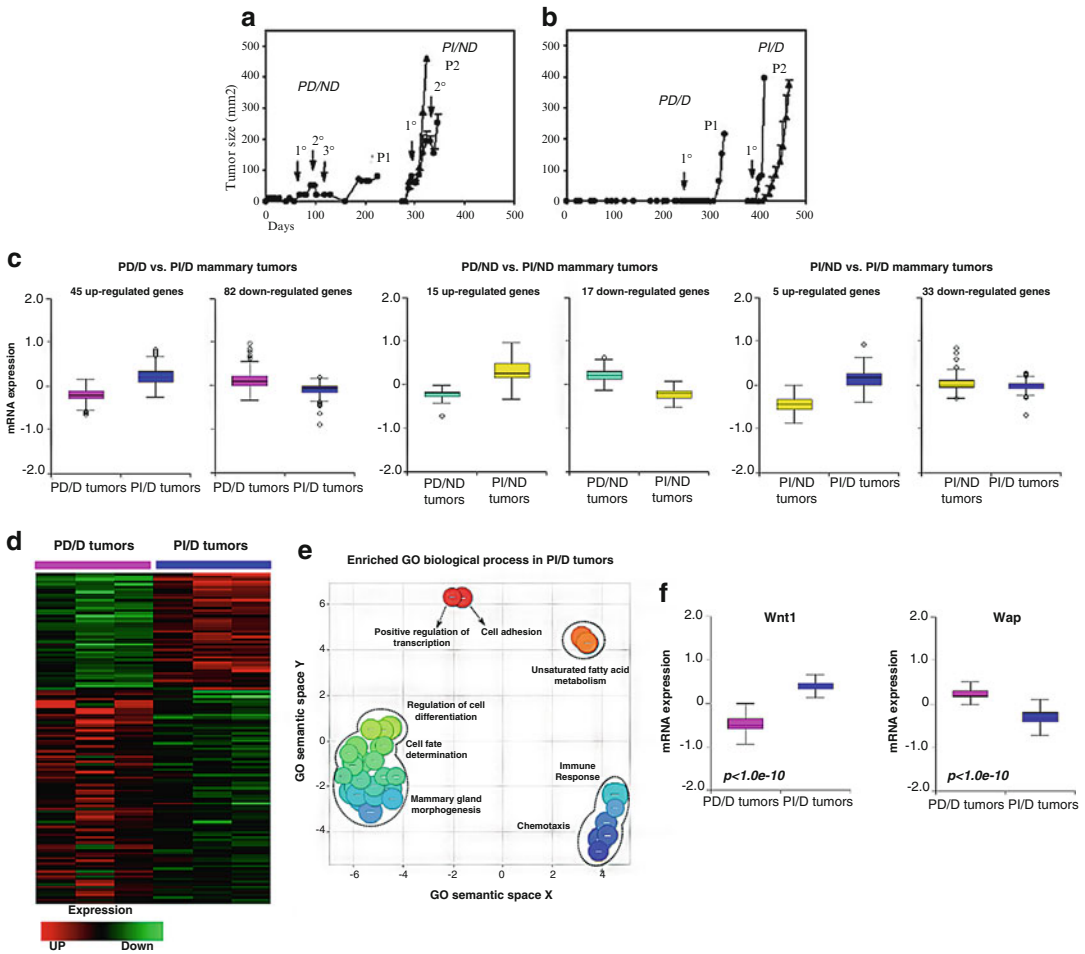
Fig. 6.1 Impact of hormone-dependency and dormancy on the expression profile of MMTV-induced mouse mammary tumors. (**a**) Growth profile of a PD/ND (pregnancy-dependant without dormancy) tumor and the following PI/ND (pregnancy-independent without dormancy) passage. (**b**) Growth profile of a PD/D (pregnancy-dependant with dormancy) tumor and the following PI/D (pregnancy-independent with dormancy) passage. *Arrows* with 1^0, 2^0, 3^0 indicate deliveries of pregnant female mice. *Circles*: parous females. *Triangle*: virgin females. (**c**) Comparison of gene expression profiles obtained from the MMTV-induced tumors shown in Table 6.1: PD/D, PI/D, PD/ND and PI/ND. (**d**) Heatmap of 127 differentially expressed genes of PD/D vs. PI/D mouse mammary tumors (q-value < 0.05).

Color scale at *bottom* of picture is used to represent expression level: low expression is represented by *green*, and high expression is represented by *red*. (**e**) Scatterplot graph showing the representative clusters, after redundancy reduction of the statistical significant GO terms (p < 0.025) enriched in the PI/D gene expression signature, in a two dimensional space related to GO terms' semantic similarities. *Bubble color* indicates the p-value of GO terms (expressed as Log10 p-value) and *bubble size* indicates the frequency of the GO term in the underlying GOA database (bubbles of more general terms are larger). (**f**) Box plots showing increased expression of *Wnt1* and decreased expression of *Wap* mRNA expression levels in PI/D tumors when compared to PD/D tumors (p < 0.00001)

Array (Affymetrix) according to the standard Affymetrix protocols. For the analysis, two to three biological replicates of each tumor pattern: PD/ND, PD/D, PI/ND and PI/D were used (Table 6.1). A total of ten tumor samples were analyzed. Importantly, these tumors belonged to ten different independent tumor lines. Before to perform the microarray, the quality of the RNA from tumors was analyzed using Eukaryote Total RNA Nano assay (2,100 Expert). Briefly, we carried out QC and normalization procedures in R/Bioconductor using the simpleaffy package

Table 6.1 MMTV-induced tumors used in the gene expression profiling analysis

	Tumor	Status
1.	LA13	PD/ND
2.	LA0	PD/ND
3.	LACAYB	PD/D
4.	LA4	PD/D
5.	LA	PD/D
6.	2216	PI/ND
7.	LA45	PI/ND
8.	2312	PI/D
9.	2178	PI/D
10.	2314	PI/D

(Wilson and Miller 2005). We employed the Robust Multichip Average algorithm for background adjustment, quantile normalization and probe set values summarization (Irizarry et al. 2003). To compare the mammary gland tumor expression profiles, we utilized the Rank Products test (Breitling et al. 2004). Statistical analysis and heat-map visualization of differentially expressed transcripts ($p < 0.01$; $q < 0.05$) were done with the MultiExperiment Viewer software (MeV 4.8) (Saeed et al. 2003).

For automated functional annotation and gene enrichment analysis, we used the Database for Annotation, Visualization and Integrated Discovery (DAVID) (Huang da et al. 2008). The DAVID resource calculates over-representation of specific biological themes/pathways with respect to the total number of genes assayed and annotated. REViGO resource was employed to summarize and visualize the enriched GO terms in an scatterplot graph based on the p-values obtained by DAVID. This allows the identification of biological themes/pathways within a specific list of differentially expressed genes.

To identify the molecular pathways that were mainly affected in hormone-independent tumors that evolved after long dormancy periods (PI/D), we looked for protein/gene interaction networks using the STRING resource ('Search Tool for the Retrieval of Interacting Genes/Proteins') (von Mering et al. 2005). The database STRING aims to collect, predict and unify most type of protein-protein associations, including direct and indirect associations. STRING runs a set of prediction algorithms, and transfers known interactions from model organisms to other species based on predicted orthology of the respective proteins. In order to identify each gene in the database, we used both mouse gene name and Entrez gene ID in the 'protein-mode' application. The analysis input options were 'co-occurrence', 'co-expression', 'experiments', 'databases', and 'text mining' data.

To perform a comparative analysis of PI/D associated genes in human breast cancer, we analyzed 1,114 primary carcinomas obtained from four independent studies available in public databases. The frozen RMA preprocessing algorithm was applied to the Affymetrix HGU133 Plus2 platform based studies (GSE26639 n = 226, GSE21653 n = 266 and GSE20685 n = 327) to generate a compiled dataset of 819 breast carcinomas. In addition, we analyzed the van de Vijver dataset that included 295 early-stage breast cancer samples. This gene expression profile was derived by researchers from the Netherlands Cancer Institute and Rosetta Inpharmatics—Merck using Agilent Hu25K oligonucleotide (60mer) microarray (Agilent Technologies, Palo Alto, CA-USA).

Results and Discussion

Transcriptomic Changes Affected by Dormancy in Mouse Mammary Tumors

Our first goal was to identify gene expression changes that occurred during transition from hormone-dependent tumors that remained dormant in the absence of pregnancy and resumed growth upon impregnation (PD/D) to those that arose after transplantation of PD/D tumors showing hormone-independent behavior (PI/D). The statistical analysis of the gene expression profiling data identified 127 genes differentially expressed between these tumor types (q < 0.05; 2 fold changes). Among the 127 transcripts, 45 were up-modulated and 82 were down-modulated

in the PI/D tumors (Fig. 6.1c, d). Interestingly, when we examined the transcripts that were differentially expressed in hormone dependent tumors that did not go through long latency periods (PD/ND) and the hormone-independent passages that evolved from other PD/ND tumors (PI/ND), our statistical analysis revealed that only 32 genes were differentially expressed (Fig. 6.1c). Among these transcripts, 15 were up-modulated and 17 were down-modulated in the PI/ND tumors.

We used the DAVID resource for automated annotation and enrichment analysis of the differentially expressed genes based on GO database. In addition, we employed REVIGO resource for summarization and visualization of the significant GO term semantic similarities (p<0.05). Among the statistically significant over-represented categories under "Biological Process," we found the mammary gland morphogenesis/development, cell fate determination, differentiation and cell adhesion related genes (Fig. 6.1e). In addition, categories of genes found in the immune and inflammatory response were also highly enriched related genes in the PI/D mammary tumor expression profiles. Table 6.2 shows the top statistically significant deregulated transcripts in dormancy during the progression from hormone dependency to the independency. Interestingly, the most significant up-regulated gene was *Wnt1* (*Wingless-related MMTV integration site 1*) (Fig. 6.1f). We have also identified up-modulation of *Stra6* (*Stimulated by retinoic acid gene 6*) and Enpp2 (*Ectonucleotide pyrophosphatase/phosphodiesterase 2*). *Stra6* and *Enpp2* genes encode cell surface antigens that are known to be synergistically induced by Wnt-1 and RAR signaling in the mouse mammary epithelial cell line C57MG/Wnt-1 (Tice et al. 2002). *Stra6* mRNA up-modulation was also observed in hyperplastic mammary gland and mammary tumors from transgenic mice expressing Wnt-1 compared with normal mammary tissue (Szeto et al. 2001). Enpp2 (also known as *Autotaxin*) has been implicated in human cancer progression as factors contributing to the motility, angiogenic properties, and metastatic spread of cancer cells (Nam et al. 2000). *Enpp2* mRNA is over-expressed in

human breast carcinomas compared to adjacent normal breast tissue, with the lowest frequency of expression in luminal A breast cancer subtype (Popnikolov et al. 2012). It is important to point out that none Wnt-induced gene was identified as deregulated tumors that did not go through dormancy periods were compared (*i.e.* in PD/ND vs. PI/ND analysis), suggesting that Wnt pathway is particularly affected during progression of hormone dependent tumors that resume growth after dormancy.

Interestingly, in PI/D tumors a systematic down-modulation of previously described mediators of normal pubertal mammary gland development (McBryan et al. 2008) were identified. For example, *Areg* (*Amphiregulin*), *Cited1* (*Cbp/p300-interacting transactivator 1*), *Cxcl15* (*Chemokine C-X-C motif ligand 15*), *Foxa1* (*Forkhead ox A1*), *Lalba* (*Lactalbumin alpha*), *Mmp3* (*Matrix metallopeptidase 3*), *Pdk4* (*Pyruvate dehydrogenase kinase isoenzyme 4*), *Igfals* (*Insulin-like growth factor binding protein acid labile subunit*), *Sftpd* (*Surfactan associated protein D*), *Lpl* (*Lipoprotein lipase*), *Retnla* (*Resistin like alpha*), *Bche* (*Butyrylcholinesterase*), *Efemp1* (*Epidermal growth factor-containig fibulin-like extracellular matrix protein 1*), *Ubd* (*Ubiquitin D*), *Car13* (*Cabonic anhydrase 13*). Therefore, these genes would be involved in hormone-controlled (mainly estrogen and progesterone) mammary epithelial proliferation. In the transition to hormone-independent behavior their participation in tumor development, together with expression of ER and PR, would be lost.

Noteworthy, several proteins involved in the Wnt signalling pathway (e.g. *Wnt2*, *Wnt5a*, *Fzd1*, etc.) have been identified as modulators of pubertal mammary gland development (Kouros-Mehr and Werb 2006). However, differently from pregnancy-associated mammary development during which Wnt family members are thought to act as paracrine mediators of progesterone signaling (Brisken et al. 2000), it has not been proposed the mechanism by which ovarian hormones and wnt-related factors are related during mammary development of pubertal mice.

The development of the mouse mammary epithelium in virgin females is regulated by

Table 6.2 Top up-regulated and down-regulated transcripts in mammary cells from PI/D tumors

| Gene name	Entrez ID	Fold change	p-value
Up-modulated transcripts			
Wnt1 (Wingless-realted MMTV integration site 1)	22408	+2.48	<1.0e-8
Hbbt1 (Hemoglobin subunit beta-1-like)	100503605	+2.48	<1.0e-8
Serpinb11 (Serine peptidase inhibitor, clade B 11)	66957	+2.39	<1.0e-8
Lrrn1 (Leucine rich repeat protein 1 neuronal)	16979	+2.38	<1.0e-8
Serpinb10 (Serine peptidase inhibitor, clade B 10)	241197	+2.34	<1.0e-8
Krt6b (Keratin 6B)	16688	+2.24	6.31e-5
Dsg3 (Desmoglein 3)	13512	+2.23	7.53e-6
Hbb-b1 (Hemoglobin beta adult major chain)	15129	+2.22	1.88e-6
Lhfpl3 (Lipoma HMGIC fusion partner-like 3)	269629	+2.21	1.41e-6
Gabrg1 (Gamma-aminobutyric acid receptor 1)	14405	+2.20	2.59e-5
Dact1 (Dapper homolog1)	59036	+2.19	9.42e-7
Gabrg3 (Gamma-aminobutyric acid receptor 3)	14407	+2.19	3.29e-5
Nlgn1 (Neuroligin 1)	192167	+2.19	7.06e-6
Snord61 (Small nucleolar RNA C/D box 61)	353374	+2.18	1.32e-5
Cst6 (Cystatin E/M)	73720	+2.18	6.97e-5
Lemd1 (LEM domian containing 1)	213409	+2.18	3.58e-5
Asxl3 (Additional sex combs like 3)	211961	+2.17	8.01e-6
Cyp2b19 (Cytochrome P450 family 2b)	13090	+2.17	1.18e-5
Ptprz1 (Protein tyrosine phosphatase receptor Z1)	19283	+2.17	1.13e-5
Pde3a (Phosphodiesterase 3A)	54611	+2.16	7.06e-6
Stk32b (Serine/threonine kinase 32B)	64293	+2.16	6.97e-5
Down-modulated transcripts			
Cxcl15 (Chemokine C-X-C ligand 15	20309	−2.42	<1.0e-8
Tph1 (Tryptophan hydroxylase 1)	21990	−2.36	<1.0e-8
Igf2 (Insulin-like growth factor 2)	16002	−2.35	<1.0e-8
Olah (Oleoyl-ACP hydrolase)	99035	−2.34	<1.0e-8
Saa2 (Serum amyloid A2)	20209	−2.33	<1.0e-8
Wap (Whey acidic protein)	22373	−2.32	<1.0e-8
Wfdc12 (WAP four-disulfide core domain 12)	192200	−2.32	9.44e-7
Peg10 (Paternally expressed 10)	170676	−2.30	<1.0e-8
Lao1 (L-amino acid oxidase 1)	100470	−2.29	<1.0e-8
Csn1s2b (Casein alpha s2-like B)	12992	−2.28	<1.0e-8
Ttc6 (Tetratricopeptide repeat domain 6)	100503562	−2.28	3.15e-5
Hepacam2 (HEPACAM family member 2)	101202	−2.27	4.7e-6
Foxa1 (Forkhead box A1)	15375	−2.26	9.42e-7
Glycam1 (Glycosylation dependent cell adhesion 1)	14663	−2.26	<1.0e-8
Tcfap2b (Transcription factor AP-2 beta)	21419	−2.26	1.41e-6
Hpgd (Hydroxyprostaglandin dehydrogenase 15)	15446	−2.25	<1.0e-8
Defb1 (Defensin beta 1)	13214	−2.25	1.41e-5
Retnla (Resistin like alpha)	57262	−2.23	6.36e-5
Ubd (Ubiquitin D)	24108	−2.20	1.77e-4
Cxcl13 (Chemokine C-X-C motif ligand 13)	55985	−2.19	5.93e-5
Itih2 (Inter-alpha trypsin inhibitor 2)	16425	−2.19	5.32e-5
Areg (Amphiregulin)	11839	−2.18	4.15e-5

estrogen, progesterone and prolactin, together with a myriad of co-regulators and downstream paracrine effectors. Cited1 is one of these factors since it is a co-regulator of estrogen (McBryan et al. 2008) signaling during puberty and it is also a mediator of pubertal ductal growth. Importantly, it has been demonstrated that Cited1 is primarily regulated in vitro and in vivo by the Progesterone receptor (Sriraman et al. 2009), a gene whose expression resulted down-modulated in the PI/D mammary tumors.

One of the most early gene expression changes induced by estrogen during pubertal mammary gland development is the induction of *Areg*. Anphiregulin is a member of the epidermal growth factor receptor family of ligands that binds exclusively to ErbB1. The AREG secretion activates ERK and AKT intracellular pathways to regulate cell proliferation (Kariagina et al. 2011). In normal mammary glands, estrogen and progesterone induce the expression of *Areg* involving the transcriptional co-activator Cited1. *Areg* and *Cited1* KO mice phenocopy ER KO mice as ablation of these genes result in severely impaired ductal development during puberty, suggesting that they are essentials for ductal elongation as well as estrogen-induced proliferation and terminal end-bud formation. Furthermore, *AREG* and *CITED1* genes are co-expressed with ER-alpha receptor in a subgroup of primary breast carcinomas from women exhibiting good prognosis (McBryan et al. 2008). This observation is consistent with the well-established correlation between well-differentiated carcinomas (low tumor grade) and good outcome.
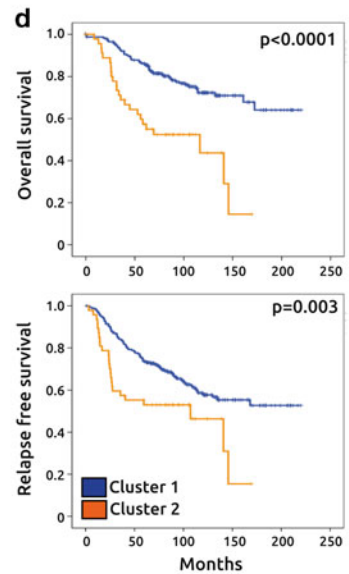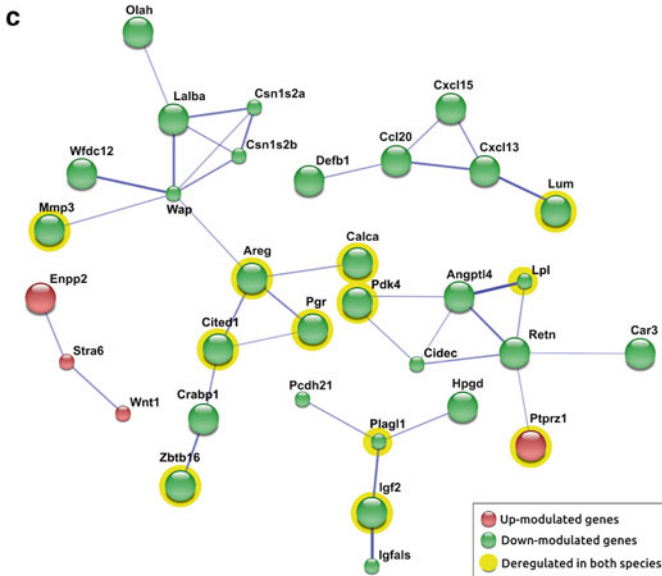
Another common PI/D down-regulated gene encodes the whey acidic protein, WAP (Fig. 6.1f). This mouse milk protein, was widely described as a typical marker of mammary gland differentiation that plays a negative regulatory role in the cell-cycle progression of mammary epithelial cells through an autocrine/paracrine mechanism (Ikeda et al. 2004). Interestingly, expression of WAP and β-casein was reduced in *Areg* KO mice (McBryan et al. 2008) and overexpression of the WAP transgene in the mammary gland depressed proliferation and advanced functional differentiation of mammary epithelial cells. In addition, it

has been shown that WAP exerted an inhibitory function in the degradation of laminin, which depressed proliferation, tumorigenesis, and invasion of human breast cancer cells (Nukumi et al. 2007). These data suggest that WAP down-regulation in the PI/D tumors would be associated not only to the loss of terminal differentiation, but also to an increase in the proliferative capacity and invasiveness of these tumors.

To further explore the relevance and the prognostic value of PI/D gene expression signature, we analyzed a total of 1,114 human primary breast carcinomas derived from 4 independent publicly available gene expression-profiling studies. We used hierarchical clustering (HCL) analysis to classify the patients into groups according to the human orthologous genes of the mouse PI/D gene expression signature and then determined the overall and relapse-free survival rates for these groups. This procedure allowed us to identify a specific group of breast carcinomas with significant up- or down-modulation of genes deregulated in PI/D mammary tumors (Fig. 6.2a, b). Interestingly, this group of samples was mainly composed by patients with basal-like breast carcinomas ($p < 0.0001$). The heat maps shown in Fig. 6.2 display 28 transcripts commonly deregulated (in the same direction) in human breast carcinomas and PI/D mouse mammary tumors. Among these transcripts, 19 were down-modulated and 9 were up-modulated in both species. Within the first list, we found some genes of which little is known as well as genes previously described to be associated with the pubertal mouse mammary development (*AREG*, *CITED1*, *FOXA1*, *MMP3*, *PDK4*, *LPL* and *BCHE*), as described above. In addition, Fig. 6.2c shows a protein-protein interaction network associating the common core of modulated genes across human and PI/D mouse mammary tumors. Kaplan–Meier analysis revealed that breast carcinomas that expressed the PI/D signature (cluster 2) were particularly associated with shorter overall ($p < 0.0001$) and relapse-free ($p = 0.003$) survival comparing with the rest of patients (cluster 1) (Fig. 6.2d). Taking together, these data suggest that tumors, in which pathways relevant to normal mammary physiology and development are maintained, may have a

a

Compiled dataset (n=819 primary breast carcinomas)

b

van de Vijver's dataset (n=295 primary breast carcinomas)
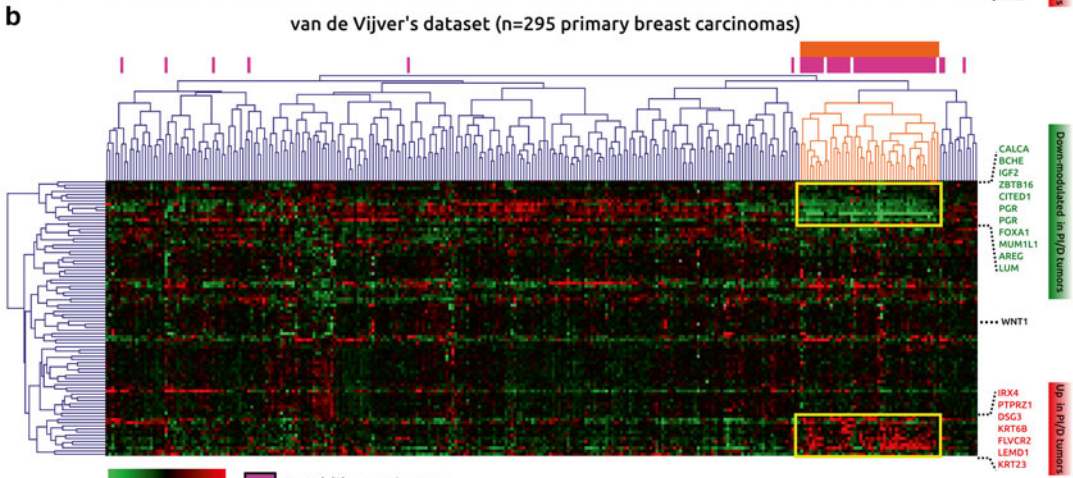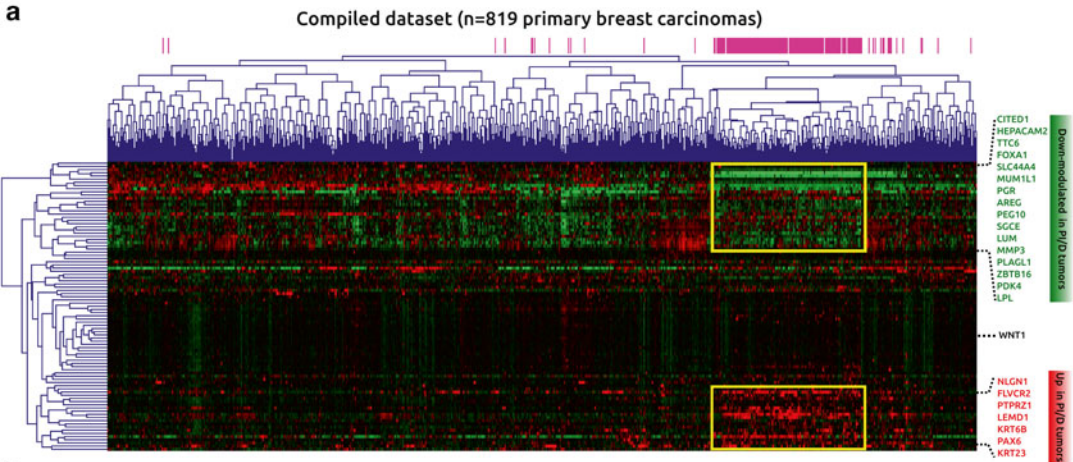
Down ▮▮▮ Up   ▮ Basal-like carcinomas

c

d

significantly better patient outcome than those tumors in which these pathways have been lost such as occurs in basal-like breast carcinomas and in PI/D mammary tumors.

In summary, the results showed herein confirm our previous observations about the relevance of the wnt pathway activation for the progression of ER+PR+ mammary cancer cells towards a more aggressive hormone-independent behavior upon resuming growth after long dormancy periods (PI/D tumors). In addition, here we demonstrate that these mouse mammary tumors display a transcription profile that resembles the pattern of expression found in basal human breast cancer with bad prognosis. In both cases, genes associated with puberal, hormone-dependent mammary growth are down-regulated. Although the list of genes commonly regulated do not include wnt pathway activators, based on the previously reported data cited herein, we believe that the cluster of co-regulated genes in basal human breast cancer and PI/D mouse mammary tumors might be mechanistically associated to the activation of wnt pathway, which would play a significant role in re-activating tumor growth from progenitor basal cells after long dormancy periods.

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Fig. 6.2 PI/D gene expression signatures in human breast cancer. (**a**, **b**) Heatmaps of the PI/D gene expression signature in 1,114 human primary breast carcinomas obtained from four independent studies available in a public database. (**c**) Graph of interactions among the core of genes modulated in PI/D mammary tumors. Pathways are discriminated by different colors based on up-modulated (*red node*) or down-modulated (*green node*) transcripts. (**d**) Kaplan–Meier curves of overall and relapse-free survival among the 295 early-stage breast cancer patients obtained from van de Vijver et al. study (2002) based on groups of carcinomas with (cluster 2) or without (cluster 1) expression of the PI/D gene signature

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