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HER2-Driven Carcinogenesis: New Mouse Models for Novel Immunotherapies

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1. Introduction

The immune system might well be the best weapon against cancer, since immune defense is programmed to recognize and destroy abnormal cells infected by viruses or affected by transforming genetic or epigenetic alterations. If properly activated, a specific immune attack can be amplified and maintained long-term. These powerful mechanisms must be harnessed to fight against invading microorganisms and against cancer cells, which subvert the circuits that normally control cell proliferation and survival by displaying an anarchic behavior.

Even at the start of the 20th century, the physician Paul Ehrlich proposed the potential ability of the immune system to continually survey and destroy newly arising cancer cells [1]. This hypothesis represented the first version of the immune surveillance theory, formulated in 1957 by Burnet [2, 3]. However, this theory was long-discredited by evidence that while the immune system can fight tumors, it is often unable to eradicate them. In the last decade, many cancer immunologists have shown renewed interest in immune surveillance theory, with the goal of generating novel immunotherapies against cancer, such as cancer vaccines. Advances in cancer biology, increased knowledge of immune mechanisms, and the availability of new animal models that recapitulate several human cancers have all helped to elucidate the critical issues that influence the efficacy of an immune attack against cancer [4]. This information is crucial for the rational design of cancer vaccines.

Cancer cells elaborate many defenses against immune attack. For example, they try to evade recognition by T cells and, in turn, T cell-mediated cytotoxicity, one of the major mechanisms to control tumor growth, by decreasing the expression of glycoproteins of the

major histocompatibility complex (MHC) on the cell membrane [5]. In fact, the T-cell receptor (TCR) recognizes antigen only when it is displayed on the surface of the target cell as peptide fragments by the class I and class II molecules of the MHC. Moreover, the increasing instability of the genome of transformed cells favors the emergence of clones with low immunogenicity no longer expressing tumor antigens. Thus, tumors can evade immune recognition, an ability that appears to increase as a tumor grows. Finally, cancer cells suppress immune reactivity through direct release of transforming growth factor (TGF)-beta, interleukin (IL)-10, and indoleamine 2,3-dioxygenase (IDO), or through the activation of myeloid-derived suppressor cells, tumor-associated macrophages and dendritic cells to secrete these molecules. As a consequence, a tumor favors the activation and the expansion of adaptive regulatory T (Treg) cells, leading to the generation of a tolerogenic environment [6].

In these conditions, the choice of antigen is a crucial factor in deriving a cancer vaccine. Recently, Lollini et al. [7] defined the “oncoantigens” as ideal targets since these antigens are indispensable for tumor progression and thus cannot be lost, and since, depending on their localization, they can be targeted by both cytotoxic cells and antibodies. Among oncoantigens, HER2 represents a very attractive target in light of its direct association with the malignant transformation of epithelial cells and its shared presence in several human carcinomas. Indeed, vaccines targeting HER2, designed as whole cells, peptides, as well as DNA expression plasmids, are able to hamper cancer progression when used at early stages of the disease [8]. However, the promising results obtained in preclinical models are difficult to reproduce in advanced cancers, when the immune system is already severely weakened. New molecular strategies are required to generate effective cancer vaccines able to awaken the immune responses against established tumors.

1.1. HER2 protein structure and function

HER2, also known as ErbB2 or neu in rat, is a 185-kd transmembrane receptor with tyrosine kinase activity and initially identified in a rat glioblastoma model. HER2 belongs to the epidermal growth factor receptor (EGFR) family, which also includes HER1 (EGFR, ErbB1), HER3 (ErbB3) and HER4 (ErbB4). Each of these receptors consists of an extracellular binding domain (ECD), a single transmembrane-spanning domain, and a long cytoplasmic tyrosine kinase domain. The ECD is about 630 amino acids long and contains four subdomains arranged as a tandem repeat of a two-domain unit. The first and third subdomains (I/L1 and III/L2) have a β -barrel conformation, and the second and fourth subdomains (II/CR1 and IV/CR2) are cysteine-rich [9] (Figure 1).

Generally, binding of ligand to the extracellular region induces receptor dimerization and activation of the cytoplasmic kinase, which in turn lead to autophosphorylation and initiation of downstream signaling events. Among the EGFR family members, HER2 and HER3 are exceptional since HER3 is kinase-inactive and HER2 has no identified ligand. Although HER2 is the only receptor without a known ligand, it is the preferred partner in heterodimer formation with other HER members.

Figure 1. The structure of HER2. Ribbon diagram of HER2 ECD: Subdomains I (dark red), II (green), III (yellow) and IV (red), are indicated. Adapted from Cho H.S. et al., 2003 [9].

Crystallographic studies have helped to elucidate the structural basis for the differences in HER receptor function. There are two conformations of the ECD, the closed configuration and the open configuration. In the closed configuration, a dimerization arm located on domain II makes an intramolecular contact with a pocket on domain IV, preventing its association with the dimerization arms of other HER receptors and maintaining the receptor in an auto-inhibited form. Binding of its native ligand to the receptor brings domains I and III close together, switching the receptor's conformation to the open configuration (active state), in which the dimerization arm is free to participate in receptor dimerization. Unlike the three other HER receptors, HER2 can adopt a fixed conformation resembling a ligand-activated state but permitting it to dimerize in the absence of a ligand (Figure 2). The constitutive open structure of HER2 helps to explain its readiness to interact with the ligand-activated HER receptors [10].

Receptor homo- and heterodimerization leads to the activation of downstream signaling pathways associated with cell proliferation, differentiation, survival and angiogenesis. Activation of the kinase domains by receptor dimerization and the subsequent transphosphorylation of tyrosine residues in the carboxy-terminal tails creates binding sites for several key proteins. These specific proteins activate intracellular signaling pathways, including the mitogen-activated protein kinases (Ras/Raf/MEK/MAPK) pathway, which mainly regulates cell proliferation, and the phosphoinositide 3-kinase (PI3K)-activated Akt (PI3K/Akt) pathway, which is important for cell survival [10].

Figure 2. Schematic overview of the structural basis for HER receptor dimerization and activation. In the ligand-free state, HER1, HER3, and HER4 have a closed conformation. Binding of ligand, involving subdomains I and III, creates an extended conformation, allowing for receptor homo- and heterodimerization. Receptor dimerization leads to C-terminal tyrosine phosphorylation, creating phosphotyrosine binding sites for binding of adaptors, signaling molecules and regulatory proteins. HER2 is unique in that it is fixed in the active conformation ready to interact with other HER receptors. Adapted from Wieduwilt M. J. and Moasser M. M., 2008 [10].

The HER signaling network normally governs cellular programs during development and post-natal life, but its deregulation is directly involved in the pathogenesis of several human tumors. Overexpression of HER2, enhancing and prolonging signals that trigger cell transformation, has a causal role in the promotion of carcinogenesis. The absence of the auto-inhibited conformation explains, at least in part, this HER2 transforming potential. Amplification and/or overexpression of HER2 have been reported in malignancies, such as breast, ovarian, prostate, colorectal, pancreatic and gastric cancers [11].

1.2. Role of HER2 oncogene in breast carcinoma

Breast cancer, one of the most common malignancies worldwide, is a heterogeneous disease that can be classified according to the expression of the estrogen receptor, progesterone receptor, and HER2. The resulting subgroups differ not only in clinical behavior and prognosis, but also in the predicted response to targeted therapies against these receptors and the pathways they activate. Amplification of the HER2 gene and overexpression at the messenger RNA or protein level occurs in about 20-30% of patients with early stage breast cancer and predicts a poor prognosis [12]. Further support for the involvement of HER2 in the initiation and progression of breast cancer comes from studies on transgenic mice, although careful analysis of these transgenic mouse models suggests that overexpression of HER2, primarily due to gene amplification, is necessary but not sufficient to induce transformation. The expression of the oncoprotein induces tumors only when accompanied by genetic alterations, which include point mutations, deletions, and insertions. These alterations are invariably located in the juxtamembrane region of HER2 and lead to an unbalanced number of cysteines, potentially affecting cysteine-mediated dimerization [13].

Evidence for the insufficiency of wild-type HER2 expression, alone and without additional mutations, to induce full malignant transformation was reported by Finkle et al. [14], who found that transgenic mice overexpressing human HER2 under the murine mammary tumor virus (MMTV) promoter developed mammary tumors in a stochastic manner and after a long latency. Interestingly, those authors found sequence anomalies, including in-frame small deletions, in the juxtamembrane region of wild-type HER2 in more than half of the analyzed mammary tumors. The majority of these mutations affected the conserved cysteine residues and could function as a second hit in the transformation process, implying that additional genetic changes beyond HER2 overexpression are required for mammary tissue transformation and tumor formation. Accordingly, somatic mutations confined to the juxtamembranous region of *neu* have been associated with the induction of mammary tumors in *neu* protooncogene transgenic mice described by Siegel et al. [15]. The relatively long latency period for the progression of these tumors seems to reflect the acquisition of activating mutations in the transgene.

Interestingly, an alternative splice form of the human HER2 gene, $\Delta 16$ HER2, containing an in-frame deletion in the same region mutated in rat *neu* or human HER2 protooncogene transgenic mice, has been described [16]. This oncogenic isoform is clinically important and commonly coexpressed with HER2 in human breast tumors, as reported by Castiglioni et al. [17] and Mitra et al. [18], who detected $\Delta 16$ HER2 transcripts in human breast carcinomas in about 10% of total HER2 transcripts. This deletion removes the relevant cysteine residues in HER2, disrupting the disulfide bond structure of the protein and leaving the remaining unpaired cysteine residues available for intermolecular bonding. Consequently, $\Delta 16$ HER2, which can be defined as a normal byproduct of HER2, forms stable homodimers maintained by intermolecular disulfide bonds (Figure 3).

$\Delta 16$ HER2 has increased transforming potency as compared with the wild-type HER2, as first demonstrated *in vitro* [17, 18]. Ectopic expression of $\Delta 16$ HER2, but not wild-type HER2, promoted receptor dimerization and significantly enhanced the proliferation of murine NIH3T3 fibroblasts and human MCF-7 breast tumor cells. In addition, $\Delta 16$ HER2 expression potentiated MCF-7 cell migration and invasion, whereas HER2 did not. In analysis of anchorage-independent growth as an *in vitro* test for tumorigenesis, both HER2 wild-type- and $\Delta 16$ HER2-transfected MCF-7 cells showed enhanced colony formation in soft agar medium, but the $\Delta 16$ HER2-expressing cells formed significantly larger colonies [18]. We obtained similar results with human embryonic kidney HEK293 cells stably transfected with wild-type HER2 or $\Delta 16$ HER2 (Figure 4). *In vivo* analyses showed that the $\Delta 16$ HER2 variant is tumorigenic *per se* since athymic mice injected with $\Delta 16$ HER2-expressing HEK293 transfectants developed tumors, whereas mice injected with HEK293 control cells ectopically overexpressing only wild-type HER2 did not [17]. The predicted enhanced oncogenic potential of $\Delta 16$ HER2-expressing cells *in vivo* is supported by analysis of our new mouse model transgenically expressing the human $\Delta 16$ HER2 under the transcriptional control of the MMTV promoter; all of the transgenic females developed multifocal mammary tumors with a rapid onset, suggesting that the $\Delta 16$ HER2 splice variant represents the transforming form of the HER2 oncoprotein [19].

Figure 3. $\Delta 16$ HER2 splice variant. a. Schematic representation of the small region of the human HER2 genomic locus indicating the exon-intron boundaries and the alternative splicing that eliminates exon 16. The values indicated above the schematic represent nucleotide numbers corresponding to the cDNA. b. Alignment of the wild-type and alternatively spliced $\Delta 16$ HER2 mRNAs; the grey box indicates sequences removed by the splicing event.

Figure 4. Anchorage-independent growth of stable transfected HEK293 cells. Cells expressing $\Delta 16$ HER2 (b) form larger colonies than cells expressing wild-type HER2 (a).

The key role of the cysteine residues in the HER2 juxtamembrane region has been also demonstrated by Pedersen et al. [20], who described a subtype of HER2-positive tumors expressing a series of carboxy-terminal fragments collectively known as p95HER2. These fragments arise through at least two different mechanisms: proteolytic shedding of the ECD of the full-length receptor; and translation of the mRNA encoding HER2 from internal

initiation codons [21]. One of these fragments, 611-CTF, which contains a transmembrane domain and a short extracellular region including the sequence deleted in $\Delta 16$ HER2, is hyperactive because of its ability to form homodimers maintained by intermolecular disulfide bonds. Despite lacking the majority of the ECD, this HER2 fragment drives breast cancer progression *in vivo*, as shown by the development of aggressive mammary tumors in mice transgenically expressing 611-CTF and suggesting a causal role for p95HER2 fragments in tumorigenesis based on their ability to constitutively homodimerize [20].

Together, these findings demonstrate that the overexpression of full-length HER2 alone is not sufficient to drive malignant transformation of mammary glands.

1.3. Mouse models for HER2-positive breast cancer

The first direct evidence for the involvement of HER2 in the initiation and progression of breast cancer came from analysis of transgenic mice with MMTV promoter-targeted overexpression of activated neu (the rat homolog of HER2) in the mammary gland. Activated neu (neu-NT) is a mutated form with valine instead of glutamic acid at residue 664 in the transmembrane domain of the protein. Although the endogenous mouse HER2 promoter has recently been used to control mammary-specific expression of activated neu-NT [22], MMTV-based mouse models have greatly increased our knowledge of the mechanisms that control HER2-mediated mammary tumor progression (Figure 5). In 1988, Leder and co-workers [23] generated the first transgenic mice that developed mammary tumors due to MMTV-driven expression of neu-NT. The short latency (11-13 weeks) and the high multiplicity of mammary tumors arising in those MMTV-neu-NT mice suggested that overexpression of the activated neu variant could drive mammary carcinogenesis in a single step.

Figure 5. Timeline. Several mouse models transgenic for the rat or human HER2 been generated over the past 24 years, increasing our knowledge of the mechanisms that control HER2-mediated mammary tumour progression. Adapted from Ursini-Siegel et al., 2007 [33].

However only 1 year later, Jolicoeur's laboratory developed a second MMTV-neu-NT transgenic mouse model in which mammary tumor development was stochastic and with a significantly longer latency, suggesting that mammary epithelial cells require many genetic events in concert with neu-NT overexpression to undergo transformation [24]. The differences between these two transgenic mouse models probably reflect the ability of the transgene to integrate randomly into the mouse genome, which, in turn, influences transgene expression levels. In fact, transgenic animals generated even with the same construct may exhibit different tumor multiplicity and latency depending on the site of integration and on the transgene copy number. Nonetheless, subsequent studies of additional MMTV-neu-NT transgenic mice clearly demonstrated that overexpression of activated neu is sufficient to efficiently transform mammary epithelial cells [25]. To assess the dependence of cancer cells in advanced tumors or metastases on an initiating oncogenic event for maintenance of the transformed state, an inducible transgenic model for neu was obtained, using the tetracycline regulatory system to conditionally express activated neu in the mammary epithelium [26]. In these MMTV-rtTA/TetO-neu-NT mice, neu-initiated tumorigenesis is reversible: upon induction with doxycycline, multiple invasive mammary carcinomas developed that regressed to a clinically undetectable state following transgene deinduction. Interestingly, most animals eventually developed neu-independent recurrent tumors long after the apparently complete regression of their tumors, indicating that neu-induced mammary tumors typically progress to a neu-independent state [26].

Although rapid onset of multifocal mammary tumors has been observed in the majority of activated neu transgenic mice, this mutation has never been observed in human cancers, which present only amplification of the HER2 gene copy number and consequent overexpression of HER2 protein on the cell membrane. In wild-type neu-expressing mice under the MMTV promoter, focal mammary tumors arise next to hyperplastic mammary tissue after a long latency period (17-48 weeks) [27], suggesting that genetic alterations in addition to those inducing HER2 overexpression are required for mammary transformation. Notably, tumors in these transgenic mice arose only when the oncoprotein carried mutations in the ECD involving small deletions that promote neu transforming activity through formation of intermolecular covalent cysteine bonds [15, 28]. Accordingly, mammary epithelium-specific expression of two activated neu receptors harboring distinct in-frame neu deletions (NDL) (MMTV-neu-NDL mice) led to rapid induction of mammary tumors [29]. Other transgenic animals with mutated forms of neu that couple specifically with Grb2 (neu-NDL-YB) or Shc (neu-NDL-YD) adaptor proteins have been generated to address the significance of HER2-coupled unique downstream signaling pathways in induction of mammary cancers [30].

More recently, after initial failed attempts [31], transgenic mice with wild-type human HER2 have been generated. A transgenic mouse expressing human HER2 under the whey acidic protein promoter was obtained, but no mammary neoplastic transformation was ever detected in any animal [32]. While another human wild-type HER2 transgenic model under the MMTV promoter did develop HER2-overexpressing breast tumors, but with a long latency of about 28.6 weeks [14]. Sequencing of the human HER2 transcripts from primary mammary tumors developed in these transgenic mice identified an in-frame 15-bp deletion in the wild-type HER2 juxtamembrane region, potentially affecting cysteine-mediated dimerization [14].

Overall, these results point to the role of HER2-activating mutations that change the number of cysteines in mammary tumorigenesis. In this context, expression of the alternatively spliced $\Delta 16$ HER2 isoform, which is constitutively active by virtue of its ability to form disulfide-bridged homodimers, might be required to obtain an oncogenic phenotype. HER2 gene amplification in primary human breast cancer might increase the levels of this oncogenic variant above a critical threshold, allowing it to contribute to breast cancer progression.

1.4. $\Delta 16$ HER2 mice

The value of $\Delta 16$ HER2 transgenic mouse models in addressing the biological importance of this oncogenic variant in breast cancer progression and in response to targeted therapies was suggested in 2007 by Ursini-Siegal et al. [33]. Indeed, we recently generated a mouse line transgenically expressing human $\Delta 16$ HER2, established using a bicistronic vector containing an IRES sequence between the human $\Delta 16$ HER2 and the firefly luciferase gene to ensure their coordinated expression driven by the same MMTV promoter (Figure 6) [19]. Luciferase was chosen as a reporter gene since it is rapidly detectable by optical imaging in live organisms and simultaneously allows accurate quantitation in tissue extracts and immunohistochemical detection using specific antibodies. In addition, a restriction enzyme PCR-based technique [34, 35] confirmed integration of the transgene at a single site on murine chromosome 5, inside an intergenic region containing neither genes nor regulatory sequences such that the insertion itself does not affect tumorigenesis. Quantitative PCR analysis revealed a transgene copy number of 5 [36] (Figure 6).

In steps to characterize $\Delta 16$ HER2 mice, we found that the founder female developed 8 spontaneous mammary tumors starting at 18 weeks of age and, as expected, readily visualized by bioluminescence analysis even one month before tumors became palpable, suggesting that luciferase expression might be predictive of tumor onset. Whole-mount and histological analysis of the mammary glands confirmed the presence of small neoplastic masses (Figure 7a). Immunohistochemical analysis of these non-palpable tumors revealed HER2 protein expression (Figure 7c), with larger tumors displaying heterogeneous membrane staining for the human transgene, while small tumors showed homogeneous distribution of HER2 in the tumor parenchyma. In all cases, HER2 expression was detected only in the mammary gland and in strict correlation with tumor development. HER2 expression also correlated with that of PCNA, a marker for mitotic activity in transformed epithelial cells (Figure 7d).

Because the MMTV promoter is hormonally regulated and tumor development in founder females might be enhanced by increased transgene expression in the mammary gland during pregnancy and lactation, we monitored spontaneous mammary tumor development by palpation in virgin female F2 transgenic mice; all of these mice developed multiple asynchronous mammary tumors (4-5 tumors/mouse) at 12 to 19 weeks of age (Figure 8c), each reaching 1-1.5 cm³ within a short time-frame (Figure 8d). Histologically, these fast-growing tumors were classified as invasive HER2-positive adenocarcinomas. Indeed,

Figure 6. Bicistronic vector for $\Delta 16\text{HER2}$ mice generation. Schematic representation of the MMTV-driven human $\Delta 16\text{HER2}$ -LUC transgene, with the MMTV LTR promoter (pMMTV, red), the human $\Delta 16\text{HER2}$ cDNA (green), the internal ribosome entry site (IRES, yellow), the luciferase cDNA (LUC, orange), and the termination signal from the SV40 (Poly A). Relevant restriction sites are indicated. The MMTV- $\Delta 16\text{HER2}$ -IRES-LUC expression cassette (8381 bp) was isolated from the plasmid backbone by *NheI* and *Sall* digestions, purified, and microinjected into fertilized eggs from FVB females. The transgene randomly integrated at a single site (at 85.72 Mb) on murine chromosome 5 region E-1 (NT109320.4) inside an intergenic region (NCBI Build m37.1). The insertion occurred exactly 1.17 Mb downstream of the non-histone chromosomal protein HMG-17-like gene and 718 Kb upstream of the centromere protein C1 gene. Quantitative PCR analysis revealed a transgene copy number of 5.

Figure 7. Analysis of non-palpable tumors in $\Delta 16\text{HER2}$ transgenic mice. a. Whole-mount analysis of an inguinal mammary gland of a 14 week old mouse reveals non-palpable tumors (red arrows); blue arrow indicates a lymph node. Hematoxylin-eosin (b) and immunohistochemical staining for HER2 (c) and PCNA (d) of non-palpable mammary tumors at 14 weeks. Magnification: a, X6; b-d, X400.

immunohistochemical analysis confirmed the concurrent expression of the human $\Delta 16\text{HER2}$ oncogene and the luciferase gene, and revealed the specific staining of epithelial cells but not of stromal cells or adipocytes, while non-neoplastic mammary ducts were negative. Tumors consisted of cells with round nuclei and eosinophilic cytoplasm growing in solid sheets and packets traversed by delicate fibrovascular septa. Growth of these unencapsulated tumors compressed the surrounding tissues (Figure 8b). Subsequent monitoring of all generations following F2 revealed similar results, indicating that formation of $\Delta 16\text{HER2}$ -overexpressing mammary tumors is a reproducible phenotype in these transgenic mice. Furthermore, transgenic $\Delta 16\text{HER2}$ females bearing primary mammary tumors developed lung metastases starting at 25 weeks of age and present in 100% of mice at 36 weeks, suggesting particularly aggressive tumor behavior upon expression of the $\Delta 16\text{HER2}$ splice variant. The histological features of these pulmonary metastatic lesions were consistent with a primary breast tumor origin, with robust staining for HER2 demonstrating high-level transgene expression (Figure 9).

Western analysis using lysates of cells isolated *ex vivo* from $\Delta 16\text{HER2}$ mice revealed a protein expression profile consistent with the immunohistochemical data, but also the presence of some phosphorylated $\Delta 16\text{HER2}$ dimers with a higher activation status in the

Figure 8. Characterization of tumorigenesis in $\Delta 16\text{HER2}$ mice. a. Primary breast tumors just before their removal from a $\Delta 16\text{HER2}$ transgenic female mouse. b. Immunohistochemical detection of HER2, revealing strong and uniform expression of HER2 protein in the mammary tumor, while the normal duct (right) is negative. Magnification: X400. c. Kaplan-Meier disease-free survival plot for F2 generation $\Delta 16\text{HER2}$ -LUC transgenic mice. Mammary tumor incidence is 100% and tumor onset is from 11 to the 19 weeks ($n=20$). d. Tumor growth curves of five different tumors. Tumor volume was calculated as $0.5 \times d_1^2 \times d_2$, where d_1 and d_2 are the smaller and larger diameters, respectively.

Figure 9. Pulmonary metastases. Hematoxylin-eosin (left) and immunohistochemical staining for HER2 (middle) and luciferase (right) in intravascular lung metastases in $\Delta 16\text{HER2}$ transgenic mice. Tumor cell aggregates are strongly positive for both human HER2 and luciferase staining. Magnification: X400.

dimeric than the monomeric form. These findings suggest a mechanism through which the disulfide-bonded $\Delta 16\text{HER2}$ homodimer amplifies HER2 transforming potential. The same analysis showed that Src kinase and several other protein mediators involved in the signaling cascade were consistently activated (phosphorylated), implicating this pathway in neoplastic transformation and tumor progression dynamics.

In vitro analyses to elucidate the oncogenic mechanisms involving the $\Delta 16\text{HER2}$ splice variant will benefit from the availability of $\Delta 16\text{HER2}$ -positive cell lines derived from surgically excised primary breast tumors and lung metastases from $\Delta 16\text{HER2}$ transgenic mice. Preliminary data from our ongoing efforts to establish such lines show that these cells maintain transgene expression even after repeated passages.

Overall, the higher tumor incidence as well as the shorter latency period in $\Delta 16\text{HER2}$ transgenic mice as compared with that in the MMTV-wild-type HER2 transgenic mice described by Finkle et al. [14], together with the higher metastatic potential of the splice variant, strongly supports the candidacy of $\Delta 16\text{HER2}$ as the transforming form of the HER2 oncoprotein. Indeed, despite some similarities between the two different experimental models, i.e., use of the same mouse strain (FVB) to derive the transgenic lines, use of the same MMTV promoter, and the development of rapidly growing adenocarcinomas, these transgenic experimental systems appear to differ greatly in some features that determine tumor aggressiveness. Not only do $\Delta 16\text{HER2}$ females develop asynchronous mammary adenocarcinomas with a higher tumor incidence and a significantly shorter average latency compared to wild-type HER2 transgenic females (15.1 vs 28.6 weeks), but transgene expression in the model described by Finkle et al was detected in tumor tissue as well as in normal mammary gland and several other epithelial tissues, whereas our transgenic mice revealed strong staining for the $\Delta 16\text{HER2}$ human transgene exclusively in tumor mammary tissue, suggesting that overexpression of $\Delta 16\text{HER2}$ in the mammary gland is sufficient to induce malignant transformation in a single step. It is also noteworthy that only 5 transgene copies can drive neoplastic transformation of mammary epithelial cells compared to a relatively high number of wild-type HER2 transgene copies (30-50) necessary to induce mammary adenocarcinomas in about 80% of MMTV-wild-type HER2 transgenic mice. Since the $\Delta 16\text{HER2}$ splice variant represents about 10% of total HER2 transcript in human breast carcinoma, it is plausible that malignant transformation ensues when $\Delta 16\text{HER2}$ reaches a critical threshold in mammary cells presenting HER2 gene amplification.

1.5. HER2 as target of immunotherapies

The crucial role of HER2 in epithelial transformation as well as its selective overexpression in cancer tissues makes it an ideal target for cancer immunotherapy. Notwithstanding the clinically approved use of the anti-HER2 monoclonal antibody Trastuzumab, a number of concerns, including resistance, considerable costs associated with repeated treatments, and side effects, make active immunotherapies that generate polyclonal and long-lasting immune responses desirable alternative approaches.

1.5.1. Involvement of $\Delta 16\text{HER2}$ in Trastuzumab resistance

Trastuzumab, a humanized monoclonal antibody against the ectodomain of HER2, was the first rationally designed anti-HER2 therapy approved by the US Food and Drug Administration for the clinical treatment of HER2-overexpressing breast cancer. Trastuzumab uses multiple mechanisms to interfere with HER2 downstream signaling and inhibit tumor growth, including HER2 receptor downregulation and blocking cleavage of the HER2 ECD, which otherwise leads to activation of the HER2 receptor [37]. In particular, the cytostatic effect of Trastuzumab is associated with the reduction of the signaling by the PI3K/Akt pathway and the upregulation of the cyclin-dependent kinase inhibitor p27kip1, as demonstrated by Western analysis of breast tumor cell lines treated with Trastuzumab *in vitro* [38]. The consequences of these Trastuzumab actions are G1 arrest, reduction in cell proliferation, and apoptosis. Trastuzumab also has cytotoxic properties, such as antibody-dependent cell-mediated cytotoxicity (ADCC) against HER2-overexpressing tumor cells. ADCC is mainly due to natural killer (NK) cells which express the Fc γ receptor that binds the Fc domain of the IgG1 Trastuzumab [38].

Overall, Trastuzumab is clinically effective, but a significant proportion of HER2-overexpressing breast cancer patients either do not respond to initial Trastuzumab treatment (*de novo* resistance) or eventually become resistant after continuous treatment (acquired resistance). In fact, an objective response (complete + partial) when Trastuzumab is used alone is observed in only about 26% of patients with HER2-positive tumors, and many of the initial responders develop resistance in less than 6 months [39]. While higher response rates (50–80%) have been reported when Trastuzumab is used in combination with standard chemotherapy for metastatic disease, primary and acquired resistance to this reagent remains a significant clinical problem.

Understanding the molecular mechanisms of Trastuzumab resistance is crucial for the development of new therapeutic strategies and for improved survival of HER2-positive breast cancer patients. Several mechanisms have been proposed in Trastuzumab resistance [38], including steric hindrance of HER2-antibody interaction by membrane-associated glycoproteins [40], PTEN deficiency (phosphatase and tensin homolog deleted on chromosome 10) [41], increased PI3K/Akt pathway activation [42], and HER2 crosstalk with other HER members or with insulin-like growth factor-I receptor (IGF-IR) [43]. In the latter context, Huang et al. [43] showed that a heterotrimeric complex of IGF-IR, HER2 and HER3 forming exclusively in Trastuzumab-resistant cells plays a key role in resistance, since knockdown of HER3 or IGF-IR by short hairpin RNA-mediated strategies upregulates p27kip1, inactivates downstream receptor signaling, and resensitizes resistant cells.

While these mechanisms may explain Trastuzumab resistance in tumors expressing only the full-length HER2 receptor, there is increasing recognition that HER2 altered forms, including p95HER2 fragments and the $\Delta 16\text{HER2}$ splice variant commonly coexpressed with wild-type protein in human tumors, play a significant role in Trastuzumab resistance. In a series of patients with HER2-positive advanced breast cancer and treated with Trastuzumab, Scaltriti et al. [44] reported that the presence of p95HER2 fragments, which

lack the Trastuzumab binding domain but retain kinase activity, was associated with clinical resistance to Trastuzumab, whereas tumors expressing only the full-length receptor exhibited a high response rate to Trastuzumab. Analysis of cell lines expressing either full-length HER2 or p95HER2 confirmed the ineffectiveness of Trastuzumab on cells expressing p95HER2 fragments [44]. Using a similar experimental approach, Mitra and coworkers [18] found that ectopic expression of $\Delta 16$ HER2, but not wild-type HER2, promotes Trastuzumab resistance in NIH3T3 fibroblasts and MCF-7 breast tumor cells; both $\Delta 16$ HER2-expressing cell types were refractory to Trastuzumab treatment, as shown in both cell proliferation and invasion assays, and displayed sustained oncogenic signaling. It seems likely that $\Delta 16$ HER2 expression above a critical threshold in wild-type HER2-overexpressing human breast cancer contributes to Trastuzumab resistance, consistent with clinical evidence of an inverse correlation between increased HER2 FISH ratios (>8) and Trastuzumab responsiveness [45].

Although the Trastuzumab binding site is amino-terminal to the exon 16 deletion of $\Delta 16$ HER2, Trastuzumab resistance might reflect the inefficient targeting of the splice variant receptor by the antibody because of the stable disulfide-bonded HER2 homodimers or the activation of alternative compensatory signaling pathways. In fact, $\Delta 16$ HER2 harbors an in-frame deletion which promotes constitutive dimerization of the receptor and the coupling of $\Delta 16$ HER2 to unique oncogenic signaling pathways mediated by Src kinase. Indeed, Mitra and coworkers [18] proposed Src kinase as the “master regulator” of $\Delta 16$ HER2 protein signal transduction, based on the cooperation between $\Delta 16$ HER2 and Src kinase as demonstrated by the ability of the Src family inhibitor dasatinib to induce Src inactivation, destabilization of $\Delta 16$ HER2, and suppression of tumorigenicity. Consistent with this proposal, a recent study by Zhang et al [46] identified Src activation as a key convergence point of several Trastuzumab resistance mechanisms, since targeting Src in combination with Trastuzumab resensitized multiple resistant cell lines and eliminated Trastuzumab-resistant tumors *in vivo*. The association between Src-mediated transduction pathways and the transforming ability of Δ HER16 in our transgenic model [19] points to the value of this model in preclinical studies to elucidate the *in vivo* mechanisms underlying Trastuzumab resistance as well as the role of this variant in HER2-targeted drug responsiveness. Moreover, $\Delta 16$ HER2 transgenic mice may recapitulate the clinical spectrum of Trastuzumab resistance associated with HER2-positive tumors and thus serve in testing innovative immunotherapies.

1.5.2. DNA vaccines

The idea of generating DNA vaccines comes from the pioneer work of Wolff and colleagues [47], who first showed that direct injection of naked DNA into the muscles of mice led to expression of the encoded reporter proteins. DNA vaccines are simple circles of DNA derived primarily from bacterial plasmids and contain a cDNA encoding the full-length or truncated target antigen, a strong viral promoter to drive antigen expression in mammalian cells, and a polyadenylation signal (usually from bovine growth hormone or from SV40) to terminate transcription. In addition, DNA vaccines contain sequences necessary for the cloning procedures (a multiple cloning site) and for plasmid production in bacteria,

Figure 10. DNA vaccine backbone. pVAX1 (Invitrogen) is a 3.0 kb plasmid vector designed for use in the development of DNA vaccines and approved for such use by the US Food and Drug Administration. Features of the vector allow high-copy number replication in *E. coli* and high-level transient expression of the protein of interest in most mammalian cells. The vector contains the following elements: human cytomegalovirus immediate-early (CMV) promoter for high-level expression in a wide range of mammalian cells; bovine growth hormone (BGH) polyadenylation signal for efficient transcription termination and polyadenylation of mRNA; kanamycin resistance gene for selection in *E. coli*; and origin of bacterial replication (pUC ori) (<http://products.invitrogen.com:80/ivgn/product/V26020>).

consisting of the origin of replication (usually from *E. coli*) and an antibiotic-resistance gene to permit selective amplification of the vaccine [48] (Figure 10).

After purification (Figure 11), the DNA vaccine is commonly delivered into the skin or muscle using the biolistic system, in which compressed helium propels DNA-coated microparticles through a gene gun [49], or by simple intradermal or i.m. injection. While both delivery methods permit the introduction of DNA vaccines, the efficacy of DNA vaccination is strongly increased if it is followed by an *in vivo* short electric pulse, i.e., electroporation [50], which enhances DNA transfection into normal tissues by inducing transient permeability of biological membranes through the opening of microscopic pores.

Once DNA vaccines enter mammalian cells, antigen synthesis and presentation occur [48], with professional antigen-presenting cells (APCs), such as dendritic cells, presenting the transcribed and translated antigen in the context of major histocompatibility complex (MHC) and costimulatory molecules. If DNA vaccine-coded antigens are processed as endogenous intracytoplasmic proteins, the peptide fragments are presented on cell surface MHC I molecules to cytolytic T lymphocytes (CD8+ T cells). If the antigens are secreted from the cells, they can be taken up by APCs, processed through the exogenous pathway, and presented by MHC II molecules for the activation of specific helper T cells (CD4+ T cells) which produce 'helpful' cytokines. For antibody responses, B cells recognize and respond to extracellularly exposed antigens, both secreted and transmembrane proteins [48]. Through these mechanisms, DNA vaccines can elicit both cellular and humoral responses, and this combined immunity may be more effective than either arm alone.

Figure 11. DNA vaccines production in bacteria. Once the antigen of interest is cloned into the plasmid, the vaccine is introduced into bacterial cells, where it replicates as the bacterium multiplies. The presence of a “relaxed origin of replication” and an antibiotic-resistance gene allows efficient plasmid replication in bacterial cells and their selection, two key aspects for high-scale plasmid production. Finally, the vaccine, i.e., RHuT, can be purified using commercial kits.

The ability to induce cytotoxic responses is a distinctive property of DNA vaccines, representing the only approach other than the use of live viruses for the activation of CD8⁺ T cells. In addition, unlike mammalian DNA, bacterial plasmids are rich in unmethylated CpG dinucleotides, which act as a “danger signal” that warns of bacterial infection and activates the innate immune response through recognition of CpG motifs by Toll-like receptor 9 expressed on B cells and APCs. Thus, DNA vaccines are effective even when administered without adjuvants [51].

DNA vaccines also have further distinct advantages over other vaccine prototypes, such as stability and ease of manipulation. Since the subcellular localization of the recombinant antigen dictates the type and the intensity of the immune response, it is possible to change the intracellular fate of a protein to induce selective immune responses [52, 53]. For example, signal (leader) sequences can be added or deleted from an antigen to modulate its immunogenic performance. Signal peptides, consisting of amino-terminal extensions that direct the insertion of proteins into the membrane of the endoplasmic reticulum, can be removed from secreted or membrane proteins to target them into the cytoplasm to improve antigen presentation by the MHC class I pathway and thereby enhance cytotoxic T cell

induction. To optimize cytotoxic responses, an antigen can also be ubiquitinated; when expressed in fusion with a target protein, ubiquitin can promote rapid proteasomal degradation of the protein, leading to MHC I peptide presentation [54]. Alternatively, CD4+ T-cell responses can be enhanced by a DNA vaccine designed to direct endogenously synthesized proteins to the lysosomal compartment of APCs, where the proteins are degraded into peptides that can be eventually loaded into the pockets of MHC class II molecules. For this purpose, a fusion construct of the full-length protein and the 20-amino acid C-terminal tail of lysosomal integral membrane protein-II (LIMP-II) has been used [55]. To favor the antibody response, signal sequences, usually of 16 to 30 amino acid residues comprising a central hydrophobic core and a C-terminal region with the cleavage site for signal peptidase, can be cloned in-frame with the sequence encoding a cytoplasmic protein, driving it toward the plasma membrane.

Recently, we have conceived a new kind of DNA vaccine that combines antigen expression with the silencing of molecules involved in the immunosuppression exerted by growing cancers. This double action is associated with two distinct modules: one is the conventional antigen expression cassette, while the other generates short hairpin (sh)RNA under the control of a polymerase III promoter [56, 57]. The RNA interference with synthesis of negative immune regulators, such as IDO or IL-10, is expected to ensure optimal presentation of the encoded antigen by APCs.

1.5.3. DNA vaccination targeting HER2

While the success of vaccination in preventing infectious diseases is uncontested, the derivation of efficient vaccines against cancer represents a more difficult challenge. Although cancer cells express antigens in a way that distinguishes them immunologically from normal cells, most tumors are only weakly immunogenic because most tumor antigens are "self" proteins and generally tolerated by the host. Thus, an effective cancer vaccine must activate the immune system to react against tumor-associated molecules and, in some cases, overcome immunological tolerance to such molecules. This implies a vaccine-stimulated immune reaction in patients showing no or only a weak pre-existing immune response against the tumor antigen [58].

The HER2 oncoantigen is considered an ideal target for DNA vaccination because it is directly involved in cancer progression and because it plays a causal role in the transformed phenotype, restricting the emergence of antigen-loss variants. HER2 overexpression in several carcinomas with an aggressive course, unlike its expression in normal tissues, ensures a specific anti-cancer response and minimal risk of an autoimmune attack on healthy tissues. Finally, HER2 is exposed on the cell membrane and can thus be readily targeted by antibodies and cell-mediated immunity [5]. On the other hand, HER2 is a "self" molecule, such that triggering a response to it must circumvent tolerance mechanisms.

An abundance of experiments in preclinical models demonstrates the promise of DNA vaccination as an effective approach to prevent the development of HER2-positive tumors, eliciting immune protection against spontaneous mammary carcinomas in mice transgenic

for the rat HER2 oncogene as well as in transplantable rat and human HER2-expressing tumors [59-65]. As we specifically documented [63], anti-HER2 antibody production after vaccination represents the main mechanism responsible for the anti-tumor response. In fact, anti-HER2 antibodies are able to downmodulate the expression of this growth factor receptor causally implicated in carcinogenesis. Indirect reactions, such as ADCC and complement-mediated cytotoxicity, are also crucial in preventing the onset of a tumor and controlling its progression.

As mentioned above, DNA vaccines are easily manipulated to optimize immune activation using recombinant DNA technologies. In one of the first studies of HER2-targeted DNA vaccination, the plasmid encoding the extracellular and transmembrane (EC-TM) domains of this molecule proved to be far superior to plasmids encoding only the extracellular domain (secreted form) or the full-length protein [59]. Most subsequent studies, performed in both wild-type BALB/c mice and cancer-prone BALB-neuT transgenic mice, confirmed the unique ability of this vaccine to trigger protective immunity toward rat HER2-positive tumors [60-63]. BALB-neuT mice transgenically expressing the rat activated neu oncogene under the control of the MMTV promoter are genetically predestined to develop lethal invasive carcinomas in the mammary glands at high multiplicity (all mammary glands are affected) and with relatively short latency [66]. About 50% of BALB-neuT mice electroporated with EC-TM plasmid when the mammary glands display atypical hyperplasia, at 10 and 12 weeks of age, remained free of autochthonous mammary tumors up to at least 1 year of age, whereas all unvaccinated mice succumbed to mammary cancer within 22–27 weeks [62].

In efforts to define the minimal antigen portion still able to elicit protective immunity, we carried out molecular dissection of the HER2 molecule through sequential deletions of multiples of 240 bp, corresponding to 80 amino acids, starting from the amino-terminal of the extracellular sequence [63]. The resulting seven cut-down fragments were cloned into a recipient expression vector downstream of the leader sequence, which drives the proteins through the endoplasmic reticulum toward the plasma membrane (Figure 12).

A first series of DNA vaccination experiments with these seven cut-down plasmids was performed in wild-type BALB/c mice transplanted with syngeneic rat HER2-positive adenocarcinoma cells, established from a mammary tumor of BALB-neuT mice (TUBO cells). Significant protection was obtained in mice immunized with the first four cut-down plasmids, while protection declined in mice immunized with shorter fragments. In particular, EC4-TM, which lacks almost half of the EC domain and exposes only 344 amino acids, protected all vaccinated mice through the induction of anti-rat HER2 antibodies at levels comparable to those in mice vaccinated with the whole EC-TM [63]. However, in wild-type BALB/c mice, vaccination triggered a strong immune response because the rat HER2 protein target is a foreign, xenogeneic antigen. It is much more difficult to induce immunoprotection in cancer-prone BALB-neuT mice, which are tolerant to rat HER2 protein because they express the transgene in the thymus early in life [67]. In those mice, only electroporation with EC-TM or EC4-TM led to a significant delay in the progression of mammary lesions, whereas the other cut-down plasmids were completely ineffective.

Interestingly, EC4-TM induced a stronger ADCC response than did the whole EC-TM, suggesting that EC4-TM provides accessible critical determinants that may be partially masked in the whole EC-TM [63]. Together, the results of these experiments suggest that the first 390 amino acids of HER2 are those responsible for triggering the protective immunity induced by EC-TM vaccination.

Figure 12. HER2 cut-down vaccines. The seven cut-down HER2 DNA fragments were inserted downstream of the leader sequence using EcoRI and XbaI restriction enzymes. The truncated protein encoded by the first cut-down plasmid (EC1-TM) displays an EC domain lacking the first 70 NH₂-terminal residues. All the other truncated proteins, encoded respectively by the EC2-TM, EC3-TM, EC4-TM, EC5-TM, EC6-TM, and EC7-TM cut-down plasmids, display EC domains progressively shortened by 80 NH₂-terminal residues. All of these truncated proteins have an identical TM domain.

In a strategy aimed at breaking the tolerance to HER2 and further improving the elicited protection in BALB-neuT mice, we constructed two new DNA vaccines, RHuT and HuRT, encoding rat and human HER2 chimeric proteins. Containing both syngeneic and xenogeneic portions of the protein antigen, they ensure specificity as well as a tolerance break [64, 65]. In particular, HuRT was derived by cloning the human cDNA fragment encoding the first 390 amino-terminal residues into the rat EC5-TM cut-down plasmid to regenerate the whole EC domain. Almost symmetrically, RHuT encodes a protein in which the 410 amino-terminal residues are from the rat HER2 and the remaining residues from human HER2 (Figure 13 and Figure 14).

Figure 13. RHuT electroporation. RHuT encodes for a chimeric protein in which the 410 NH₂-terminal residues are from the rat HER2 extracellular domain and the remaining residues from the human protein. After i.m. injection of 50 µg of DNA plasmid, two low voltage pulses of 150V of 25 ms with a 300 µs interval were applied through the insertion of Cliniporator needles (Igea, Carpi, Italy) into the mouse quadriceps muscles.

Figure 14. Presentation of xenogeneic peptides by dendritic cells (DC) contributes to an antibody response to both the tolerated and non-tolerated moieties of the antigen. Following DNA electroporation (a) with RHuT plasmid encoding for a rat (orange) and human (blue) chimeric HER2 protein, T cells (T) recognizing the xenogeneic peptides proliferate. The expanded T cells interact and provide helper signals to B cells, leading to the production of antibodies (Y) to both the xenogeneic and tolerated moieties by plasmacells (PC). Adapted from Iezzi M. et al, 2012 [68].

Chimeric vaccines displayed superior performance in tolerant BALB neu-T mice [64, 65]. While control mice vaccinated with empty pVAX plasmid developed HER2-positive mammary tumors within 27 weeks of age, all mice electroporated at 10 weeks and at 12 weeks of age with RHuT or fully rat EC-TM (RRT) remained tumor-free at 40 weeks. However, 10 weeks later, the protection of mice vaccinated with RRT decreased to about

50%, while 80% of RHuT vaccinated mice remained tumor-free (Figure 15a). In both cases, the tumor rejection pattern correlated with higher titers of anti-rat HER2 antibodies (Figure 15b). In addition, RHuT protection could be further extended by repeated boosting to maintain immunological memory [64].

Preclinical data obtained with RHuT provided the rationale for its use in an ongoing phase I clinical trial (EudraCT 2011-001104-34) approved by Italian Ministry of Health in HER2-positive head-and-neck cancers (protocol code: IOV-HN-1-2011). Moreover, preliminary data have shown that chimeric vaccines used in $\Delta 16$ HER2 transgenic mice are also able to counteract the aggressive breast carcinogenesis driven by the $\Delta 16$ HER2 splice variant.

Figure 15. Vaccination with RHuT effectively protects Balb-neuT mice from HER2-driven mammary carcinogenesis. a. Mammary tumor incidence of BALB-neuT vaccinated mice with RHuT (dotted red line, $n = 7$ mice), RRT (continuous red line, $n = 8$ mice), and empty control pVAX (dotted black line, $n = 6$ mice). Differences in tumor incidence were analyzed by the log-rank (Mantel-Cox) test. b. Mouse sera collected 2 weeks after the second vaccination were analyzed for anti-rat HER2 antibody titer by flow cytometry (b). Data are mean MFI $\pm$ SEM (* $p = 0.02$, Student's t test).

2. Conclusions

Breast cancer remains one of the major causes of morbidity and mortality in Western Countries, despite progress in both knowledge and treatment. A deeper understanding of the underlying biology of breast cancer is necessary for the identification of new molecular targets and development of novel targeted therapeutics.

In the last three decades, a large number of transgenic mice have been generated that demonstrate the direct involvement of the HER2 receptor in mammary carcinogenesis. In these cancer-prone mice transgenically expressing the rat or human HER2 molecule, the development of autochthonous tumors recapitulates several of the molecular and genetic features of human cancer progression. Some of these animal models support the hypothesis that overexpression of HER2 alone is not sufficient to generate mammary tumors and requires activating mutations.

Emerging evidence indicates that the $\Delta 16$ HER2 splice variant plays a key role in tumor progression and refractoriness to Trastuzumab treatment. Currently, a new mouse model transgenic for the human $\Delta 16$ HER2 isoform is available. While no single genetically engineered mouse can offer a complete model of the wide assortment of human neoplasms found in human breast cancer, the $\Delta 16$ HER2 mouse represents a novel tool to test the ability of drugs and vaccines to inhibit the progression of HER2-driven cancer and to investigate Trastuzumab resistance.

Among targeted therapies being developed for breast cancer, anti-HER2 cancer vaccines seem particularly promising.

However, the generation of an effective vaccine able to trigger a long-lasting immunity that prevents tumor recurrence in cancer patients implies the understanding of how tolerance, immunity and immunosuppression regulate antitumor immune responses. Equally important for the rational design of cancer vaccines is the development of new biotechnological tools for the identification of the most immunogenic portions of a molecule and for the selection of the key epitopes within a protein.

In Steven Spielberg's "War of the Worlds", mankind prevailed over extraterrestrial invaders thanks to immunity, because "For neither do men live nor die in vain." The challenge is to redirect the powerful mechanisms of the immune response, so effective against outside invaders such as microorganisms, against an inside enemy, i.e., cancer.

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