Aberrant Splicing of Estrogen Receptor, HER2, and CD44 Genes in Breast Cancer

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ABSTRACT: Breast cancer (BC) is the most common cause of cancer-related death among women under the age of 50 years. Established biomarkers, such as hormone receptors (estrogen receptor [ER]/progesterone receptor) and human epidermal growth factor receptor 2 (HER2), play significant roles in the selection of patients for endocrine and trastuzumab therapies. However, the initial treatment response is often followed by tumor relapse with intrinsic resistance to the first-line therapy, so it has been expected to identify novel molecular markers to improve the survival and quality of life of patients. Alternative splicing of pre-messenger RNAs is a ubiquitous and flexible mechanism for the control of gene expression in mammalian cells. It provides cells with the opportunity to create protein isoforms with different, even opposing, functions from a single genomic locus. Aberrant alternative splicing is very common in cancer where emerging tumor cells take advantage of this flexibility to produce proteins that promote cell growth and survival. While a number of splicing alterations have been reported in human cancers, we focus on aberrant splicing of *ER*, *HER2*, and *CD44* genes from the viewpoint of BC development. *ERα36*, a splice variant from the *ER1* locus, governs nongenomic membrane signaling pathways triggered by estrogen and confers 4-hydroxytamoxifen resistance in BC therapy. The alternative spliced isoform of *HER2* lacking exon 20 (Δ16HER2) has been reported in human BC; this isoform is associated with transforming ability than the wild-type HER2 and recapitulates the phenotypes of endocrine therapy-resistant BC. Although both *CD44* splice isoforms (*CD44s*, *CD44v*) play essential roles in BC development, CD44v is more associated with those with favorable prognosis, such as luminal A subtype, while CD44s is linked to those with poor prognosis, such as HER2 or basal cell subtypes that are often metastatic. Hence, the detection of splice variants from these loci will provide keys to understand the pathogenesis, predict the prognosis, and choose specific therapies for BC.

KEYWORDS: alternative splicing, breast cancer, tumorigenesis, metastasis, estrogen receptor, HER2, CD44, signal transduction, stem cell, prognosis

CITATION: Inoue and Fry. Aberrant Splicing of Estrogen Receptor, HER2, and CD44 Genes in Breast Cancer. *Genetics & Epigenetics* 2015:7 19–32 doi:10.4137/GEG.S35500.

TYPE: Review

RECEIVED: September 24, 2015. **RESUBMITTED:** November 1, 2105. **ACCEPTED FOR PUBLICATION:** November 3, 2015.

ACADEMIC EDITOR: Christian Bronner, editor in chief

PEER REVIEW: Four peer reviewers contributed to the peer review report. Reviewers' reports totaled 919 words, excluding any confidential comments to the academic editor.

FUNDING: K. Inoue was supported by NIH/NCI 2R01CA106314, ACS RSG-07-207-01-MGO, and KG080179. The authors confirm that the funder had no influence over the study design, content of the article, or selection of this journal.

COMPETING INTERESTS: Authors disclose no potential conflicts of interest.

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Paper subject to independent expert blind peer review. All editorial decisions made by independent academic editor. Upon submission manuscript was subject to anti-plagiarism scanning. Prior to publication all authors have given signed confirmation of agreement to article publication and compliance with all applicable ethical and legal requirements, including the accuracy of author and contributor information, disclosure of competing interests and funding sources, compliance with ethical requirements relating to human and animal study participants, and compliance with any copyright requirements of third parties. This journal is a member of the Committee on Publication Ethics (COPE). Provenance: the authors were invited to submit this paper.

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Introduction

Alternative splicing (AS) is a mechanism through which cells generate multiple messenger RNAs (mRNAs) with different functions from a single genomic locus. This is conducted by the inclusion or exclusion of specific exons in pre-mRNA processing. It occurs in nearly all the mammalian genes that consist of multiple exons and is catalyzed by the spliceosome, a protein complex that consists of five small nuclear ribonucleoproteins.[1,2] It is assisted by numerous transacting factors that recognize cis-regulatory sequences within the pre-mRNAs and direct splice variants generated from different mechanisms, including alternative promoters, preferential usage of exons or splice sites, and/or alternative sites for polyadenylation.[3]

AS gives a significant evolutionary advantage by providing proteomic diversity.[4] It is often regulated in a tissue-specific manner and contributes to the remodeling of protein–protein interaction networks.[5] The functional classes of genes that are regulated by AS include both those with wide-spread homeostatic activities and those with cell type-specific functions. AS can drive determinative physiological change or can have a permissive role by providing mRNA variability that is used by other regulatory mechanisms.[6] AS is pervasive in stem cells and has a fundamental impact on stem cell differentiation by regulating different isoforms of the core pluripotency transcription factors. Additionally, splicing factors can regulate pluripotency by affecting stem cell-specific AS. Thus, the crosstalk between AS and other gene regulatory networks has a fundamental effect on the maintenance and differentiation of stem cell pluripotency.[7,8]

A common signature of cancer cells is a general loss of splicing fidelity with the concomitant reorganization of splicing profiles and even switching to specific splicing isoforms usually expressed in other cell types to bestow incipient cancer cells a growth advantage; thus, specific splicing errors are detectable in fully developed cancer cells than pathologically normal-looking tissues. Indeed, genome-wide studies have revealed the existence of cancer-specific splicing alterations.[9–13] The ability to regulate AS could be beneficial to emerging cancer cells at their early stage of development if splice isoforms encode proteins that stimulate cell proliferation and inhibit apoptosis, driving their uncontrolled cell growth. This switch in splicing preference can be critical as numerous genes

possess splice variants that have dominant-negative or even antagonistic activities. Typical examples for these are aberrant splicing for *p63* and *p73*, where oncogenic splice variants are generated from tumor-suppressive loci by aberrant splicing,[14] contributing to solid tumors. Splicing abnormalities are also common in hematopoietic malignancies. Yoshida et al[15] performed whole-exome sequencing of myelodysplasia specimens and found novel pathway mutations involving multiple components of the RNA splicing machinery. These splicing pathway mutations were frequent (45%–85%) in myeloid neoplasms showing features of myelodysplasia.[16]

Many onco- and tumor suppressor genes are aberrantly spliced in cancer.[9] They include genes that control cell cycle progression (eg, cyclin D1b[17,18]), proliferation (fibroblast growth factor [FGF] receptor, telomerase[19]), differentiation (C/EBP[20]), signal transduction (Ha-Ras, Rac1, Ron[9]), cell death (Bcl-x, Fas1, caspase 2[9]), angiogenesis (VEGF-A[9]), tumor suppression (p53, p63, p73, DMP1[14,21]), and invasion and metastasis (ASF/SF2, SRp20, hTra2β1, YB-1, MDM2[9–13]).

Breast cancer (BC) is the most common malignance in women in the US[22] and industrialized countries. Although there has been significant progress in the diagnosis and treatment in the past decades, significant number of patients die of relapsed disease, thus improved diagnosis including gene expression and microRNA profiling and stem cell evaluation to decide therapeutic strategy therapy is expected.[23–25] BC is categorized into five groups (luminal A, luminal B, human epidermal growth factor receptor 2 [HER2] type, triple negative, and normal like) dependent on the cell surface and other molecular markers, which are critical in predicting the prognosis and deciding therapies (see Refs. 26–30 for review). Aberrant splicing of genomic loci for estrogen receptors (ERs), *HER2/neu*, *Cyclin D1*, *BRCA1*, *BARD1*, *Tenscin-C*, and *CD44* has been shown to contribute to breast carcinogenesis. This review focuses on the splicing mechanisms for *ER*, *HER2*, and *CD44* genomic loci, which play essential roles in breast carcinogenesis and development.

Estrogen Receptor

Estrogens play an important role in the development and progression of BC. ERα and ERβ are encoded by two distinct genes, *ESR1* (Fig. 1A) and *ESR2*, that are on

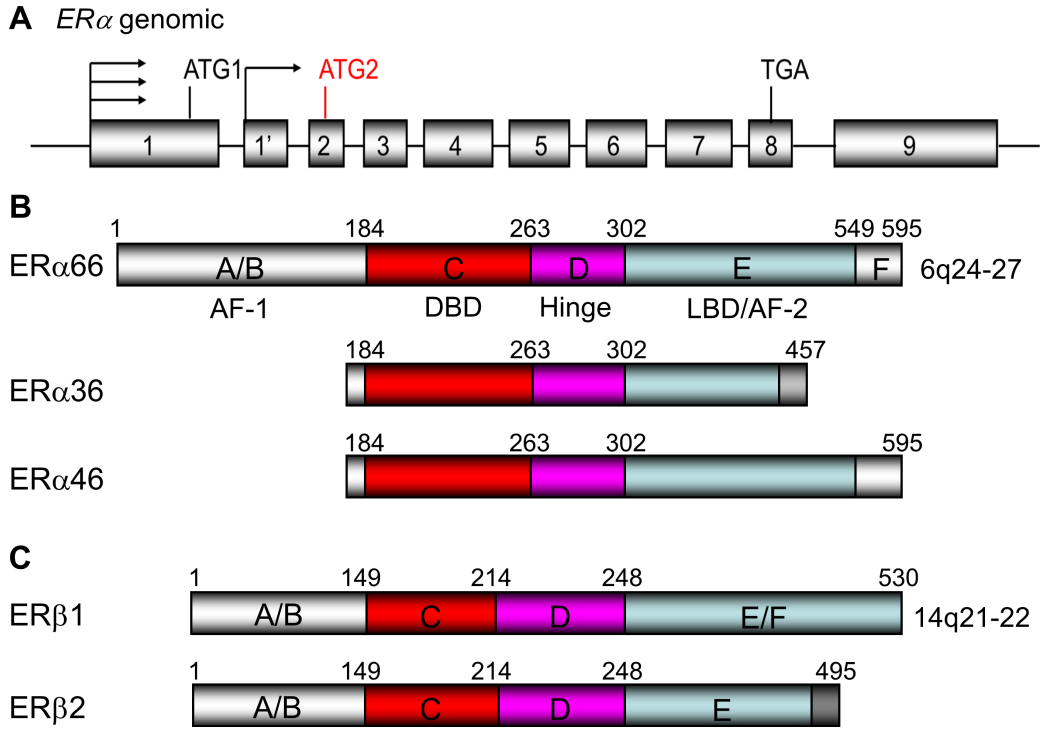

Figure 1. Human estrogen receptor domain structures and their variants. (**A**) The genomic structure for the human *Estrogen Receptor 1* (*ESR1*) locus. The human *ESRα* locus consists of 10 exons with two different promoters. The ATG for ERα66 exists in exon 1, while that for ERα36 and 46 exists in exon 2. The stop codon is in exon 8. The second promoter for *ERα36/46* is repressed by ERα66. (**B**) The domain structure for human ERα66 and its splice variants ERα36 and ERα46 (38, Sotoca 2012). ERα contains two transactivation functions, a weak constitutive activation function (AF-1; A/B domains) and a hormone-dependent activation function (AF-2). AF-2 (E domain) works by recruiting a large coactivator complex, composed of one or more p160s, CBP/p300, and P/CAF via direct contacts with the p160s. The DNA-binding domain (C domain) binds specific EREs on genomic DNA. The D domain is a bridging region that connects the C and E domains. The F domain of ERα is critical for attenuation of E2β-induced receptor dimerization and transcriptional activity. Both ERα36 and ERα46 lack domains A and B responsible for transactivation. ERα36 lacks the C-terminal domain E but has 27 unique amino acid residues (gray). ERα46 has the same domains E and F as those in ERα66. (**C**) The domain structure for human ERβ1 and its splice variant ERβ2.[71] Although ERβ1 binds to E2β, ERβ2 does not bind to it, but blocks the activity of ERα through heterodimerization.

human chromosomes 6q24–27 and 14q21–22, respectively. Hormone-activated ERs form homo- or heterodimers, namely, ERα (αα), ERβ (ββ), and ERαβ (αβ). ERα and ERβ show significant overall sequence homology, and both are composed of six domains A–F (Fig. 1B and C). In the absence of estrogen, ERs are largely located in the cytosol. Hormone binding to the receptor triggers a number of events starting with the migration of the receptor from the cytosol into the nucleus, dimerization of the receptor, and subsequent binding of the receptor dimer to specific sequences of DNA known as estrogen-response elements (EREs). The DNA/receptor complex then recruits other proteins that are responsible for gene transcription, resulting in a change in the cellular function. When the ERα and ERβ are coexpressed, each of them mediates specific effects with estrogens in the BC cells (reviewed in Refs. 31–33).

Both ERα (consisting of 595 amino acids [aa]) and ERβ (530 aa) are hormone-responsive nuclear receptors.[33] ERα contains two transactivation domains, such as a weak, constitutive activation function (AF-1) and a hormone-dependent activation function (AF-2; Fig. 1B). AF-2 (E domain) works by recruiting a large coactivator complex, composed of one or more p160s, CREB-binding protein (CBP)/p300, and p300 and CBP-associated factor (P/CAF) via direct contact with the p160s.[34] The DNA-binding domains (DBDs, C domains) have 96% homology between ERα and ERβ and bind most EREs on genomic DNA. The D domain is a bridging region that connects the C and E domains. The F domain of ERα is critical for the attenuation of 17β-estradiol (E2β)-induced receptor dimerization and transcriptional activity.[35]

Genomic versus nongenomic (membrane signaling) pathways governed by estrogen. Estrogen-stimulated cell proliferation by E2β is largely mediated by the activation of ERα66 localized in the nucleus. However, earlier studies also reported that estrogen binds to a cell surface receptor and stimulates a rapid generation of cyclic adenosine monophosphate (cAMP).[36,37] Subsequently, other reports of a plasma membrane-localized ER that transduces membrane-initiated estrogen signaling appeared; this membrane signaling (ie, nongenomic pathway) was found to activate different cytoplasmic proteins, including adenylate cyclase, G proteins, protein kinase C δ, phospholipase C, and mitogen-activated protein kinase, and phosphatidylinositide 3-kinase/protein kinase B (PI3K/Akt) pathways.[38–41] These nongenomic pathways control more genes that are involved in the regulation of cell growth, survival, motility, invasion, and apoptosis than the genomic pathway.

ERα splicing—ERα46 and ERα36. There are at least two physiologically relevant splice variants of full-length ERα (ERα66), ERα46, and ERα36 (Fig. 1). Transcription of *ERα36* initiates from a previously unidentified promoter in the first intron of ERα66 (Fig. 1A, ATG2), which continues from exon 2 to exon 6 and skips the final exons 7 and 8 of *ERα66*. Transcription for ERα46 also starts from ATG2 but uses the same exons 2–8 as those for ERα66. Both ERα46 and ERα36 are truncated in the amino-terminus (173 aa) and lack the first transcriptional activation domain (AF-1). ERα36 lacks the second transcriptional activation domain in AF-2 and has a unique 27-aa sequence at the C-terminus. This extra sequence may change the ligand-binding spectrum of an ERα in a way that it can interact with other factors than estrogens. Conversely, ERα46 is identical to ERα66 in the amino acid sequences for the C–F domains (Fig. 1B). Both ERα46 and ERα36 make homodimers or heterodimers with ERα66. ERα46 has a twofold higher binding affinity to the EREs than ERα66.[42] While 74% of ERα66 is in the nucleus, ERα46 is localized in the plasma membrane (26%), cytoplasm (30%), and nucleus (26%), possibly by palmitoylation.[43] Overexpression of ERα46 in Michigan Cancer Foundation-7 (MCF-7) cells reduces E2-stimulated endogenous pS2, cyclin D1, nuclear respiratory factor-1, and progesterone receptor (PR) transcription.[44] Transfection with ERα46 changed the pharmacology of E2 regulation of oncomiR *miR-21* expression from inhibition to stimulation, suggesting that ERα46 inhibits the ERα66 activity.[44]

Wang et al[39] showed that ERα36 is primarily localized on plasma membrane (50%) and cytoplasm (40%) rather than on the nucleus in HEK293 cells. Since ERα36 has three potential myristoylation sites near the N-terminus,[38–41,45] instead of the nuclear location signals of ERα66, it may be modified by palmitoylation and located in the plasma membrane/cytoplasm. ERα36 inhibits the traditional nuclear estrogen signaling mediated by ERα66 in a dominant-negative fashion since it has the DBD but lacks the two transactivation domains (Fig. 1B).

The molecular mechanisms for the differential expression of ERα66 and α36 have been studied. ERα36 is highly expressed in the majority of ERα66(–) BCs; overexpression of ERα36 occurs with a decrease of ERα66, indicating that the expression of ERα66 and ERα36 is mutually exclusive.[41] This is because, at least in part, ERα66 negatively regulates the promoter activity of *ERα36* (Fig. 1A).[46] The Wilms' tumor gene *WT1* encodes a zinc-finger protein WT1 that functions as a dual transcription regulator to activate or suppress gene transcription (reviewed in Refs. 47–49). Although the gene has been cloned as a tumor suppressor for the gene in Wilms' tumor, high levels of wild-type (the phenotype of the nonmutated form of a species as it occurs in nature) *WT1* are found in acute leukemias and solid tumors with poor prognosis,[50–52] involved in cell proliferation[53] and block cellular differentiation when the wild-type is overexpressed.[54] High passage ER-positive BC MCF-7 cells were found to express ERα66 and WT1 at higher levels and ERα36 at a very low level.[55] MCF-7 cells depleted for *WT1* expressed a reduced level of ERα66 with an increased level of ERα36, suggesting that WT1 increases ERα66/ERα36 ratio. WT1 directly activated the promoter of the *ERα66* gene while repressing the *ERα36* promoter. Thus, WT1 stimulates genomic estrogen signaling (mediated by ERα66) while inhibiting nongenomic

(by ERα36) estrogen signaling.[55] A similar finding was reported by Nasomyon et al,[56] who reported that the WT1 expression correlated with the high expression of ERα and HER2, leading to cell proliferation and might be involved in cancer development and progression.

Another factor that affects the ERα66/ERα36 ratio is synuclein γ (SNCG), which binds to ERα66 or ERα36 depending on the estradiol concentration.[57] SNCG is highly expressed in cancer cells but not in normal epithelium. Shi et al showed that SNCG expression enhanced estrogen-induced activation of ERK1/2 and mechanistic target of rapamycin (mTOR). Heat-shock protein 90 (Hsp90) acts as molecular chaperone, a group of proteins that assist the covalent folding/unfolding and the assembly/disassembly of other macromolecular structures, together with several cochaperone molecules.[58] Hsp90 binds to its client proteins such as steroid receptors, Cdks, and Akt that regulate cell cycle, survival, and death and promotes their proper protein folding, assembly, and transportation across different cellular compartments.[59] Disruption of Hsp90 with 17-N-allylamino-17-demethoxygeldanamycin (17-AAG) significantly reduced ERα36 expression and membrane-initiated estrogen signaling, which was recovered by the expression of SNCG.[57] Expression of SNCG also rendered tamoxifen (TAM) resistance, consistent with the clinical observation that ERα36 expression was associated with TAM resistance explained later. In summary, their study indicates that ERα36 mediates membrane-initiated estrogen signaling and that SNCG can replace the function of Hsp90, a molecular chaperone ERα36, to stimulate ligand-dependent cell growth.[57]

Expression of ER36 in primary BC samples and their roles in TAM resistance. Under normal conditions, ERs bind to estrogen and then translocate to the nucleus, subsequently binding to specific EREs and regulating transcription of downstream gene expression. With the dynamic regulation of ERα66 and ERα36, genomic and nongenomic estrogen signaling pathways should be coordinated to maintain a balance. An imbalance between ERα66 and ERα36 may result in abnormal proliferation and differentiation, leading to BC and other neoplastic disorders.

The role for ERα36 in nongenomic, membrane signaling of estrogens has been studied in BC cell lines. To understand the role of ERα36 in breast carcinogenesis and drug resistance, it is essential to study the expression of the protein in a primary BC specimen. Lee et al[60] studied 31 tissue samples of patients with BC for ERα36 and ERα66 protein expression status by immunohistochemistry and six additional patient tissue samples by Western blotting using an antibody specific to each ERα isoform. They found a cytoplasmic and plasma membrane-associated expression pattern of ERα36 in both ERα66-positive and -negative BC samples. Furthermore, ERα36 expression was associated with decreasing nuclear/cytoplasmic ERα66 expression, suggesting its potential use as a diagnostic and prognostic marker. In conclusion, ERα36 is frequently expressed in ERα66-negative BCs, which may provide additional information for better diagnosis and prognosis of BC.[60]

Antiestrogens such as TAM have provided a successful treatment for ER-positive BC for the past four decades. However, BCs eventually acquire resistance to TAM therapy.[61] The molecular mechanisms for the TAM resistance have been extensively studied to overcome the problem.[62–68] It was reported that BCs expressing high concentrations of ERα36 benefited less from TAM therapy than those with low levels, indicating that increased ERα36 levels are one of the underlying mechanisms of TAM resistance.[45] Zhang and Wang[40] reported that TAM increased ERα36 concentrations, and TAM-resistant BC cells expressed high levels of ERα36. Depletion of ERα36 in TAM-resistant BC cells with short hairpin RNA restored TAM sensitivity. They also found that cells with high concentrations of ERα36 protein were hypersensitive to estrogen, activating ERK phosphorylation at a picomolar range. Thus, elevated ERα36 is one of the mechanisms by which ER-positive BC cells escape TAM therapy and provided a rationale to develop novel therapeutic approaches for TAM-resistant patients by targeting ERα36.

The structure of ERβ. ERβ (Fig. 1C) has been identified as the major form of ER in the normal breast that is localized in the luminal epithelium, myoepithelium, and also in the stroma.[69–71] Similar to the ERα, ERβ1 binds to E2β with high affinity through its ligand-binding domain (LBD), but they share only moderate homology at the protein level (58% in humans) at the LBD.[69] ERβ2 lacks the C-terminal LBD (Fig. 1C) and thus does not bind to E2. Since the homology for the DBD for ERα and ERβ is very high (96% in humans[69]), they interact with specific EREs and transactivate common ER target genes.[72]

The biology of ERβ: a tumor suppressor in BC? Approximately 58% of BCs express both ERα and ERβ, 14% express ERα only, and 18% express ERβ only.[73] Ectopic expression of ERβ inhibits E2β-stimulated proliferation of the BC cells,[74] reduces cell motility and invasion,[75] and thus inhibits tumor development in mice.[76] Thus, ERβ antagonizes the tumor-promoting activities of ERα. Honma et al[77] showed that the ERβ was associated with better survival in patients with HER2-positive and triple-negative breast cancer (TNBC) with a good response to TAM. Generally, ERβ1 expression is associated with small tumor, lower histological grade, lymph-node negativity, and longer disease-free/overall survival of BC,[77–81] suggesting that ERβ1 expression has positive impact on BC survival. Consistently, epidemiologic studies demonstrated a loss of ERβ expression in higher grade BC tissues.[82,83] ERβ expression was inversely correlated with Ki67, particularly in high-grade ductal carcinoma in situ.[84] Although there is no correlation between loss of heterozygosity (LOH) at 14q22–24 (genomic locus at the *ERβ*/*ESR2* locus) and ERβ1 expression in primary BCs, a negative correlation between *ERβ1* mRNA expression and methylation status was observed for the *ERβ* promoter in BC cells,

which was reversed by 5-aza-deoxycytidine and trichostatin A.[85] Together, these studies indicate that ERβ functions as a tumor suppressor to inhibit BC initiation and progression.

Interestingly, ERβ1 is often expressed in TNBC, the most aggressive type of BC with limited treatment options because of the lack of expression of a biological target (ERα, PR, HER2). Clinical data have demonstrated a clear correlation between ERβ1 positivity and improved disease-free and overall survival in those patients treated with TAM.[86] It was also shown that ERβ1 inversely correlates with PTEN/PI3K/AKT pathway and predicts a favorable prognosis in TNBC.[87] Thus, ERβ1 may be worth considering as a potential therapeutic target, particularly in TNBC.

Notwithstanding with these findings that suggest tumor-suppressive roles for ERβ, other studies with BCs lacking ERα demonstrated a positive correlation between high ERβ expression and poor prognosis associated with increased proliferation[88,89] since ERβ is widely expressed in basal myo- and luminal epithelium in normal breast. Hou et al[90] reported that ERβ increased the proliferation and invasion of MDA-MB-435 cells (ERα-negative) significantly in estradiol-independent fashion in culture. In vivo studies showed that ERβ(+) MDA-MB-435 cells grew much faster and had more pulmonary metastasis than control cells. Thus, ERβ shows differential effects on BC growth and metastasis dependent on ERα levels, which needs further investigation at the molecular level.

ERβ splice isoforms and BC prognosis. The human *ERβ* locus on chromosome 14q21–22 generates two splice variants, namely, ERβ1 and ERβ2 (Fig. 1C). The mode of dimerization for ERβ1 and ERβ2 is similar in heterodimerization but different in homodimerization. ERβ1 forms homo- and heterodimers with other ERβ isoforms as well as with ERα and quenches ERα signaling.[91,92] Conversely, ERβ2 molecules do not form homodimers but inhibits ERα signaling through heterodimerization and proteasome-dependent degradation of ERα since ERβ2 has undetectable affinity for E2 and other tested ligands.[72,92,93] ERβ isoforms are differentially expressed in BC cells and in normal epithelial and nonepithelial components of breast tissues,[94,95] indicating that they have different biological effects on both normal and transformed cells. ERβ1 antagonizes the tumor-promoting activities of ERα and thus is a favorable prognostic marker at least in ERα(+) BC as described earlier. Conversely, recent studies show that ERβ2 expression in BCs, especially when it is expressed in the cytoplasm, is associated with worse (disease-free) survival of patients regardless of the ERα status.[96–100] Thus, ERβ1 and β2 isoforms have distinct impacts on BC survival.

HER2

The transmembrane *HER2/ErbB2/neu* gene encodes an 185-kDa glycoprotein with protein tyrosine kinase activity.[101–103] It belongs to the epidermal growth factor receptor (EGFR) family together with HER1, HER3, and HER4 (for reviews, see Refs. 104, 105). Overexpression and gene amplification of *ErbB2* are frequently observed in human malignancies, in particular in ~30% of primary BCs,[106] which correlate with enhanced tumor aggressiveness, lymph node metastasis, and poor clinical outcomes of patients. It is widely accepted that wild-type *HER2* gene amplification is necessary but not sufficient to induce transformation.[107]

Bargmann et al[101] isolated complementary DNA (cDNA) clones of the normal and transforming *neu* gene through NIH 3T3 focus forming assay of neuro- and glioblastomas of BXID rats. Then they created constructs in vitro between the normal and transforming cDNAs for Rat *neu* to determine the mutation responsible for the activation of the *neu* gene, which was the substitution of Val664 to Glu664.[108] The Val664 was in the transmembrane domain of the predicted neu protein p185. Segatto et al[109] then mutated the corresponding valine to glutamine in HER2 and demonstrated that this mutant protein had dramatically increased protein tyrosine kinase activity with 15-fold increase in transforming efficiency. Consistently, *MMTV-neu* mice with point mutation of *neu* develop aggressive mammary carcinomas at the latency of seven months, while *MMTV-ErbB2* mice (wild-type) develop mammary carcinomas at the latency of 15 months,[110] indicating increased oncogenicity of HER2/neu by the transmembrane point mutation. The oncogenic potential by mutant HER2/neu is quenched by the Arf–p53 pathway since it transactivates both *Dmp1* and *Arf* promoters[111–113] (for Dmp1, see Refs. 114–121). Although mutations at the transmembrane region have not been reported in human BCs, several studies have reported the expression of an *HER2* alternatively spliced isoform in normal mammary cells and in human breast carcinomas.[122–124] The in-frame deletion of 16 amino acids at exon 20 (aa 619–634; Fig. 2B) in the extracellular domain by aberrant splicing induces the formation of ΔErbB2 that displays a stronger transforming activity than wild-type ErbB2. This has been demonstrated by the development of ER(–), high-grade, and metastatic mammary tumors in ΔHER2 transgenic mice with short latency, driven by the *MMTV* promoter.[125,126]

Although extensive research has been done to identify/isolate the ligand(s) for c-ErbB2, it is now called an orphan receptor due to the lack of any known ligands.[127] A structural biological study for HER2 revealed that HER2 by itself had an activated conformation similar to that of the EGFR–ligand complex, which was very different from that seen in the unligand forms of HER1 or HER3.[128] The electrostatic repulsions possibly prevent homodimerization of HER2 explaining its inability to bind known ligands, which also explains why HER2 fails to form homodimers.[128] Interestingly, HER2 makes heterodimer with HER3 that has an authentic ligand heregulin but is kinase-dead.[127,129] Vaught et al[130] demonstrated the importance of HER3 in all stages of HER2-mediated mammary epithelial transformation and metastasis through the analyses of gene-engineered mouse models. HER2:HER3 heterodimerization is critically important in the progression of HER2(+) BC since

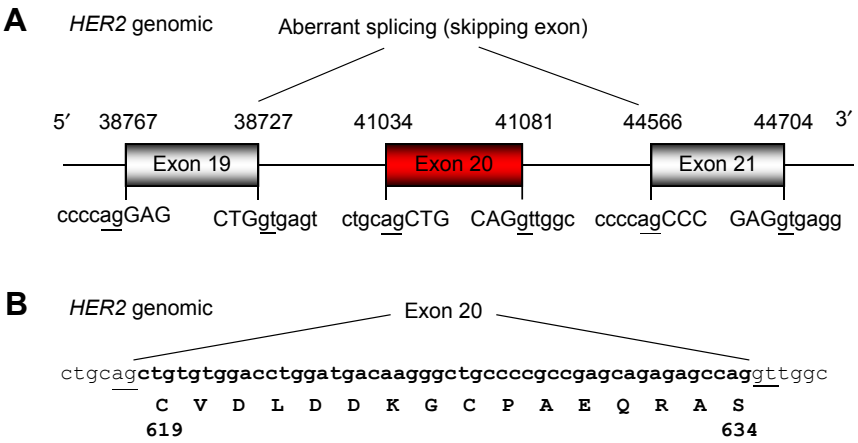

Figure 2. Activation of the *HER2* gene by alternative splicing at exon 20. (**A**) *HER2* splicing and generation of *Δ16HER2*.[202] Constitutive expression of a human *HER2* alternative splice isoform carries an in-frame deletion in the same mutated region of the rat *neu* proto-oncogene.[122] This splice variant produces an aberrant receptor that lacks exon 20 encoding 16 amino acids (Δ16HER2). (**B**) The 16 amino acids (aa 619–634) in the HER2 extracellular domain deleted in Δ16HER2 include two relevant cysteine residues close to the T-binding epitope. This deletion results in stable, constitutively active homodimer formation, enhanced multisignaling activity, and accelerated transformation.

heregulin–HER3 binding initiates HER2:HER3 dimerization, causing epithelial–mesenchymal transition (EMT) via phosphorylation of AKT-heat shock factor 1 (HSF1)-SLUG, eventually leading to cancer metastasis.[131]

Splicing in *HER2* is supposed to trigger the kinase activity by promoting intermolecular disulfide bonding, which, in turn, forms homodimers capable of transforming cells.[132] Since the levels of the *HER2* splice variant represent only 9% of those observed with the wild-type receptor, HER2 protein overexpression caused by gene amplification (or other mechanisms) in primary human BCs will therefore increases the levels of this oncogenic variant above the critical threshold, allowing it to contribute to BC progression.[124] Δ16HER2 is expressed in many HER2-positive BCs, where it has been linked with resistance to the HER2-targeting monoclonal antibody trastuzumab in metastatic BC,[133] but the impact of Δ16HER2 on tumor pathobiology and therapeutic response in patients with operable BC remains to be determined. Castagnoli et al[134] provided genetic evidence in transgenic mice that expression of Δ16HER2 was sufficient to accelerate mammary tumorigenesis and improve the response to trastuzumab (Herceptin®), a monoclonal antibody that interferes with the HER2 receptor. A comparative analysis of effector signaling pathways activated by Δ16HER2 and wild-type HER2 revealed that Δ16HER2 was optimally functional through a link to SRC activation (pSRC). Clinically, HER2-positive BCs from patients who received trastuzumab as an adjuvant therapy showed a positive correlation in Δ16HER2 and pSRC abundance, consistent with the results from the mouse study. Moreover, patients expressing high pSRC or Δ16HER2 received the greatest benefit from trastuzumab therapy with BC patients in an adjuvant setting.[134] Thus, the Δ16HER2 and pSRC abundance should be predetermined in tumors when making therapy decisions. They speculate that although HER2-positive primary BCs expressing high levels of pSRC are initially dependent on HER2 and all its potential driver isoforms and are, thus, responsive to trastuzumab, the progression of such BCs due to a high HER2-dependent growth rate might lead to the accumulation of genetic alterations that result in less HER2 dependency, which, in turn, results in significantly less or even no responsiveness to trastuzumab as reported by Zhang et al.[133]

Cittelly et al[135] studied the mechanisms of resistance with endocrine therapy in BCs with Δ16HER2 in relationship to microRNA and BCL-2. They showed that Δ16HER2 was expressed in >30% of ER-positive BCs, which promoted TAM resistance and estrogen independence of MCF-7 xenografts. MCF-7/Δ16HER2 cells evade TAM through upregulation of BCL-2, which was targeted by miR-15a and miR-16. Reintroduction of miR-15a/16 reduced TAM-induced BCL-2 expression and sensitized MCF-7/Δ16HER2 to TAM. Hence, their preclinical models of BC with Δ16HER2 overexpression recapitulate numerous phenotypes of endocrine-resistant human breast tumors.[135]

Huynh and Jones[136] also studied the contribution of altered microRNA expression in Δ16HER2-mediated tumorigenesis and trastuzumab resistance. Using a gene array strategy comparing microRNA expression profiles of MCF-7 with MCF-7/Δ16HER2 cells, they found that Δ16HER2 caused a fivefold suppression of the miR-7 tumor suppressor. Re-expression of miR-7 in the MCF-7/Δ16HER2 cell line caused a G1 cell cycle arrest and reduced both colony formation and cell migration to levels of parental cells. MiR-7 inhibited MCF-7/Δ16HER2 cell migration through EGFR and the inactivation of the SRC kinase. Together miR-7- and -15a/16-regulated signaling pathways involving BCL-2, EGFR, and/or SRC kinase can be future targets for therapeutic intervention of Δ16HER2-driven BC.

After the discovery of Δ16HER2, another mechanism was proposed to mediate resistance to trastuzumab: a truncated form of the HER2 receptor, p95-HER2.[137,138] The amino terminal-truncated p95-HER2 is a constitutively active kinase that can form heterodimers with other HER family proteins and activates the downstream signaling pathways. Since p95-HER2 lacks the trastuzumab-binding site, its expression is associated with trastuzumab resistance and poor prognosis but maintains sensitivity to the HER2 kinase inhibitor lapatinib.[137,138] Thus, it is essential to determine the levels of p95-HER2 levels in BC with HER2 overexpression before making decisions in therapy. We do not discuss this issue further since this mutant is not considered to be a splice variant for *HER2*.

CD44

BC is characterized by a remarkable biological heterogeneity within tumors, which has been demonstrated by GeneChip microarray analyses of gene expression.[139,140] Early studies have identified a subpopulation of cells with stem cell activity in $CD44^{+}/CD24^{-/low}$/lineage(–)[25,141] fraction, and more recently, aldehyde dehydrogenase (ALDH) activity was shown to mark normal as well as malignant human mammary stem cells.[142,143] These cancer stem cells (CSCs) have enhanced invasiveness,[144] resistance to radio-[145] or chemotherapy,[146] and are associated with poor prognosis.[142,147,148] The presence of $CD44^{+}/CD24^{-/low}$/lineage(–) tumor cells has been associated with the basal-like subtype of BC, especially those with hereditary mutations for *BRCA1*.[149] The role of CD44 as a marker for CSCs will be discussed later in this section.

The CD44 gene and splice variants. The human *CD44* gene is located on chromosome 11p13 and consists of 20 coding exons of which 10, located between constant exons 5 and 6 (colored pink), can be alternatively spliced into many different isoforms with tissue- and differentiation-specific expression (Fig. 3A).[150,151] The standard isoform of CD44 (CD44s) contains none of the 10 variable exons, whereas the CD44v2–v10 isoform includes all of them (exon v1 is not expressed in humans[152]). The protein products for CD44 are shown in Figure 3B. The CD44v3–v10 isoform has one less exon, and the CD44v8–v10 isoform includes only the last three of the variable exons. Other isoforms are formed by AS, and various posttranslational modifications further increase the heterogeneity of the CD44 proteins.

CD44 protein structure. CD44 is a multifunctional transmembrane glycoprotein that participates in many cellular processes including cell division, survival, migration, and adhesion[153] through the binding of its major ligand, hyaluronic acid (HA; Fig. 4). HA is a polymer of disaccharides, themselves composed of D-glucuronic acid and D-*N*-acetylglucosamine, linked via alternating β-1,4- and β-1,3-glycosidic bonds.[154,155] HA is synthesized by a class of integral membrane proteins called hyaluronan synthases, that is, HAS1, HAS2, and HAS3. These enzymes lengthen HA by adding glucuronic acid and *N*-acetylglucosamine repeatedly to the growing polysaccharide, and the final products are extruded into the extracellular space via ATP-binding cassette (ABC) transporter through the plasma membrane. CD44 can act as a coreceptor to mediate signaling of receptor-type protein tyrosine kinases (RTKs) by making functional complexes, which

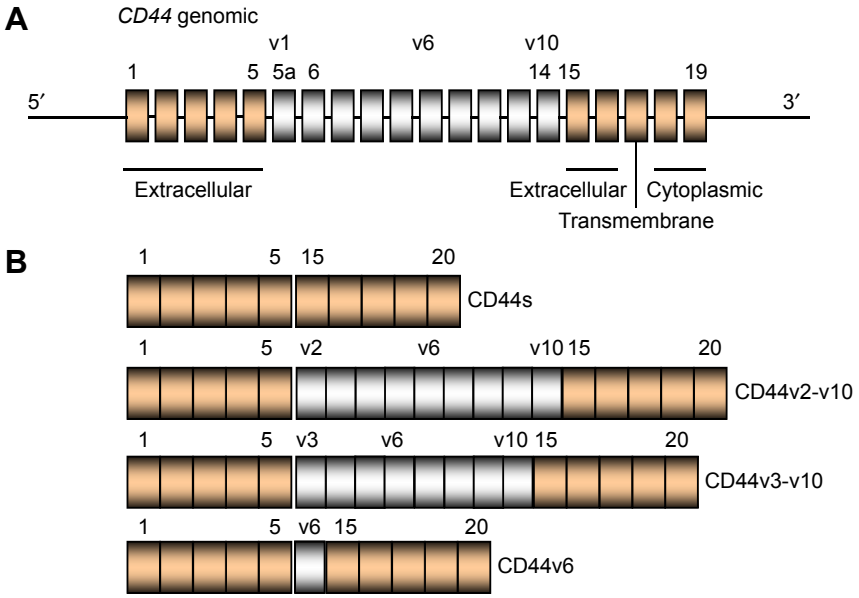

Figure 3. Alternative splicing for the human *CD44* locus and its protein products. (**A**) The genomic structure for the human *CD44* locus. It has 10 constant exons (exons 1–5 and 15–19) shown in tan that encode the extracellular, transmembrane, and cytoplasmic tail sequences and 10 variable exons for the extracellular domain (shown in silver). (**B**) Protein structures for the standard (CD44s) and variant CD44 (CD44v) proteins. CD44v has insertion of amino acid sequences between those encoded by exons 5 and 15 (exons 5a–14). The exon 5a (=exon v1) is not expressed in human tissues.

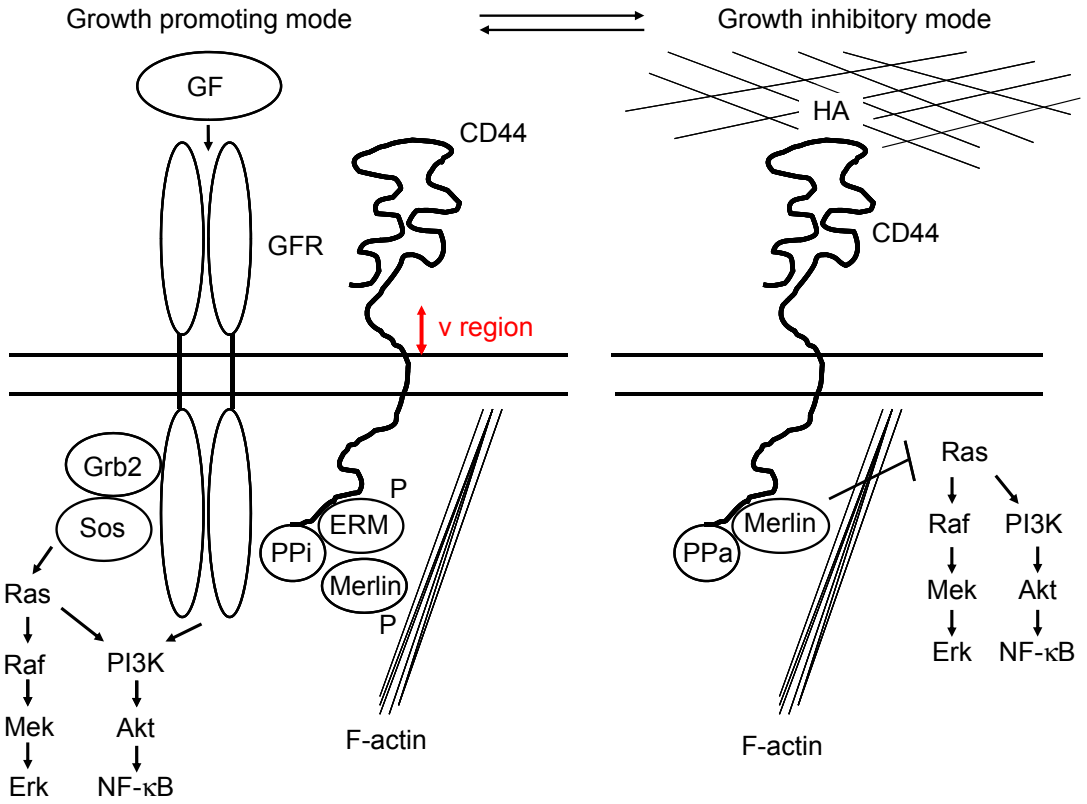

Figure 4. The model for CD44 action in logarithmic (growth-promoting mode) and confluent (growth-inhibitory mode) growth conditions.[157] Specific ligands determine two functional states of CD44 that influence the cytoplasmic complexes. The ligands of the growth mode have not been defined for CD44. It is, however, known that CD44s, and particularly the larger splice variants CD44v, serve as a platform for the activation of growth factors (GF). GFR, growth factor receptor; PPi, inactive protein phosphatase; PPa, active protein phosphatase; ERM, ezrin, radixin, and moesin. Merlin (NF2), which is an inhibitor for the Ras–Raf–Mek–Erk pathway, is inactivated by phosphorylation in the growth-promoting mode for CD44.

will be explained later (see "CD44 promotes tumorigenesis" section). CD44 also provides a link between the plasma membrane and the actin cytoskeleton, modulating cellular shape and motility (Fig. 4).[155,156]

The CD44 molecule consists of an amino-terminal extracellular and LBD, a membrane-proximal stem loop including the variable region (shown in red) and a transmembrane region, and a cytoplasmic tail that attaches to actin, ankyrin, ezrin, radixin, and moesin (ERM) in the cytoskeleton (Fig. 4).[150,151] The epitope recognized by the CD44 monoclonal antibodies commonly used for the isolation of CSCs is located in the amino-terminal region of CD44 consisting of the nonvariable exons 1–5, indicating that all CD44 isoforms should be detected by these antibodies.[156]

CD44 contributes to both cell proliferation (Fig. 4, growth-promoting mode, left) and growth inhibition (growth-inhibitory mode, right), dependent on the biological conditions of cells. The *neurofibromatosis-2* (*NF2*) gene encodes merlin, an ERM-related protein that functions as a tumor suppressor that inhibits the Ras–Raf–Mek–Erk cell growth pathway.[157] At low cell density, merlin is phosphorylated and growth permissive and exists in a complex with ERM and CD44 (Fig. 4, left). CD44s- or CD44v-mediated activation of mitogenic and antiapoptotic proteins is initiated through their association with RTKs. At high cell density, merlin becomes hypophosphorylated and inhibits cell growth in response to HA in the extracellular matrix (Fig. 4, right). Merlin's growth-inhibitory activity depends on specific interaction with the cytoplasmic tail of CD44 that interacts with an active protein phosphatase. The hypophosphorylated merlin will directly associate with CD44 and inhibit the Ras–Raf–Mek–Erk and PI3K–Akt mitogenic pathways (Fig. 4, right).

CD44 promotes tumorigenesis. CD44 promotes tumorigenesis through a variety of major signaling pathways, including the Ras–Raf–Mek–Erk–cyclin D1 and PI3K–Akt pathways for stimulating cell growth, survival, and invasion, and Rho GTPases for cytoskeletal remodeling and invasion.[151] CD44 makes complexes with growth factor receptors such as EGFR (HER1), HER2, HER3, and HER4. The association of CD44 with HER2 and HER3 mediates heterodimerization and activates the receptor in response to neuregulin, which strongly endows apoptosis resistance in cancer-initiating cells. ERBB protein activation stimulates growth factor receptor-bound protein 2 (GRB2) and son of sevenless (SOS) proteins, which subsequently activate the Ras–Raf–Mek–Erk (proliferative) and PI3K–Akt–NF-κB pathways (antiapoptotic; Fig. 4, left).

CD44 regulates other RTKs through physical association. CD44 promotes MET phosphorylation via CD44v3-bound hepatocyte growth factor (HGF), which is important in colorectal cancer tumorigenesis.[151] CD44v6 also initiates MET activation through HGF binding.[158] Likewise, CD44 interacts with insulin-like growth factor 1 receptor, platelet-derived growth factor receptor, and transforming growth factor beta (TGFβ) receptor,[151] demonstrating its broad activity in receptor-mediated signaling.

In addition to these RTK-related activities, CD44 serves as a docking molecule for matrix metalloproteases (MMPs), which are matrix-modifying enzymes that degrade basement membrane and promote cell migration.[159] MMP2 and 9, in turn, cleave TGFβ for activation, which promotes angiogenesis and invasion.[160] Interestingly, Kuo et al[161] later showed that TGFβ induced membrane type 1 MMP expression in MDA-MB-435s BC cells, which caused CD44 cleavage. Cleaved CD44 then promoted the migration of tumor cells, indicating the significant role of the CD44–MMP–TGFβ axis in cancer invasion and metastasis. CD44 also interacts with multidrug resistance 1 to confer drug resistance.[151] Although crosstalk between the p53 tumor suppressor pathway and CD44 has not been extensively studied, Godar et al[162] showed that p53 inhibited expression of the CD44 cell-surface molecule via binding to a noncanonical p53-binding sequence in the *CD44* promoter. In the absence of p53 function, increased CD44 expression accelerated the growth and tumor-initiating ability of highly tumorigenic human mammary epithelial cells. Thus, CD44 is a key tumor-promoting agent in transformed tumor cells lacking p53 function.

Several groups have assessed the role of CD44 in BC progression in vivo using mouse models. Ouhtit et al[163] developed a tetracycline-regulated CD44s expression system in the weakly metastatic BC cell MCF-7. Induction of CD44s alone increased their abilities to proliferate, migrate, and invade in vitro. They then developed a doxycycline (DOX)-repressed CD44s BC xenograft model.[164] Although induction of CD44s did not affect the growth rate or local invasion of the primary tumor, 8 of 11 mice from the DOX(–) group expressing CD44s developed secondary tumors to the liver. They showed that TGFβ2 was a novel target for CD44 that promoted BC invasion.[164] Consistently, treatment with a CD44-blocking monoclonal antibody P245 dramatically inhibited tumor growth and prevented recurrence in human BC xenografts after treatment with doxorubicin and cyclophosphamide, demonstrating growth-promoting activities of CD44 in vivo.[165]

CD44 as a marker for CSCs—role of EMT in cancer metastasis. Normal stem cells renew themselves through asymmetrical cell division while simultaneously generating committed progenitor cells whose descendants will eventually differentiate and execute tissue-specific functions.[166,167] More recently, studies of cancer cells have provided evidence of self-renewing, stem-like cells within tumors, which have been called CSCs (reviewed in Refs. 25, 168–170). CSCs were first identified in hematopoietic malignancies;[171,172] later, they have also been discovered in solid tumors, such as those found in the breast, colon, and brain.[141,173–176]

The process of tumor metastasis is often enabled by EMT,[177,178] where cancer cells require self-renewal capability. This raises the possibility that the EMT process, which enables cancer cell dissemination, may also bestow a self-renewal capacity to the cancer cells. EMT is transcriptionally regulated by a family of transcription repressors that suppress E-cadherin expression and by microRNAs (reviewed in Refs. 179–184). Successful colonization of cancer cells in secondary sites requires the ability of the cells to avoid the mechanisms that interfere with metastasis where CD44 plays a critical role. Yae et al[185] showed that orthotropic transplantation of a CD44v(+) subpopulation of 4T1 BC cells, but not that of a CD44v(–) subpopulation, in mice results in efficient lung metastasis accompanied by expansion of stem-like cancer cells proving the role of the variant isoform in cancer metastasis.[185] Other studies support the finding of the Yae study concluding that some CD44v isoforms mediate cancer metastasis.[186,187]

In good contrast to these studies, other groups demonstrated the importance of CD44s rather than CD44v in cancer progression. Brown et al[188] demonstrated the role of CD44s in BC progression. Most importantly, they showed that CD44v and CD44s were differentially regulated during EMT, resulting in a switch from CD44v isoform to CD44s.[188] The expression of CD44s accelerated both EMT and BC progression through activation of Akt. Although both CD44s and CD44v were upregulated in BCs in comparison to normal tissues, the expression of CD44s was significantly higher in advanced tumors and correlated with N-cadherin expression. Thus, the regulation of AS for *CD44* constitutes a critical mechanism in controlling EMT and cancer progression.[188] Xu et al[189] showed that the RNA-binding protein heterogeneous nuclear ribonucleoprotein M (hnRNPM) promoted BC metastasis by activating the switch of AS from the CD44v to CD44s isoform during EMT. Genome-wide deep sequencing analysis showed that hnRNPM potentiated TGFβ signaling and identified CD44 as a key downstream target of hnRNPM.[189] They also showed that the hnRNPM expression was associated with aggressive BC in primary samples. Thus, tumor metastasis is accelerated by the hnRNPM-mediated splicing program, which increased relative expression of CD44s over CD44v.[189]

EMT, which causes invasion and metastasis of carcinoma, is driven by the transcription factor ZEB1 that promotes tumor-initiating capacity. Remarkably, EMT-induced repression of epithelial splicing regulatory protein 1 (ESRP1) controls AS of *CD44*, causing a shift in the expression from the variant CD44v to CD44s isoform.[190] Intriguingly, CD44s itself activates the expression of ZEB1, resulting in a self-sustaining ZEB1 and CD44s expression. Activation of this CD44s–ZEB1 regulatory loop has functional impact on tumor cells, as evident by increased tumor sphere initiation, drug resistance, and tumor recurrence. Their study again

emphasizes the importance of CD44s in tumor cell stemness independent of external stimuli as ZEB1 downregulates ESRP1, further promoting CD44s isoform synthesis.[190]

Olsson et al[191] studied the correlation between CD44 isoforms and BC subtypes. They found that BCs with a strong expression of the CSC marker ALDH1 had elevated expression of CD44s. A high expression of the CD44v2–v10 and CD44v3–v10 isoforms (Fig. 3B) correlated with positive hormone receptor (ER, PR) status, low proliferation, and luminal A subtype. High expression of CD44v8–v10 correlated with positive EGFR, negative/low HER2 status, and basal-like subtype. High expression of CD44s was associated with strong HER2 expression and also a basal-like phenotype. Thus, individual CD44 splice isoforms can be associated with particular BC subtypes and clinical markers.[191] Taken together, although both CD44 splice isoforms have been reported to play essential roles in BC development and progression, CD44v (especially CD44v2–v10, v3–v10) is more likely to be associated with BCs with good prognosis, such as luminal A, while CD44s is linked to BCs with poor prognosis, such as HER2 or basal cell subtypes that are often metastatic.

Inhibition of cancer progression by CD44. Although the majority of in vitro researches described earlier suggest the role of CD44 in cancer progression, other reports have shown that CD44 can respond to signals from the microenvironment, often in response to high molecular weight (HMW, >500 kDa) HA, to inhibit growth and invasion in cancer cells (Fig. 4, right). Consistent with the tumor-suppressive role of CD44, loss of *CD44* has been reported in Burkitt's lymphoma, neuroblastoma, prostate cancer, and BC.[192] CD44 binding to merlin acts as a growth/arrest sensor in response to signals from the microenvironment and plays a role in contact inhibition, which is lost in cancer cells.[192] Similarly, Louderbough et al[193,194] showed that collagen-embedded HMW HA interfered with the activation of EGFR and prevented filopodia formation on collagen in a BC cell line, inhibiting the invasion of tumor cells. CD44 has also been implicated in the inhibition of angiogenesis, particularly by HMW HA,[195] suggesting its role in the inhibition of metastasis.

Tumor-suppressive activities for CD44 have been demonstrated in vivo using *CD44*-deficient mice. SV40-transformed *CD44*-null fibroblasts injected subcutaneously into nude BALB/C mice were highly tumorigenic, whereas the introduction of CD44s into these cells resulted in a dramatic inhibition of tumor growth.[196] In a mouse model of spontaneously metastasizing BC (*MMTV-polyoma middle T*), Lopez et al[197] found that loss of *CD44* promoted tumor metastasis to the lung, but not the onset, suggesting its suppressive role for tumor progression. CD44 was expressed in the myoepithelium of the developing mammary gland in mice. The loss of *CD44* resulted in defective luminal–myoepithelial cell–cell adhesion and promoted the mixing of luminal and myoepithelial layers, disrupting epithelial bilayer organization.[198] The ductal outgrowth and terminal end bud formation were delayed/impaired in *CD44*-null mice. In BCs, CD44 was expressed in the basal cells of early-stage tumor cells but exhibited altered localization with the development of the disease. Collectively, global depletion of all the *CD44* isoforms leads to acceleration of tumorigenesis and metastasis, suggesting its tumor-suppressive role in vivo.

Possible mechanisms for the dual roles of CD44 in cancer. Research findings described earlier indicate that CD44 has dual roles, that is, it either promotes or inhibits cancer progression dependent on the experimental conditions used.[194] In the case of HA–CD44 signaling, the molecular weight of HA will decide the biological consequences, that is, HMW HA inhibits metastasis-promoting activity of CD44, whereas LMW HA does the opposite. Thus, environmental factors significantly influence the biological activity of CD44 in cell growth.

Contradictory roles of CD44 in cancer progression can also be attributed to the expression of the standard and alternatively spliced isoforms with different activities. Indeed, a high expression of the CD44v2–v10 and v3–v10 isoform correlated with positive ER/PR status, low proliferation, and luminal A subtype, while a high expression of CD44v8–v10 correlated with positive EGFR, negative/low HER2 status, and basal-like subtype in BCs.[191] In advanced ovarian cancer, CD44v6 was associated with peritoneal dissemination and poor prognosis.[187] Thus, each splice variant has different biological activity. It is hypothesized that CD44s and CD44v have to be expressed at certain ratio/level in a relatively narrow range in each cell type/tissue to maintain the normal homeostasis, and any events that affect the *CD44* transcription, pre-mRNA splicing, translation, or posttranslational modifications result in increased predisposition to cancer.

The third possibility to explain the dual roles of CD44 in cancer is its crosstalk with the TGFβ pathway since TGFβ has both tumor-suppressive (early stage) and tumor-promoting (advanced stage) activities dependent on the level of tumor development.[199,200] Indeed, published studies have shown the importance of the CD44–MMP–TGFβ axis in tumor cell invasion and metastasis.[159–161] It will thus be essential to elucidate the molecular interactions between CD44 and TGFβ signaling cascades since both of these molecules are cleaved and activated by MMPs.

Conclusive Remarks and Future Directions

Estrogen has both genomic and nongenomic pathways for signaling. Published studies have shown that ERα36 is a potential regulator for membrane-initiated mitogenic signaling and is a promising diagnostic/prognostic biomarker for therapy-resistant cancer. Conversely, ERα66 expression is generally associated with good prognosis of cancer. Thus, molecular characterization of signaling cascades that regulate ERα36/66 ratio will have significant impacts on cancer

therapy. It will also be needed to characterize the signaling pathways governed by ERα46 that has different C-terminal structure from ERα36.

Although the *HER2* mutation that corresponds to *neu* has not been reported in human cancers, the Δ16HER2 variant has revealed its oncogenic activity. Since the levels of Δ16HER2 significantly affect the therapeutic response of BC patients to the monoclonal antibody therapy, Δ16HER2 level should be predetermined using tissues obtained by biopsy or surgery before making therapeutic decisions. Investigation for microRNA-regulated signaling pathways will give novel therapeutic modalities to treat BC refractory to the antibody therapy.

The molecular mechanisms for the role of CD44 in cancer development look very complicated since CD44 has multiple isoforms that may have conflicting activities in tumor initiation or progression. Although Schmits et al[196] demonstrated the tumorigenicity of *CD44*-deficient fibroblasts in vivo, the interpretation of their results is difficult since both Rb and p53 tumor suppressors had already been inactivated by the SV40 T antigen, which does not frequently happen in naturally occurring human cancers. Interestingly, the same laboratory later reported that the absence of *CD44* prevented sarcoma metastasis using the more physiological *min* mutation model for colon cancer, demonstrating the pro-metastatic potential for CD44.[201] There has been no report on increased spontaneous or carcinogen/irradiation-induced tumor incidence in *CD44*-deficient mice. Since CD44 has multiple splice variants with possible conflicting functions, it will be necessary to establish Dox-inducible transgenic mouse models for each variant or create isoform-specific knockout mice to elucidate the role of each CD44 isoform in cancer development or prevention.

Acknowledgments

We thank all members of Dr Inoue's laboratory for sharing research findings on BC.

Author Contributions

Contributed to the conceptual designing, writing of the text, creating figures, and collecting literatures: KI and EAF. All authors reviewed and approved of the final manuscript.

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