

Regulatory Nodes That Integrate and Coordinate Signaling as Potential Targets for Breast Cancer Therapy

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Abstract

Blockade of the estrogen receptor (ER) with antiestrogens and aromatase inhibitors is effective in the treatment of breast cancer. Why ER plays such a dominant role in breast cancer and represents such an excellent target remains to be defined. The ability of ER to respond to multiple inputs and to control expression of multiple downstream genes may be one of the reasons why ER is such a powerful target for breast cancer treatment. The recent modest performance of a number of targeted therapies in breast cancer has raised the question whether we will ever develop therapies that have such success as antiestrogens. Targeted therapies tend to inhibit a single pathway that is probably altered in only a subset of patients. Even within this subset, only a limited number of patients respond. The evidence that virtually all pathways can cross-talk and that they exhibit several layers of redundancy reveals a complexity of signaling networks that may defy the generation of targeted therapies with efficacy similar to antiestrogens. However, there are clearly regulatory nodes that can integrate multiple upstream inputs and elicit diverse downstream outputs. We provide evidence and rationales for integrins, insulin receptor substrates (IRSs), and cyclin D1 as potential therapeutic targets. These proteins, similar to ER, can integrate and coordinate multiple signals in breast cancer cells and thus mediate diverse aspects of breast cancer progression. New treatment targets will emerge in light of more global models of signal transduction that fully integrate all aspects of cell biology such as the role of the extracellular matrix and will hopefully result in the development of targeted therapies that show efficacy similar to antiestrogens.

Introduction

Approximately 211,300 women in the United States will be diagnosed with invasive breast cancer in 2003—about 39,800 women will die from the disease. Breast cancer death rates declined significantly in the last decade, probably because of

earlier detection and improved treatment. Multiple clinical trials have demonstrated that systemic chemotherapy, hormone therapy, and immunotherapy after surgery and radiation therapy substantially reduce the risk of recurrence and metastases and prolong relapse-free and overall survival rates. Therapeutic strategies directed at inhibiting the action of the ER, using antiestrogens or aromatase inhibitors, represent one successful example of targeted therapy for clinical breast cancer (1).

This raises an interesting question of why the ER-targeting treatment has been so successful compared with other new targeted therapies? A simple answer is that *in vitro* and *in vivo* studies have demonstrated that estrogen receptor (ER) plays a key role in normal breast development and breast cancer progression. It is estimated that 75% of carcinoma-*in situ* breast lesions and similar percentages of invasive breast cancers express elevated levels of ER protein, whereas in normal breast epithelial cells, only 10% of cells express ER and only at low levels (2).

A molecular understanding of ER action may also highlight why ER is such a dominant player in breast cancer. ER can be activated by multiple upstream signals, including growth factors and other hormone receptors (3). In addition, ER can elicit multiple downstream signals via estrogen response elements in the promoters of estrogen-responsive genes but also via interaction with signal transducers and activators of transcriptions (signal transducers and activators of transcriptions), AP-1, SP-1, and so on. The ability of ER to respond to so many signals may be a result of its evolution as the first ancestral steroid receptor (4).

The transcriptional profile of ER-regulated genes is very apparent in recent microarray analysis. Several recent studies have shown that unsupervised hierarchical clustering of breast tumors results in the clustering of two major groups of breast cancer (5). One of the major differences between these groups is ER status and a group of genes that are coexpressed with ER. This suggests that ER may dictate a dominant gene signature in breast cancer, although there may also be other dominant players in this cluster of genes.

Research from different laboratories has revealed that ER can regulate or affect the six acquired capabilities or hallmarks of cancer cells (6). For example, ER can up-regulate cyclin D (Ref. 7; proliferation), insulin-like growth factor (IGF) signaling components (Ref. 8; proliferation and apoptosis), human telomerase activity (Ref. 9; replicative potential), and vascular endothelial growth factor (Ref. 10; angiogenesis) while down-regulating Rb (Ref. 11; antiproliferation). Thus, it is possible that antiestrogens may actually block cell cycle, replicative capacity, and angiogenesis while inducing apoptosis in breast cancer.

As with estrogen, growth factors also play an important role in breast cancer initiation and progression. Components of cell signaling pathways are frequently altered in breast cancer. It has been found that synergism and coordination exist between ER and growth factor signaling. ER can modulate growth factor

Presented at the Third International Conference on Recent Advances and Future Directions in Endocrine Manipulation of Breast Cancer, July 21–22, 2003, Cambridge, MA.

Grant support: NIH Grant R01CA94118, Department of Defense Award DAMD 17-02-1-0286, and an AstraZeneca research award (to A. L.) and Department of Defense Postdoctoral Fellowship Award DAMD 17-01-1-0133 (to X. C.).

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signaling by increasing the levels of growth factor signaling components and directly interacting with these proteins (12). On the other hand, growth factors can induce ligand-independent ER-mediated transactivation through phosphorylation of ER and its coactivators, which has been suggested to be one mechanism of antiestrogen resistance in breast cancer (13). Thus, growth factor signaling components represent promising therapeutic targets.

One challenge of developing signaling inhibitors is that distinct pathways can integrate or cross-talk at multiple levels and that redundancy exists in many systems. Although many inhibitors are currently in clinical trials or under development, only some show modest efficacy. One obvious reason is that distinct signaling pathways cross-talk with each other. Hence, a compensatory pathway can emerge or selectively strengthen after inhibitors block one specific pathway. Different signaling pathways can control or affect the same cellular function, and one single signaling pathway can regulate different cellular functions. This seemingly redundant cell signaling network actually may reflect a fine-tuning mechanism for cells to respond and adjust to combined effects of simultaneous or sequential stimulation by many extracellular or internal signals and to control duration and intensity of each signal.

Although the complexity and the redundancy of this signaling network create obstacles, there exist signal-converging proteins or signal integrators which receive or relay diverse signals and/or elicit distinct downstream signaling cascades. We call these proteins signaling nodes and propose that targeting these proteins may impair cellular processing of various signal inputs and, correspondingly, the six capabilities of cancer cells.

Membrane Nodes

The Extracellular Matrix (ECM). Coordination of cell-matrix adhesion is essential for the development and progression of diseases like cancer. Integrins are cell adhesion molecules that link the ECM to the intracellular cytoskeleton, and they can regulate cell proliferation, differentiation, apoptosis, angiogenesis, and migration [reviewed by Bissell *et al.* (14)]. Altered interaction between cells and the surrounding ECM is a common feature in a variety of tumors. How cancer cells adjust to and modify their surrounding ECM may be the key to render them invasive. Integrin engagement can trigger diverse downstream signaling pathways, which are at least as complex as growth factor signaling. In addition, the cross-talk between integrins and growth factor receptors is thought to play a relevant role in transformation and tumor progression. In some cases, integrins enable growth factor signaling, *i.e.*, normal growth factor signaling does not occur unless cells adhere to ECM. For example, IGF function depends upon ECM (15). Binding of primary mammary epithelial cells to laminin is required for insulin signaling (16). Not only can integrins enhance ligand-induced activation of growth factor receptors, but they also induce ligand-independent phosphorylation and activation of growth factor receptor through complexes containing both integrins and growth factor receptors (17). Importantly, integrin attachment can also regulate ER levels (18). Thus, we propose that integrins are membrane nodes that integrate ECM and other extracellular signals in breast cancer. Targeting inte-

grins may block cell communication with the extracellular environment and growth factor signaling simultaneously, leading to growth inhibition. Vitaxin, a $\alpha_v\beta_3$ integrin inhibitor that interferes with blood vessel formation by inducing apoptosis in newly generated endothelial cells, has been evaluated in a Phase I breast cancer clinical trial (19). Initial data suggest that this integrin represents a clinically relevant antiangiogenic target for prolonged cancer therapy. It is anticipated that accumulating data on the role of integrins and the mechanism of action of their pharmacological antagonists will help to develop and apply a powerful anti-integrin cancer therapeutic strategy.

Growth Factor Receptors. Growth factor receptors may also represent regulator nodes. An example of this is ErbB2, a receptor that is central to signaling by the ErbB family of receptors. Amplification of the ErbB2 gene, resulting in overexpression of the protein, is found in 20–25% of invasive breast cancers (20). Up-regulation of ErbB1 is also present in some breast tumors (21). Because of the frequency of aberrant ErbB family member signaling in breast cancer, therapeutic strategies targeting these proteins have been pursued intensively. Clinical trials of trastuzumab (Herceptin), directed against the extracellular domain of ErbB2, have shown that its response rate ranges from 12 to 40% when it is used as a single agent (22). Additional increase of its treatment efficacy is limited by two facts. First, it has been found that only patients with tumors overexpressing ErbB2 benefit from trastuzumab therapy. In addition, a considerable problem with trastuzumab treatment is *de novo* resistance and the development of acquired resistance in some breast cancers. Because ErbB family members share common signaling pathways, blocking one of them may not stop tumor growth as other members may compensate for the lost one. On the basis of this rationale, a pan-ErbB inhibitor has been developed to inhibit all ErbB members (22).

However, resistance to trastuzumab treatment in ErbB2-overexpressing breast tumors may be caused not only by other ErbB family members but also by non-ErbB extracellular signals. In breast cancer cell models that overexpress ErbB2, an increased level of IGF-I receptor (IGF-IR) signaling appears to interfere with the action of trastuzumab (23), enabling breast cancer cells to become resistant to trastuzumab. Additional studies have shown that cotargeting ErbB2 and IGF-IR causes strong synergistic inhibition of growth in ErbB2-overexpressing breast cancer cells (24). Thus, strategies that target IGF-IR signaling may be therapeutically useful to prevent or delay development of resistance to trastuzumab. Previous work in which IGF was found to indirectly activate ErbB1 and its downstream mitogen-activated protein kinase signaling (25) suggest that IGF-IR cross-talks with ErbB receptors. This was also substantiated by the finding that IGF-IR null cells do not respond to epidermal growth factor (EGF) stimulation (26) and that IGF-I is required for EGF-mediated cell cycle progression in mammary epithelial cells (27). Similarly, there is also a hierarchical interaction between IGF-IR and ErbB2, by which IGF-IR directs ErbB2 phosphorylation (28). Suppression of IGF-IR expression results in complete abrogation of phosphorylation of ErbB2 in breast cancer cells, whereas blockade of ErbB2 did not affect IGF-IR phosphorylation. Association of IGF-IR and ErbB2 can be induced by heregulin and IGF. These data imply that IGF-IR may serve as a membrane node to

coordinate EGF, heregulins, and IGF signaling. Targeting IGF-IR may affect different growth factor signaling in breast cancer, and thus, IGF-IR may be an ideal candidate for breast cancer treatment.

Cytoplasmic Nodes

Extracellular signals, via membrane receptors, can elicit diverse cytoplasmic signaling cascades, which constitute a complex signaling network. Different signaling pathways can interact or cross-talk at multiple sites, and each pathway possesses precise feedback control mechanisms at multiple locations along the cascade. Extensive research has established phosphatidylinositol 3'-kinase (PI3K) and Ras as major signaling integrators in the cytoplasm and accordingly potential targets for breast cancer therapy. However, there are two problems in selecting these proteins as targets. First, these proteins have redundant functions—both PI3K and Ras can regulate cell proliferation, apoptosis, and invasion. PI3K can be activated by Ras or by Ras-independent pathways, for example, by receptor tyrosine kinases, integrins, and heterotrimeric G-proteins (29). The effect of inhibition of either one in tumors may be compromised by activity of the other one. Indeed, it is also possible that inhibition of either pathway may add selective pressure in tumors for increased activity of the other protein and consequently nullify the inhibitor efficacy. Second, mutations in Ras, although occurring frequently in human cancers, are found in <5% of all breast tumors (30). Despite this, overexpression of Ras has been detected in some aggressive breast cancers. Similar to studies on Ras, PI3K, and Akt are rarely mutated or amplified in human breast cancer, but both may be overexpressed (31). Interestingly, the PI3K-negative regulator PTEN exhibits loss of heterozygosity in 40% of invasive breast cancers (32); thus, PI3K may indeed be activated by a decrease of PTEN expression in breast cancer. Given the above observations, it will be hard to predict the results from clinical trials using inhibitors of Ras and PI3K.

Then what about other cytoplasmic nodes? Scaffolding proteins or adaptor proteins, which bind multiple elements of a signaling cascade and coordinate signaling, may be ideal candidates. An example are the insulin receptor substrates (IRSs). These constitute a family of proteins that contain no intrinsic activity but harbor multiple tyrosine phosphorylation sites and thus, after phosphorylation by upstream signaling cascades, allow the binding and recruitment of an assortment of downstream signaling intermediates. Indeed, similar to the ER, there seem to be few signaling pathways that cannot interact with IRSs. They are activated by integrins, hormones, interleukins, growth factor receptors, and oncogenes. In response, they can themselves bind and/or activate PI3K, grb2, FAK, *Bcr/II*, 14-3-3 proteins, calmodulins, Rho-associated kinase, and many other proteins (33). Thus, IRSs may represent regulatory nodes that coordinate and integrate multiple different signaling pathways, many of which are important in tumorigenesis.

Several observations render IRS family proteins promising therapeutic targets in breast cancer. The importance of IRS-1 in breast cancer growth has been demonstrated through experiments using IRS-1 overexpression and antisense strategies (34), whereas IRS-2 has been associated with breast cancer cell motility and invasion (35, 36). Recently, IRS-1 was found to be

constitutively activated in breast cancers (37), and we have shown that high-levels of IRS-1 in breast tumors are associated with shorter disease-free survival (8).

Intriguingly, IRSs levels are modulated in many ways. It has been reported that EGF can elevate IRS-1 protein stability (38), and integrins can increase IRS-1 transcription (39). Both estrogen and progesterone can potentiate growth factor signaling in breast cancer cells or prime cells to growth factor signals (40). The observed synergism between estrogen and IGF effects may be attributed to estrogen-induced increase in IRSs levels (12). Recently, we have also found that progesterone may enhance IGF signaling via induction of IRS-2 expression in breast cancer cells (41).

Taken together, these findings indicate that IRSs represent a unique and valuable target for breast cancer therapy, given their critical role in various signaling pathways. Although there are other adaptor proteins such as Grb-2 and Crk, the latter are more selectively involved in specific signaling pathways. Hence, they may not be appropriate nodes in breast cancers.

Nuclear Nodes

As mentioned before, ER is a dominant regulator of breast cancer growth, perhaps because of its ability to respond to multiple inputs and control transcription of a large set of downstream genes. Cyclin D1 is also an attractive target for breast cancer therapy for many reasons. First, it is rate limiting for progression of cells through the G₁ phase of the cell cycle and is a stringent gatekeeper for cell proliferation. Genetic deletion of cyclin D1-impaired mammary gland development (42), whereas its overexpression causes mammary hyperplasia and carcinoma (43). Second, potent breast cancer mitogens such as estrogen, EGF, and the IGFs can induce cyclin D1 expression through the transcriptional mechanism and protein stabilization (44). The synergism between estrogen and the IGFs on breast cancer cells can also be attributed to their effect on cyclin D1 (45). Moreover, >50% of breast tumors exhibit up-regulation of cyclin D1, and the transforming properties of ErbB2 overexpression rely on intact cyclin D1 function (46). Surprisingly, cyclin D1 has been shown to have a novel role in growth regulation of estrogen-responsive tissues by activating ER-mediated transcription in the absence of estrogen and enhancing transcription in its presence (47). This cyclin D1 effect is not inhibited by antiestrogens, which may suggest that cyclin D1 plays a role in antiestrogen resistance. On the basis of these facts and rationales, it would be reasonable to expect that cyclin-dependent kinase inhibitors currently under preclinical development may offer new successful treatment in breast cancer patients.

Summary

Tremendous progress has been made in the treatment of breast cancer patients. A wealth of information gained in the last decade in the field of molecular endocrinology and tumor biology has advanced our understanding of the mechanisms of antiestrogen action and has led to substantial improvements in the efficacy of hormonal therapy and chemotherapy. Future increases in the survival rate of breast cancer patients largely depend upon the development of novel therapeutic strategies,

which in turn requires elucidation of sophisticated cellular signaling networks. The signaling nodes described here only represent some well-studied proteins. With the rapid progress of protein functional study using biochemistry, cell biology, and genetics tools, the scope of those multifunctional nodes will definitely be expanded. Because these signal-converging proteins control so many critical cell functions, to target them in treatment may cause complications and toxicities in patients. However, breast cancer cells frequently overexpress one or several of these signaling nodes and may evolve to highly depend on them for deregulated and unlimited growth. Thus, by selecting appropriate concentrations, treatment time, and combinations with other agents, it is possible to minimize side effects of targeted therapy. We believe that signaling nodes represent attractive targets for the treatment and prevention of breast cancer.

Open Discussion

Dr. Jeffrey Green: What is the status of the IRS in ER-negative tumors? Is it phosphorylated?

Dr. Lee: IRS-1 levels correlate with hormone receptor status, so if you just look at it across the board, it is much lower in ER-negative than it is in ER-positive tumors. We haven't looked at its phosphorylation status. Chang did, and if I remember rightly, in all of the tumors it was consistently phosphorylated (Q. Chang *et al.*, *Cancer Res.*, 62: 6035–6038, 2002). This was in both ER positive and ER negative. So I don't really know. We now have phospho-specific antibodies and we are going to look at them in paraffin sections, rather than going back to our original immunoblot studies.

Dr. Robert Nicholson: If there is such a tight relationship between ER and IRS-1, why isn't IRS-1 just considered part of the ER signaling cascade that promotes tumor cell growth? Why is it necessary to distinguish between the two signaling rafts?

Dr. Lee: Should we target the things that are completely independent? I think that is the question. I think we always want to try and add things to the drugs that we already have that are quite well tolerated. We know there is a synergism when we add anti-IGF therapies to already established hormone therapy, since we get an additional response. I think all hormone therapies dramatically knock down IGF signaling. The question is will it add to anti-IGF therapy? Maybe in the hormone-resistant disease.

Dr. Myles Brown: I guess one problem is, at this point, that the IRS may be involved in some of the oncogenic events in breast. It may be causing activation of downstream critical events. We don't find mutations in the Ras and PI3 kinase pathways, so what do we have? We have PTEN loss. We don't have Ras mutations or Raf mutations. So most changes seem to be directly upstream.

Dr. Lee: So then blocking IRS and taking it out would be a mechanism of blocking downstream signaling via PI3K and MAPK.

Dr. Daniel Medina: Is there any specific inhibitor of the IRSs?

Dr. Lee: That is complicated; IRS-1 doesn't have enzymatic activity, so it is not a great initial target. One could block its binding to receptors—it binds via PTB domains. However,

these types of interactions are very hard to interrupt with small molecule inhibitors, for example. You could target its phosphorylation and interaction with downstream signaling molecules, but again, those interactions are hard to break up with small molecule inhibitors. There are too many bonds there. There is an effort now underway to try to find inhibitors to receptors binding the IRS.

Dr. Matthew Ellis: If you look at the basal type in profile experiments, is there anything with cluster analysis or anything else you know that might suggest the node to target there?

Dr. Lee: There are lots of genes in those clusters. It is going to take a long time to weed that out and find the dominant one.

Dr. Ellis: With siRNA technology, one can presumably test and find these dominant molecules, and we have cell lines that have basal-like mimicry. The definition of a node is that it has a dominant effect, whether you inhibit its expression or you inhibit ER. With an orchestrated approach you can start looking for those nodes.

Dr. Lee: Exactly, and that's why we will go for a global screen. Hopefully, that will pick out those critical ones.

Dr. Brown: IRS-1 can feed into the MAP kinase and PI3K kinase pathways. Which one do you think is dominant? Would we know whether it would be targeting Raf or targeting PI3K?

Dr. Lee: At the moment we don't know.

Dr. Carlos Arteaga: Why not try blocking IRS phosphorylation with an IGF1 receptor kinase inhibitor or an IGF1 receptor antibody?

Dr. Lee: That's a possibility; the problem is that it's not just downstream the IGF receptor. So that gets into the question: Is it being activated by growth hormone or by integrin signaling? The other problem always comes back to the idea of the redundancy of those growth factors.

Dr. Arteaga: What is the mechanism by which IRS-2 induces tumor cell motility—is that known?

Dr. Lee: We don't know that at the moment.

Dr. Arteaga: Is high IRS-2 expression more predominant in ER-negative tumors?

Dr. Lee: We haven't done that. We have never looked at IRS-2. We focused on IRS-1 initially because that was the dominant activated molecule. It turns out that IRS-2 seems to be very interesting with this motility issue, so now we are going back and looking at that.

Dr. Mina Bissell: Do you think that siRNA may help you understand this better, or if you put IRS-1 into a human mammary cell, what does it do? Then if you inhibit its function, can you revert this transformed phenotype? How do its levels change in the mammary gland?

Dr. Lee: We are just doing that at the moment. We've done it in breast cancer cells. We did those studies using antisense to IRS-1; they stopped growing, and they became sensitive to chemotherapy. In the mouse, yes, it is both developmentally and hormonally regulated; it is low in virgin mice, increases during pregnancy, increases about 200-fold during lactation and then within 6 hours of involution it is completely lost. This is without a change in mRNA levels, so there is a dramatic posttranscriptional regulation.

Dr. Bissell: So IRS-1 levels go down in involution and then the level comes back up?

Dr. Lee: Yes, and it needs to go away during involution, because you need to get rid of the survival signals.

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