Review
Gene therapy for carcinoma of the breast
Genetic ablation strategies
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Abstract

The gene therapy strategy of mutation compensation is designed to rectify the molecular lesions that are etiologic for neoplastic transformation. For dominant oncogenes, such approaches involve the functional knockout of the dysregulated cellular control pathways provoked by the overexpressed oncoprotein. On this basis, molecular interventions may be targeted to the transcriptional level of expression, via antisense or ribozymes, or post-transcriptionally, via intracellular single chain antibodies (intrabodies). For carcinoma of the breast, these approaches have been applied in the context of the disease linked oncogenes *erb*B-2 and cyclin D_1, as well as the estrogen receptor. Neoplastic revision accomplished in modal systems has rationalized human trials on this basis.

Keywords: antisense, dominant-negative, gene therapy, oncogene, ribozyme

Introduction
The gene therapy approach of mutation compensation involves correction of the genetic lesions that are etiologic for neoplastic transformation [1•,2–4]. For the dominant oncogene type of genetic lesion, such genetic correction approaches are designed to functionally ablate dysregulated patterns of gene expression. In considering this type of gene therapy approach for breast cancer, knowledge of the genetic mechanisms responsible for initiation and progression of malignancy is of relevance. In this regard, breast cancer, like other types of human cancer, develops via a succession of genetic alterations [5–7]. Inherited or somatic genetic changes that occur in oncogenes, tumor suppressor genes, the DNA repair machinery, and cell cycle checkpoints lead to low-risk or high-risk forms of *in situ* carcinoma, and subsequently to invasion and metastasis. For colorectal carcinoma, such changes have been well enumerated by Vogelstein and coworkers [8,9].

A similar analysis of these genetic changes in breast cancer represents a daunting task, because of the greater genetic heterogeneity that characterizes carcinoma of the breast and because few of the relevant genes appear to cause familial syndromes. In this regard, potentially, functional analysis of gene products that are known to be involved in breast cancer (eg p53, Rb, p16, BRCA-1, BRCA-2, ATM), combined with further characterization of other loci implicated by allelic loss or gene amplification, will lead to the identification of pathways that are important to the pathogenesis of many or all breast cancers. Such an understanding of the molecular pathogenesis of breast cancer will allow rational application of novel gene therapeutic and pharmacologic strategies for adjuvant therapy and the early treatment of recurrence.

On this basis, it may be understood that genetic ablation gene therapy strategies may be most successful when such strategies are designed to correct one, or more, of the spe-

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EGFR = epidermal growth factor receptor; IGF = insulin-like growth factor; sFv = single-chain variable fragment antibody.

cific genetic changes known *a priori* to be present in tumor cells. Many of these changes, including gene amplification, allelic loss, *p53* mutation, and increased expression of certain oncogenes, are found in preinvasive lesions such as ductal carcinoma *in situ* of the breast [10–15]. Early correction of these defects could prevent subsequent progression of invasion and metastasis. Indeed, such gene therapy approaches for precancer have been attempted in the context of carcinoma of the lung, and are clearly relevant in the context of carcinoma of the breast also [16]. In addition, targeting of the same alterations in more advanced tumors could prove effective in supplementing current therapies: surgery, radiotherapy, and chemotherapy.

Genetic abnormalities in breast cancer

Two major forms of genetic alteration in breast cancer are loss of specific chromosome arms and gene amplification. Loss of heterozygosity analysis of polymorphic DNA markers have implicated chromosomes and subregions of chromosome arms that probably harbor tumor suppressor genes [7]. Nonetheless, in only a few cases have specific genes relevant to allelic loss been identified. Karyotype analysis and chromosome *in situ* hybridization approaches, such as comparative genomic hybridization or fluorescent *in situ* hybridization, point to amplified chromosomal loci likely to harbor oncogenes, and facilitate loss of heterozygosity studies by identifying regions of the genome that are under-represented in tumors [6,17–21]. These studies have shown that breast cancers are unusual among human tumors because of their great degree of genetic heterogeneity, suggesting that breast cancer in reality results from multiple genetic changes. Although characterization of the many unidentified genes that are relevant to allelic loss and gene amplification will undoubtedly suggest additional gene therapy strategies for breast cancer, current knowledge of a few such targets already offers the possibility of effective intervention.

The genetic heterozygosity of breast cancer may thus predicate gene therapy approaches that are targeted to multiple dysregulated alleles. In this regard, the genetic heterogeneity of breast cancer is reflected in the various oncogenes previously implicated. Genetic alteration involving known oncogenes is restricted to six loci that undergo gene amplification. No known genes, including the *ras* family members, have been shown to undergo base mutation or translocation in primary human breast cancer. Gene amplification occurs at the following specific loci at the approximate frequencies indicated: *erb*B-2 (chromosome 17q12, 20% of tumors), c-*myc* (8p24, 20%), *PRAD1/CYD1* (11q13, 15%), the fibroblast growth factor receptors (8p12, 10–15%), *BEK* (10q26, 10–15%), and the insulin-like growth factor receptor (IGF) (15q24-25, 2%). It also involves unidentified genes at chromosomes 13q31, 17q22-24, and 20q12-13.2 [19,20]. In addition to these, other potential oncogenes that are

expressed in the absence of genetic alteration include H-*ras*, *erb*B-1/epidermal growth factor receptor (EGFR), *erb*B-3, and others. Thus, a variety of candidate oncogenes have been identified that might be approached via genetic ablation strategies.

With the exceptions of c-*myc* and *PRAD1/CYD1* (encoding the kinase-associated cyclin D_1), gene amplification in breast cancer commonly involves one of several growth factor receptors, as noted above. Although the signal transduction mechanisms of these diverse molecules are currently under study, it is likely that common elements of the signaling machinery are involved. For example, signaling by p185[c-ErbB-2/1(ErbB-2)] utilizes downstream elements such as phospholipase C-γ, phosphatidylinositol 3-kinase, guanosine triphosphatase-activating protein, and the adapter protein SHC [22]. Gene therapeutic modulation of the basal signal transduction apparatus could therefore prove effective in a majority of breast cancer cases. Thus, despite the molecular heterogeneity, common points of dysregulation can provide a limited set of rational targets.

Genetic ablation strategies for breast cancer

In this regard, the dysregulation of oncogenes by mutation, gene amplification, gene rearrangement, or overexpression contributes to oncogenesis by removing controls on normal cell cycle regulation. One approach to gene therapy for breast cancer is to 'knockout' dominant oncogenes and thereby reduce the growth or invasive potential of the tumor. Inhibition or ablation of oncogenic function can occur at three levels. First, the translation of the oncogene can be targeted. This strategy involves the use of antisense molecules to sequester and/or functionally ablate oncogene messenger RNA. Second, the function of the gene product can be targeted. This approach uses polypeptides containing dominant interfering mutations ('dominant-negative') to downregulate signal transduction in tumor cells. Third, the nascent oncogenic protein can be prevented from reaching its proper intracellular location. This approach uses intracellular antibodies ('intrabodies') to pre-empt the cellular localization machinery and sequester growth factor receptors inside the cell.

Antisense molecules

With regard to antisense inhibition of oncogene function, early studies demonstrated inhibition of lymphoma growth by administration of naked antisense DNA to c-*myc* [23]. Somewhat more recently, a *myc* antisense phosphorothioate oligonucleotide that has enhanced stability has been shown to produce cytostatic effects in estrogen-dependent and estrogen-independent breast cancer cell lines [24]. As an alternative approach to delivering antisense molecules, inhibition of K-*ras* expression and its normally potent tumorigenicity was achieved in nonsmall cell lung cancer cells *in vitro* through the use of a mammalian plasmid vector that produces antisense RNA oligo-

nucleotides [25]. The same investigators showed a dramatic reduction in tumor growth in nude mice [26]. Additional genes relevant to breast cancer have been successfully targeted by antisense oligonucleotides. Antisense molecules to cyclin Di inhibited growth and reversed the transformed phenotype in esophageal cancer cells [27], antisense RNA to type 1 IGF receptor suppressed rat prostate tumor growth and invasion [28], and transforming growth factor-α antisense messenger RNA inhibited estrogen-induced proliferation in estrogen responsive breast cancer cells [29]. In addition, antisense approaches have been developed targeting the ErbB-2 oncoprotein. In those studies, antisense oligonucleotides delivered *in vitro* to breast cancer cell lines could accomplish downregulation of ErbB-2 with modest inhibition of cellular proliferation [30]. In addition, the adenoviral protein E1A has also been shown to allow selective ErbB-2 downmodulation with reversion of the transformed phenotype [31–33].

In the context of clinical trials, a messenger RNA antisense approach for breast cancer is being entered into human trials in a protocol by Arteaga and Holt [34•], which uses disabled mouse mammary tumor virus to drive the tissue-specific expression of antisense oligonucleotides to c-*fos* and c-*myc* in late-stage breast cancer patients with lung, meningeal or peritoneal metastases. These investigators have shown that delivery of this vector encoding antisense to c-*fos* results in inhibition of breast tumor growth and increased survival in a mouse model. Similarly, the E1A approach has been translated into the clinical context [35].

The use of antisense oligonucleotides must overcome several obstacles in order to be clinically useful. These include attaining stable intracellular levels by frequent administration or by constant production internally, and the need to inhibit oncogenes that are amplified or expressed at high levels. As for other therapeutic approaches, the molecule must be delivered to nearly every tumor cell in order to be effective in inhibiting growth or invasive potential. Thus, the enthusiasm for antisense approaches to human gene therapy trials has been limited.

Dominant negative mutations

A second approach to ablation of oncogene function is expression of dominant interfering or 'dominant negative' mutant proteins. Especially attractive targets for such an approach are the receptor tyrosine kinases (eg ErbB-1/EGFR, ErbB-2, and ErbB-3), which appear to be amplified and/or overexpressed in breast cancer. Two different mechanisms have proved effective in blocking receptor function. One approach disrupts dimerization, which is required for intracellular signaling. This was accomplished by transfection of cytoplasmic domain mutants of EGFR into cells expressing wild-type receptor. The resulting heterodimers failed to show high affinity EGF

binding, receptor endocytosis, or biological signaling [36]. Alternatively, growth factor binding to the receptor can be prevented by expression of a mutant growth factor, or by sequestering the growth factor extracellularly. NIH3T3 cells that produced a mutant platelet-derived growth factor molecule showed a reverted phenotype with a reduced growth rate in culture, and reduced invasive potential [37]. Extracellular sequestering was demonstrated for IGF-1. Cells were transfected with IGF binding protein-3, which bound IGF-1 in the extracellular space. This inhibited growth of Balb/c cells even in the presence of high concentrations of insulin and IGF-1 [38]. In addition, this type of approach has been applied in the context of the estrogen receptor, whereby neoplastic reversion has been achieved [39].

Intracellular single-chain antibodies

In addition to antisense strategies and dominant-negative mutations, another strategy aims at disrupting normal subcellular localization of growth factor receptors. We have recently developed an approach that prevents growth factor receptors from reaching the cell surface. By transfecting human ovarian cancer cells with a gene encoding an anti-ErbB-2 single-chain variable fragment antibody (sFv), we were able to demonstrate downregulation of cell surface ErbB-2 and a corresponding specific growth inhibition of cells overexpressing the receptor [40,41••]. This sFv-mediated oncogene downmodulation triggered apoptotic cell death in cells that overexpress the receptor [42]. Furthermore, it could be shown that breast cancer cells that overexpressed ErbB-2 were also eradicated in this manner [43]. Interestingly, the level of ErbB-2 that characterized breast cancer tumor targets was predictive of their response to this genetic intervention. The ability to accomplish selective abrogation of oncogenes by the use of intracellular sFvs opens a wide variety of possibilities in breast cancer investigation and therapy. These results were corroborated by Hynes and coworkers, who demonstrated ErbB-2 downregulation with impairment of receptor activation in breast cancer cells [44] and reversion of the transformed phenotype in ErbB-2 overexpressing cells [45].

Strategies for gene delivery

As for all mutation compensation approaches, the delivery context is an important predicate of the viability of the genetic intervention. In this regard, contexts whereby target cells can be manipulated *ex vivo* may allow the achievement of a level of specificity and efficiency of gene delivery that is commensurate with a meaningful therapeutic outcome. Thus, in the context of carcinoma of the breast it may be rational to employ such an *ex vivo* approach to achieve genetic purging of bone marrow, as has been attempted in a variety of neoplastic contexts [46,47].

Alternatively, locoregional and disseminated disease require direct *in vivo* delivery of the knockout gene. This

delivery context imposes a greater stringency, and current generation vectors are limited in their ability to achieve this goal [48,49•,50,51]. In this regard, such *in vivo* gene delivery approaches have been limited to disease contexts whereby tumor is sequestered in a compartment context. On this basis, the relatively limited presentation of breast cancer in this manner, combined with vector limitations in this regard, have restricted direct clinical application of genetic ablation strategies for carcinoma of the breast. This recognition has led to the exploration of vector approaches that possess the capacities for efficient, and cell-specific gene delivery *in vivo*. Such systems, based on both viral and nonviral schemes, have been attempted [52–54]. Thus, despite the fact that genetic ablation strategies offer the possibility of a tumor cell-specific effect, this utility can not be realized until vector approaches can achieve a requisite level of efficiency in the stringent *in vivo* gene delivery context.

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