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ErbB-2-induced mammary tumor growth: the role of cyclin D1 and p27Kip1

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Abstract

The *neu* (c-*erbB-2, HER2*) proto-oncogene encodes a receptor tyrosine kinase that is a member of an important growth factor receptor family which includes the epidermal growth factor receptor (EGFR, ErbB1), ErbB3 and ErbB4. The *neu* is found over-expressed in 20–30% of human breast tumors [1]. The c-*erbB-2* is sufficient for the induction of mammary tumorigenesis in transgenic mice and the pathology of these mammary tumors strongly resembles human breast cancer. Murine transgenic models engineered to recapitulate human breast cancer provide an excellent and straightforward approach to dissect the molecular mechanisms governing the onset and progression of this disease. The molecular mechanisms by which ErbB-2 transforms cells involves direct effects on components of the cell-cycle regulatory apparatus. Recent studies have demonstrated a key role for components of the cell-cycle, in particular cyclin D1 and p27Kip1 (p27) in the onset and progression of ErbB-2-induced murine mammary tumorigenesis. Such studies have provided further impetus to therapeutics targeting these cell-cycle proteins.

Keywords: Transgenic mice; Breast cancer; Cyclin D1; p27; Haplo-insufficiency

1. Introduction

Neu is a member of a growth factor receptor family, which includes the epidermal growth factor receptor (EGFR), therefore Neu is also known as HER2, for human EGF receptor 2. Oncogenic activation by ErbB-2 can occur through its over-expression, point mutation within the transmembrane domain or deletion of the extracellular domain (ECD) [2]. Several signaling pathways are activated by ErbB-2, including the ERK, Akt, NF-κB, β-catenin/Tcf and cyclin-dependent kinases [3–6] (Fig. 1). Downstream transcriptional targets of ErbB-2 include E2Fs, Sp1, c-Myc and cyclin D1 [7,8]. ErbB-2 is sufficient for the induction of mammary tumorigenesis in transgenic mice. In order to dissect the molecular mechanisms by which ErbB-2 transforms the mammary gland, two different *in vivo* approaches have been pursued. In the first, murine models have been generated harboring selected genetically engineered mutations within the ErbB-2 transgene that regulate specific signaling pathways. The purpose of these models is to determine the effect these mutations have on the onset and progression of tumorigenesis. In the second approach, investigators have crossed the MMTV-ErbB-2 transgenic mice with mice deleted of candidate tumor suppressor or oncogenes to determine the role these proteins play in the onset and progression of the mammary tumors. Cell culture based studies and transgenic analysis have strongly implicated the cell-cycle regulatory protein cyclin D1 and the tumor suppressor p27 in ErbB-2 signaling and transformation. The role of these proteins in ErbB-2-induced mammary tumorigenesis is being actively explored because of the large number of human patients affected by ErbB-2 positive breast cancer.

2. Murine models of breast cancer

The *neu* (c-*erbB-2*) proto-oncogene encodes a tyrosine kinase receptor that is found amplified or over-expressed in a significant percent (>30%) of human breast tumors [9,10]. The level of *neu* amplification is inversely correlated to poor clinical outcome for breast cancer patients for

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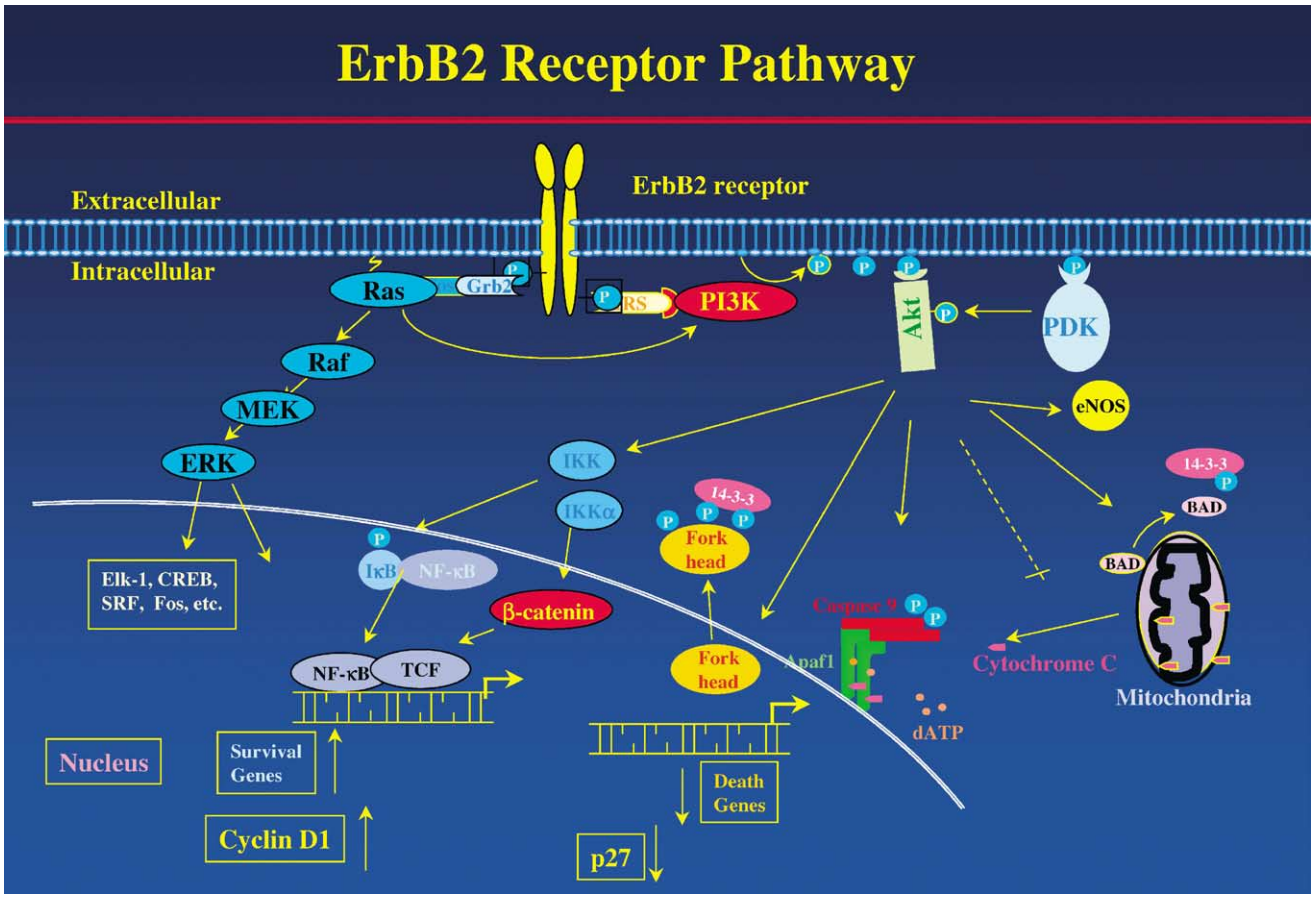

Fig. 1. Activation of the ErbB-2 receptor induces several downstream signaling pathways including Ras, ERK, PI3K and Akt. These pathways have in turn been shown to induce sequential signaling components involved in cell survival and proliferation. The specific components as shown represent an hypothetical model based on experiments in cultured cells and have not all been validated *in vivo*.

whom the cancer has not spread to the lymph nodes [11,12]. Mammary epithelial cell-targeted expression of constitutively active ErbB-2 or that of a similar mutation of human ErbB-2, is sufficient for the rapid induction of mammary tumors in mice [13]. The histopathology resembles human comedo carcinomas, which in the human are known to have a more aggressive course and express elevated ErbB-2 levels.

Research has revealed that several signal transduction pathways are induced by ErbB-2, both in cultured cells and when it is targeted to the mammary epithelium in transgenic mice. In transgenic mice expressing ErbB-2 the transgene frequently undergoes somatic mutation within the ECD. This finding is of interest as the ECD deletion mutations have enhanced transforming ability and an alternate splice form of ErbB-2, which harbors a deletion in the ECD, is found in human breast cancer.

The intracellular domain of ErbB-2 activates several important signaling modules including PI3K/Akt, ERK and JNK activity, through specific phosphotyrosine residues that dock distinct, molecules include PLCγ1, c-Src, Crk, and Grb7. Several other SH2-containing proteins bind to ErbB-2 including Grb2, Nck, Shc and Ras-GAP. Several groups have investigated the contribution of each of these specific signaling modules to cellular transformation. Functional evidence in transgenic mice supports an important role for PI3K/Akt signaling in mammary tumorigenesis and metastasis. Transgenic mice expressing the polyoma middle T antigen, under the control of the MMTV promoter, develop mammary tumors. Middle T antigen activates PI3-kinase activity. Transgenic mice possessing a middle T antigen, mutant in PI3K activation (MTY315/322F), develop mammary gland hyperplasia that are highly apoptotic but do not develop adenocarcinoma [4]. Although the specific gene products that contribute to the mammary tumor metastatic phenotype are not well understood, the PI3K pathway, RhoC, and Rac1 appear to be critical [14,15]. The PI3K pathway also appears to be important for the induction of Akt kinase activity, which is reportedly increased in tumors due either to the loss of the PTEN phosphatase, amplification of the PI3-kinase catalytic subunit, amplification of the *Akt* genes, or activation of growth factors and oncogenes including *ras* and *ErbB-2* [3,16]. The importance of the Akt pathway in ErbB-2 transformation is evidenced by several studies in transgenic mice. Mating of animals expressing constitutively active Akt to the MTY315/322F mice not only restored mammary gland cell survival but induced the formation of adenocarcinoma [5].

Downstream cell-cycle components induced by ErbB-2 are thought to contribute to the observed increase in cellular proliferation and aberrant cell survival phenotype. Cyclin D1 levels induced by transgenic over-expression of ErbB-2, were increased in mammary tumors and evidencing a key functional role for the induction of cyclin D1 expression, cyclin D1 anti-sense inhibited the growth of mammary tumors *in vivo* [8]. The induction of the *cyclin D1* gene by ErbB-2 involved the E2F and Sp1 transcription factor binding sites within the cyclin D1 promoter [8]. Indeed, ErbB-2-induced E2F transactivation [8]. As E2Fs can induce several genes involved in DNA synthesis and replication, these studies suggested one mechanism by which ErbB-2 may regulate these processes. In cultured cells, c-Myc was induced by activation of ErbB-2 [17]. It was proposed that the induction of c-Myc prevented cell-cycle control by the cell-cycle inhibitor p27. The role of p27 as an inhibitor of ErbB-2-induced mammary tumorigenesis has not formally been assessed.

3. Cyclin D1 in breast cancer

Cyclin D1 levels are induced in breast cells by both oncogenic and mitogenic signaling. In addition, cyclin D1 is required for oncogenic-driven growth by ErbB-2. The cyclins encode the regulatory subunits of a class of holoenzymes, which phosphorylate specific proteins during the cell-cycle [18–21]. Cyclin D1 encodes the regulatory subunit of the holoenzyme that phosphorylates pRB [19]. Cyclin D1 is over-expressed in greater than 30% of all human breast cancers and is associated with poor prognosis [22,23]. Cyclin D1 is involved in promoting G1 phase progression [24–26] and plays a pivotal role in cell-cycle progression in fibroblast [25,27], myocyte [28] and breast epithelial [29] cells. Inhibition of cyclin D1 expression results in cell-cycle arrest, whereas moderate over-expression accelerates G1 phase progression [24–26]. The *cyclin D1* gene is also induced by transforming mutants of Ras, Src, Rac, Dbl, β-catenin and the NF-κB signaling pathway [8,30–43].

In addition to binding cdks, cyclin D1 associates with several different intracellular proteins including the estrogen receptor (ERα), the androgen receptor (AR), P/CAF (p300/CBP associated factor) [44,45] and the cyclin D1 myb-like binding protein (DMP1) [19,46–48] (Reviewed in [21,49]). Cyclin D1 binds the ERα enhancing ERα signaling [50–52] perhaps through recruiting a co-activator SRC-1, as shown *in vitro* [53]. Cyclin D1 binds to the ERα *in vivo* and overcomes the BRCA-1-mediated repression of ERα activity [54–57]. The induction of ERα function by

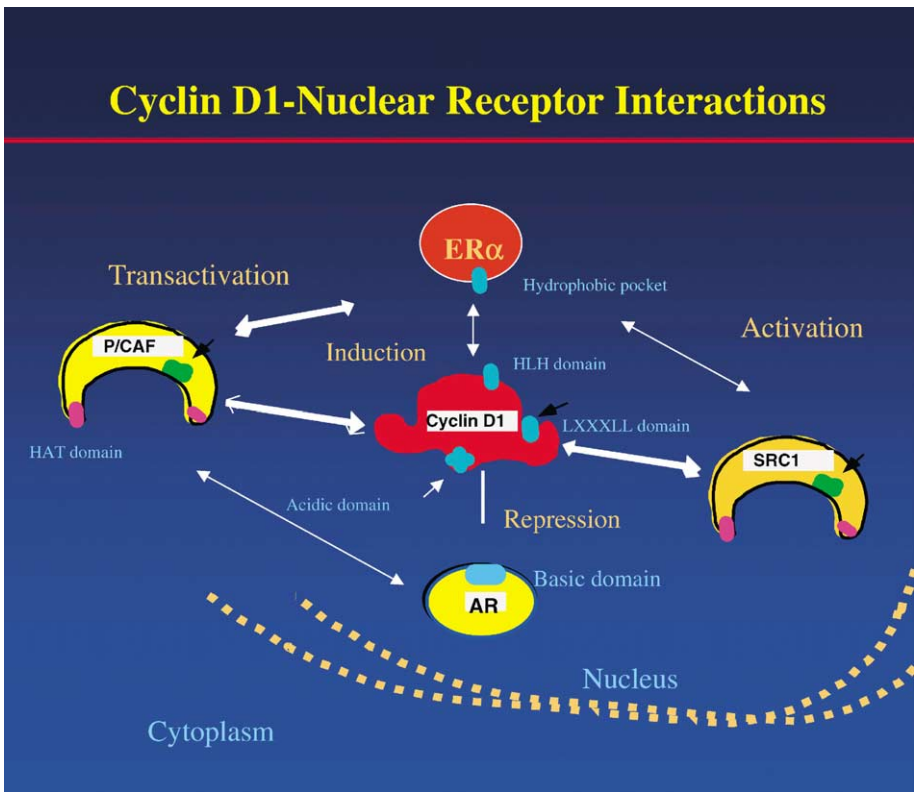

Fig. 2. The cyclin D1 regulatory subunit has been shown to form physical interactions with several proteins including the ERα, the AR, SRC1 and P/CAF. The domains of cyclin D1 involved in these physical interactions are shown based on pulldown analysis between two proteins and do not necessarily represent the site of interaction in the native complex *in vivo*. The functional significance of the interaction between each of the proteins, assessed in reporter gene assays is shown as either positive (transactivation) or negative (repression).

cyclin D1 likely contributes to estrogen's proliferative effects and this interaction is thought to contribute to cellular proliferation in a subset of breast cancers (Fig. 2).

Several lines of evidence suggest that cyclin D1 is also involved in cellular transformation. First, cyclin D1 mRNA is found over-expressed in a diverse set of neoplasms including prostate, breast, parathyroid adenomas, centrocytic lymphoma and B cell lymphomas [58,59]. Second, cyclin D1 over-expression promotes the transforming capacity of p21Ras and adenovirus E1A mutants, which alone do not have transforming capacity [47,60,61]. Third, cyclin D1 antagonized the cytostatic action of pRB [61]. Fourth, cyclin D1 anti-sense blocked contact-independent growth induced by Ras in NIH3T3 cells [62], ErbB-2 (NeuT) in Rat-1 cells [8] (Fig. 3). Ras-induced skin tumor formation was also reduced in cyclin $D1^{-/-}$ mice [63]. Fifth, over-expression of cyclin D1 in the breast of transgenic mice resulted in mammary tumor formation [64]. Finally, consistent with the reduction by cyclin D1 anti-sense of MMTV-ErbB-2-induced mammary tumors in nude mice [8], two recent studies using *cyclin D1* knockout mice suggested an important role for cyclin D1 in ErbB-2-induced mammary tumorigenesis with some caveats [65,66].

Several caveats apply to the finding that cyclin $D1^{-/-}$ mice are partially resistant to transformation by ErbB-2 *in vivo* [65,66]. First, cells derived from *cyclin* $D1^{-/-}$ mouse embryo fibroblasts conveyed reduced cellular proliferation [39] and increased basal and UV-induced apoptosis [43] as compared to wild-type litter mate control cells indicating a general effect on cell survival. Secondly, the resistance to tumorigenesis by loss of cyclin D1 was only partial, tumor formation was delayed rather than abrogated. This delay in tumorigenesis was perhaps due to cyclin E compensation [66]. Finally, the mammary glands of the cyclin $D1^{-/-}$ mice showed a developmental abnormality making interpretation difficult in accessing the role of cyclin D1 in the tumor resistance.

4. The use of *cyclin D1* knockout mice (*cyclin* $D1^{-/-}$) to study mammary tumorigenesis by ErbB-2

Both, the development of normal breast and the induction/progression of breast carcinogenesis involve a complex interplay between growth factors, steroids, activation of oncogenes and inactivation of tumor suppressor genes [9,22]. The transgenic mouse has served as a useful model in the investigation of those genes required in normal breast development and mammary tumorigenesis. By activating or inactivating a gene product, one can control and study a specific initiating event. Breast tumorigenesis can be induced by targeted over-expression of several different oncogenes including *myc*, *neu*, *ras*, *src*, *cyclin D1* and

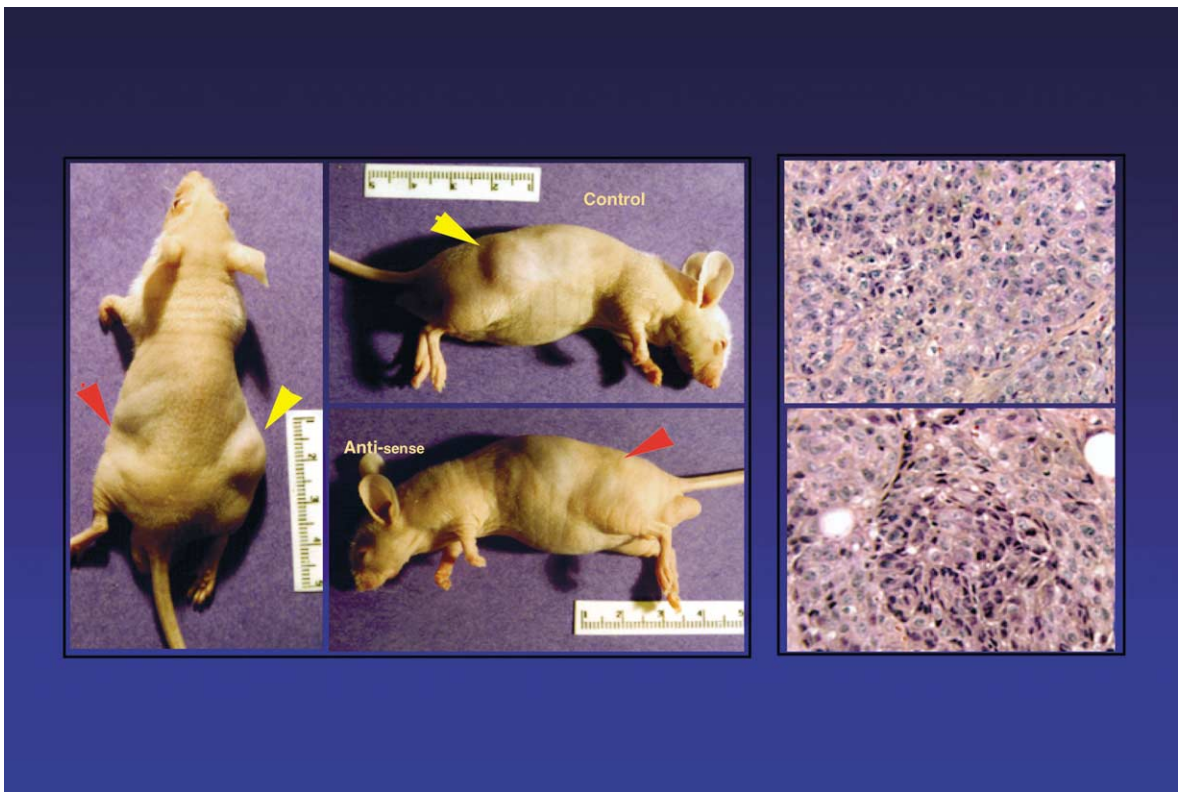

Fig. 3. Reproduced with permission from [8]. Mammary tumor epithelial cells derived from ErbB-2 over-expressing transgenic mice were infected with expression vectors encoding cyclin D1 anti-sense (left flank of animal) or control vector (right flank of animal). The tumor size is dramatically reduced with the cyclin D1 anti-sense. Histopathology of the mammary tumor is shown.

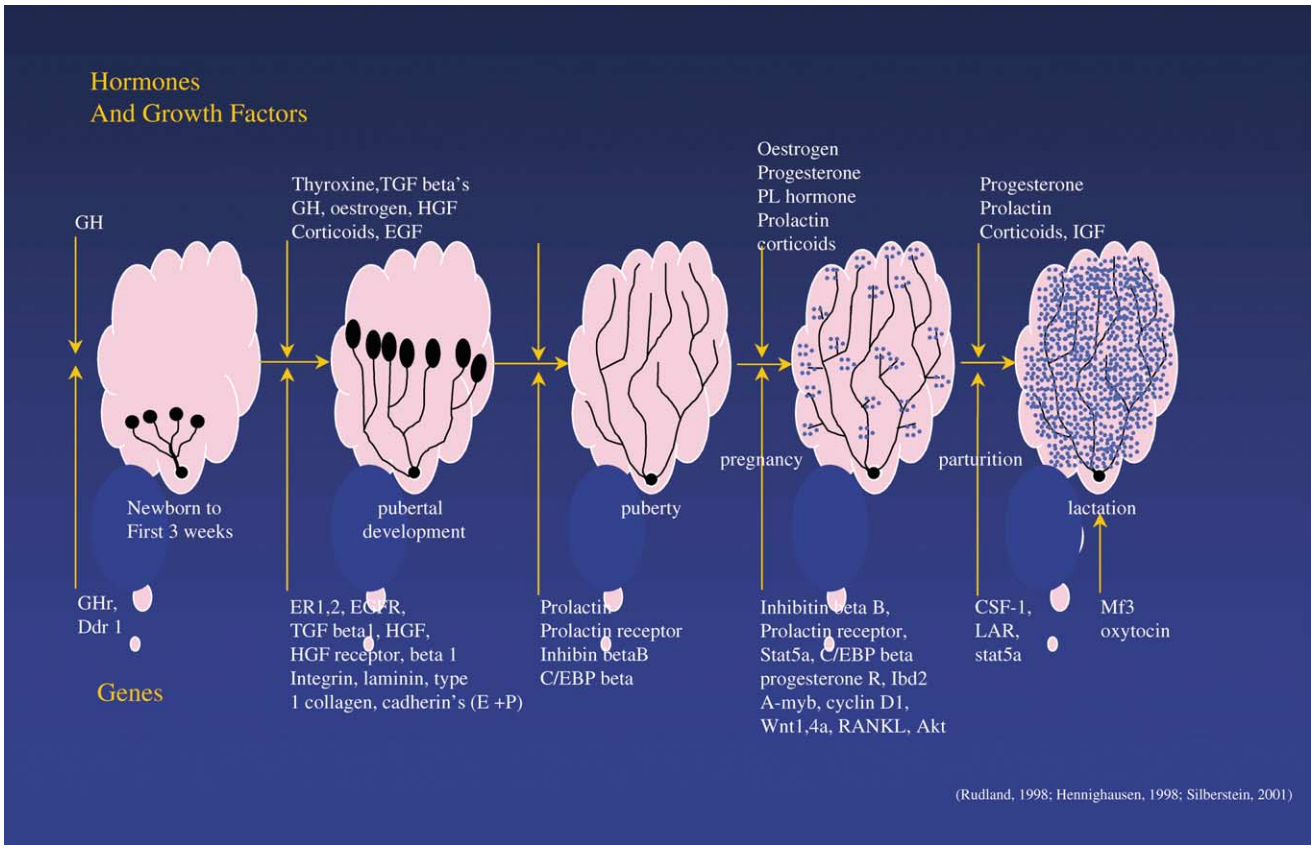

Fig. 4. Several distinct stages of mammary gland development are tightly regulated by hormonal influences (above) and require specific target genes (below) as evidenced by transgenic or knockout animals experiments. The ability to deduce the specific role of genes or hormones in breast cancer using genetically engineered "knockout mice" must be balanced with the potential interference of these gene deletions on the normal developmental program.

cyclin E. Furthermore, the homozygous deletion of specific genes has revealed a role for several distinct proteins in normal breast development including *cyclin D1*, the *prolactin receptor*, the *estrogen receptora*, and *Stat5a* (Figs. 4 and 5).

Homozygous deletion of the *cyclin D1* gene results in mice which display a developmental failure in the mammary gland terminal alveolar during pregnancy and retinal apoptosis [67,68]. The mammary gland developmental failure in *cyclin* $D1^{-/-}$ mice is quite striking, with the clubbed shaped protuberances of alveoli virtually abolished [69]. The developmental defect from cyclin D1 is cell autonomous [69] and is rescued by mating to p27 knockout mice [70,71]. The mammary gland defect is similar to the observed defect found in the C/EBPβ, Stat5a and progesterone receptor knockout mice. The reasons for the marked reduction in development of alveoli in the cyclin $D1^{-/-}$ mice are not known. However, there is an accompanying reduction in phosphorylated Stat5a, an alteration of the progesterone receptor A and B isoform ratio in the *cyclin* $D1^{-/-}$ mammary tissue [69] and evidence for attenuated induction of estrogen-responsive genes in the mammary gland [69]. The epithelial cells and the fibroblasts of the *cyclin* $D1^{-/-}$ have apparently distinct transforming responses to ErbB-2, with fibroblasts being transformed and epithelium being resistant [65]. Together these studies indicate a specific and important role for cyclin D1 in normal mammary gland development and although experimentally expedient, raise a strong cautionary note in the use of the *cyclin* $D1^{-/-}$ mice for studies on the role of cyclin D1 in mammary tumorigenesis. Nonetheless the *cyclin* $D1^{-/-}$ mice provide a useful *in vivo* testing ground to assess the requirement for cyclin D1 in tumorigenesis mediated by specific oncogenes.

5. p27Kip1 as a tumor suppressor of ErbB-2

Experiments in cultured cells have implicated the p27 protein as an inhibitor of ErbB-2 signaling [17]. The p27 protein was initially described as a protein found to be homologous to the tumor suppressor p21 [72]. When overexpressed in fibroblasts, cell-cycle progression was delayed, and anti-sense p27 experiments overcame cell-cycle arrest induced by several paradigms including mitogen withdrawal and protein kinase Cδ isoform overexpression, indicating a critical role for p27 in the establishment or maintenance of cellular quiescence [21,73]. Strong *in vitro* and *in vivo* evidence also suggests that p21 and p27 enhance cyclin D-dependent activity, where

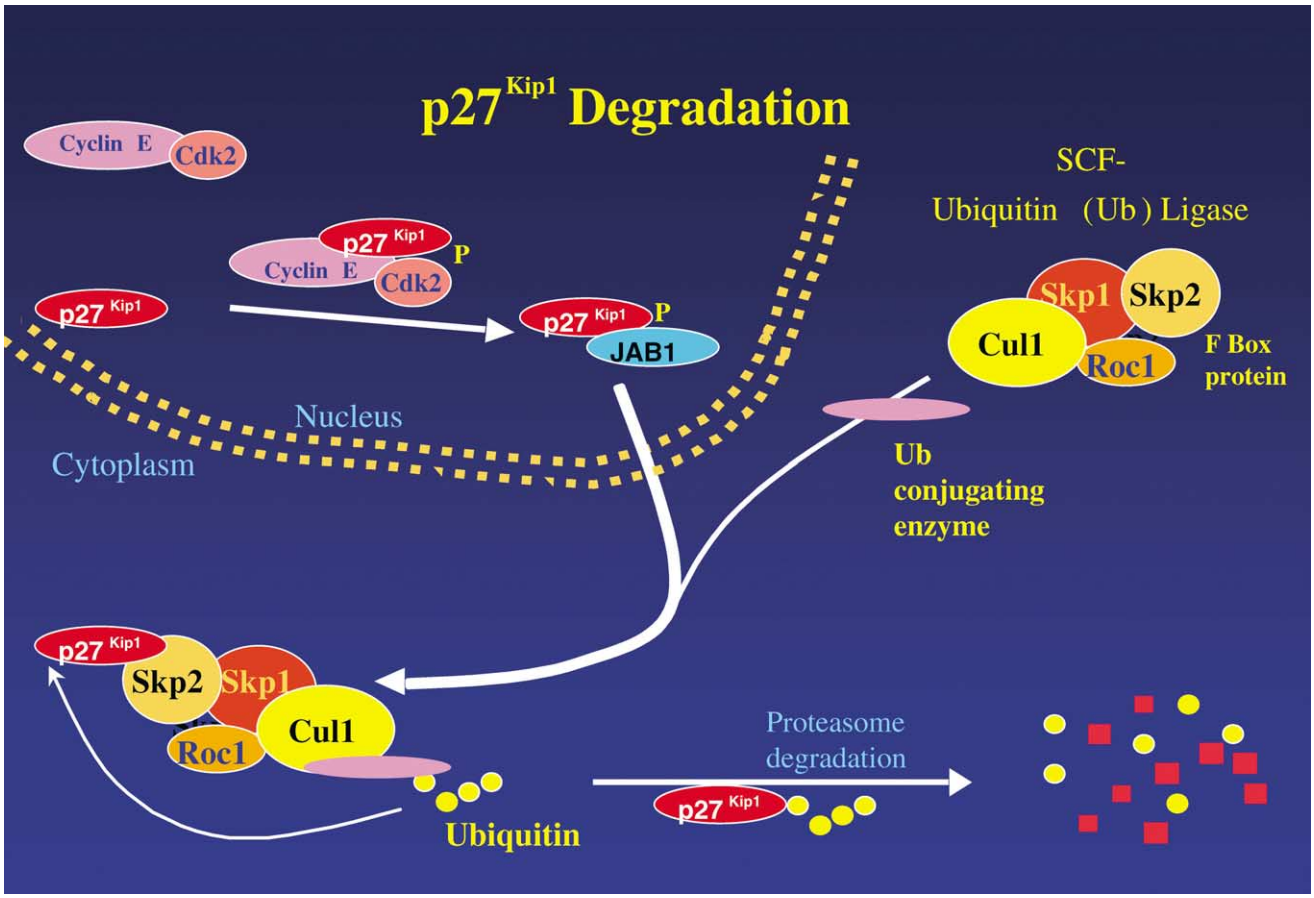

Fig. 5. The degradation of the p27 protein involves phosphorylation of Thr-187-dependent and a second less well defined mechanism [93]. JAB1 facilitates nuclear/cytoplasmic translocation of p27 and the SCF complex facilitates degradation by a proteosome-mediated mechanism.

they function as ''assembly factors'' [74]. The cyclin/cdk complex to which p27 is bound determines its functional activity. The p27 is found associated with cyclin E in a variety of cell types during quiescence. When bound to cyclin D1/cdk4, p27 may not be inhibitory, whereas cyclin E/cdk2 activity is inhibited by p27. It is thought that the removal of p27 from the cyclin E/cdk complex is an essential step for S-phase entry. Through its binding to cyclin D1/cdk4, p27 is sequestered from binding cyclin E/cdk2 thereby reducing its inhibition on this complex [21].

In a majority of studies, reduced p27 levels in tumors, including breast cancers correlates with poor prognosis although elevated levels have also been reported in a subset of tumors [75–78]. The p27 abundance is regulated primarily at the level of translation and protein turnover. Phosphorylation of p27 by cyclin-dependent kinase 2 creates a binding site for an E3 ubiquitin–protein ligase complex (SCF), which promotes proteosome-mediated degradation of p27 [79–81]. The F-box protein Skp2 is the substrate recognition factor of the SCF complex that recognizes and binds to phosphorylated p27. Skp2 is required for G1–S-phase transition in transformed cells and diploid fibroblasts [82] and Skp2-deficient cells exhibit increased p27 levels and polyploidy [83].

Reduced p27 levels are found in a variety of tumors including breast cancers and conveys an adverse prognosis in a subset of cases [84]. Reduced levels of p27 protein correlate with poor prognosis in human breast cancer raising the possibility that p27 functions as a mammary gland tumor suppressor. The role of p27 as a tumor suppressor for breast cancer gene therapy therefore requires evaluation. Although loss of a single *p27* allelle is not uncommon in human tumors, the second allelle is frequently wild type. Thus, the *p27* gene does not fit the classic tumor suppressor paradigm [85]. Recent studies in transgenic mice assessed the role of *p27* as a tumor suppressor of ErbB-2-induced mammary adenocarcinoma [86]. The rate of onset of mammary tumorigenesis was substantially accelerated by the loss of a single *p27* allelle without affecting mammary gland development [86]. These studies were the first to demonstrate that p27 functions *in vivo* as a tumor suppressor with LOH in the context of ErbB-2-mediated mammary gland transformation (Fig. 6) [86].

Surprisingly, inactivation of *Cdkn1b* (encoding *p27*) in mice does not result in enhanced spontaneous tumor onset, with the exception of pituitary adenomas [87–89], despite the widespread implication that p27 may serve as a tumor suppressor in different human cancers [90,91]. Reduced p27 may be anticipated to cooperate as a component of

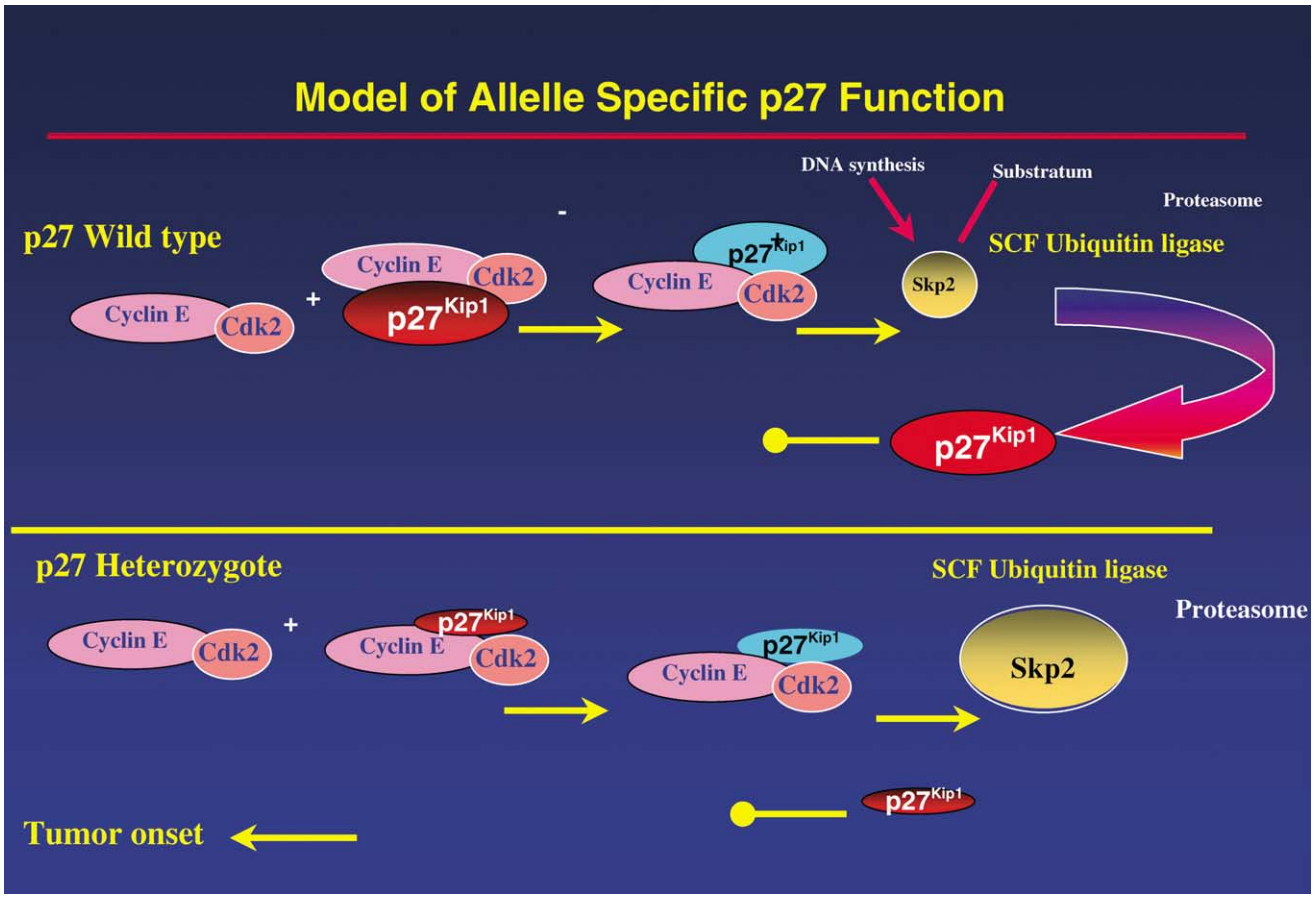

Fig. 6. Hypothetical model through which the loss of one allele of p27 promotes aberrant cellular proliferation. Upper panel: The normal degradation of p27 involves the SCF complex, in which the abundance of Skp2 is shown as regulated by contact with the substratum and DNA synthesis. Lower Panel: The loss of one allele of p27, or overexpression of Skp2 through oncogenic signals such as ErbB2, or amplification, result in the enhanced degradation of p27. Reduced 27 levels, in turn, alter the expression of genes regulating cell cycle control, integrins and cellular adhesion to promote contact-independent growth.

multistep tumorigenesis with either loss of other tumor suppressors or in the context of activating oncogenes. The inactivation of *p27* for example may be epistatic to the inactivation of *pten*, as the inactivation of one *pten* allelle together with the inactivation of one *Cdkn1b* allelle was found to enhance the rate of prostate tumor onset [92].

6. Conclusion

The induction of mammary tumorigenesis by ErbB-2 involves several distinct signaling pathways that collaborate in the induction of the invasive and metastatic tumor phenotype. Murine models have provided important information by identifying key signaling molecules and cell-cycle proteins required for the development of mammary tumors. In the case of ErbB-2-induced mammary tumors, cyclin D1 is required, in part, for the induction of tumor onset. The p27 functions as a haplo-insufficient suppressor of ErbB-2-induced mammary tumors. The further dissection of key target genes required for the onset and progression of mammary tumorigenesis is essential for the tailoring of less toxic therapies for human breast cancer.

Acknowledgments

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