—Review—

Estrogen Receptor α Isoforms Generated by Alternative Use of Cryptic Exons

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Estrogen receptor α (ERα) regulates several physiological functions. In pathophysiological conditions, ERα is involved in the development and progression of estrogen-sensitive tumors. The ERα gene contains multiple 5'-untranslated exons and eight conventional coding exons and presents multiple isoforms generated by alternative promoter usage and alternative splicing. This gene also possesses non-conventional exons in the 3'- and intronic regions, and alternative use of cryptic exons contributes to further diversity of ERα mRNAs and proteins. Recently, the genomic organization of ERα genes and the splicing profiles of their transcripts were comparatively analyzed in humans, mice, and rats, and multiple ERα isoforms with distinct structures and functions were identified. These transcripts contain cryptic sequences that encode insertion-containing or truncated ERα proteins. In particular, alternative cryptic exons with in-frame stop codons yield transcripts encoding C-terminally-truncated ERα proteins. The C-terminally-truncated ERα isoforms lack part or all of the ligand-binding domain but possess an isoform-specific sequence. Some of these isoforms exhibit constitutive transactivation and resistance to estrogen receptor antagonists. Although numerous studies have reported conflicting results regarding their functions, the critical determinant for their gain-of-function has been identified structurally. Here we review recent progress in ERα variant research concerning the genomic organization of ERα genes, splicing profiles of ERα transcripts, and transactivation properties of ERα isoforms.

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Key words: alternative splicing, chemotherapy-resistance, endocrine-resistance, estrogen receptor

Introduction

Estrogens have physiologically important roles in various organs[1]. They are involved in pathophysiological events, including hormone-dependent proliferation and development of breast and endometrial cancers[2]. Estrogens signal mainly via estrogen receptors α and β (ERα and ERβ, respectively), which belong to the nuclear receptor superfamily and function as ligand-dependent transcription factors[3].

The ERα gene is composed of multiple 5'-untranslated exons and eight coding exons[4,5]. The alternative use of promoters and 5'-untranslated exons generates ERα transcripts with unique 5'-untranslated regions[6–8]. Alternative skipping of the conventional coding exons results in several truncated ERα variants. Additionally, the gene contains cryptic exons in its 3'- and intronic regions, and the alternative use of these non-conventional exons yields transcripts encoding insertion-containing or truncated ERα proteins. Recent studies have identified multiple intronic exons and truncated ERα isoforms[9–12]. Some of the C-terminally-truncated isoforms exhibited constitutive transactivation functions. Previously, most C-terminally-truncated nuclear receptor variants were believed to act as non-functional or dominant-negative forms[13]. Several controversial results have been reported regarding the transactivation abilities of C-terminally-truncated ERα variants[14–17]. Constitutively active ERα variants are considered candidate molecules for acquiring endocrine resistance and chemotherapy resistance in estrogen-responsive cancers. Thus, the critical determinant for their gain-of-function needs to be identified. Previously, we determined the general mechanisms underlying their constitutive transactivation functions on a structural basis[10,11]. Here, we review the genomic organization of ERα

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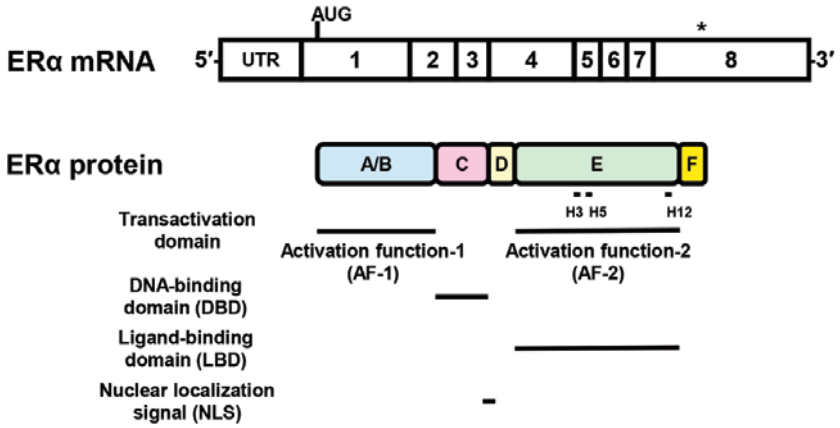

Fig. 1 Structures of full-length ERα mRNA and protein
The modular structures of ERα66 mRNA and protein are presented schematically.

genes, splicing profiles of truncated ERα isoforms, and transactivation properties of C-terminally-truncated ERα isoforms.

ERα Structures

The human, mouse, and rat ERα genes are located at 6q 25.1-q25.2, 10A1, and 1q11 in their genomes, respectively. The genes contain multiple 5'-untranslated exons and eight conventional coding exons (exons 1-8), which encode full-length ERα proteins of 66 kDa (ERα66). As is the case for other members of the nuclear receptor superfamily, ERα66 proteins have characteristic structures (**Fig. 1**). They are composed of six modules from their N- to C-termini (A-F), which are further categorized functionally as domains: the A/B, C, D, and E modules correspond to the N-terminal transactivation domain, DNA-binding domain (DBD), hinge domain (HD), and C-terminal transactivation/ligand-binding domain (LBD), respectively[18]. The N- and C-terminal transactivation domains are also called activation functions-1 and -2 (AF-1 and AF-2, respectively). The highly conserved DBD binds to estrogen response elements (EREs). The moderately conserved LBD contains 11 helices (H1 and H3-12)[19,20]. In ligand-dependent transactivation, ERα agonists induce conformational rearrangement of H3, H5, and H12, which results in the formation of co-activator binding sites to allow transcriptional transactivation with a co-activator complex[21–23]. The nuclear localization signal (NLS) is located in the N-terminal region of the HD and is constitutively active[24]. Thus, ERα variants including the NLSs can translocate into the nucleus in a ligand-independent manner.

Organization of ERα Genes and mRNAs Including Cryptic Exons

ERα pre-mRNAs are subject to alternative splicing. Alternative splicing of ERα transcripts in the regions encoding the C-termini results in truncated variants that lack part or all of the LBD but possess variant-specific C-terminal sequences. The Δexon 5 ERα (ERαΔ5)[17] and Δexon 7 ERα (ERαΔ7)[25] variants are single-exon-skipping splice variants that encode C-terminally-truncated ERα proteins. Furthermore, the ERα genes possess cryptic exons in their intronic regions, and these alternative exons generate transcripts encoding truncated or insertion-containing isoforms. Previous studies have described multiple cryptic exons in human, mouse, and rat ERα genes[9–12,26–34]. To comparatively examine their gene structures and cryptic exons, their genomic organization is presented in **Figure 2**. The nucleotide sequences of non-conventional exons have little homology among the three species. The cryptic exons function as leader, internal, or terminal exons. Human exons i12 and S, mouse exons i45c and i45d, and rat exon i45b are leader exons, and the activation of their corresponding promoters induces transcription of N-terminally-truncated ERα isoform mRNAs. Human exons "ERα clone 4" (ERC4), i34, 4_L, $i45a_L$, i45b, i56, i67, and 9; mouse exons 4_L, i45b, i45e, and i45f; and rat exons 4_L and i56e are terminal exons, whereas other cryptic exons are internal exons. Human exons $i45a_S$ and i45c, mouse exon i45a, and rat exons i45a, i45c, and i56a-d contain in-frame stop codons. Alternative splicing from conventional coding exons to the terminal exons and the internal exons with in-frame stop codons generate mRNAs encoding C-terminally-truncated ERα isoforms. Insertion-containing ERα isoforms are produced by the alternative insertion of internal exons without premature in-frame stop codons

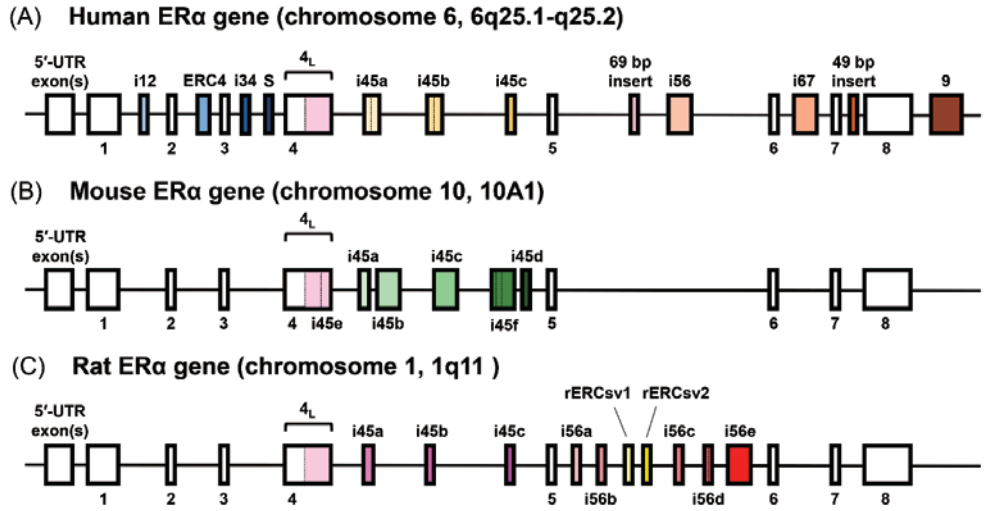

Fig. 2 Genomic organization of ERα genes

Genomic organization of human (A), mouse (B), and rat (C) ERα genes are represented schematically. The white and colored boxes indicate 5'-untranslated and conventional coding exons and cryptic exons and extensions, respectively. The images are not to scale.

(human "69 bp insert" and rat exons "rERCsv1" and "rERCsv2"). Some cryptic exons contain alternative splice sites that contribute to the diversity of ERα transcripts. To clarify the diversity of ERα mRNA and protein isoforms and characterize their species-specific differences, ERα mRNAs with cryptic exons and encoded proteins are represented schematically in **Figure 3**.

C-terminally-truncated ERα Isoforms

The alternative use of cryptic terminal and internal exons with in-frame stop codons generates transcripts encoding C-terminally-truncated ERα proteins. Most of the recently identified cryptic exons are located in the intronic regions between exons 3 and 7 or downstream of exon 8 and are spliced from exons 3 to 6. The HD and LBD are encoded in exons 4-8; therefore, the truncated ERα variant proteins preserve the N-terminal transactivation domain, DBD. Although these proteins lack the C-terminal parts of the HD/LBD, they substitute isoform-specific sequences.

Swope et al.[31] identified a cryptic nucleotide sequence downstream of exon 4 in the mouse ERα gene. Exon 4 plus this downstream sequence was named exon 4_L, and alternative use of the exon generates C-terminally-truncated estrogen receptor α product-1 (CTERP-1). An equivalent generation of mouse CTERP-1 was observed in humans and rats[10,11]. Our subsequent studies[10,12] identified additional alternative exons (exons i45a-f) between exons 4 and 5 and C-terminally-truncated ERα isoforms (CTERP-2-7) in mice. The isoforms are localized predominantly in the nuclei and exhibited constitutive transactivation of an ERE-driven promoter in transfected cells.

Several studies have reported human C-terminally-truncated ERα isoforms with cryptic exons. Dotzlaw et al.[34] were the first to describe a C-terminally-truncated ERα isoform (ERC4) generated by splicing from exon 2 to a long interspersed nuclear element-1 (LINE-1)-like sequence. Although the study did not determine the origin of the LINE-1-like sequence, our analysis using the human genome assembly revealed its localization between exons 2 and 3. As the variant's DBD is truncated, it did not exhibit modulatory effects on an ERE-driven promoter. An ERα variant with a 113-bp genomic insert was identified as the only ERα transcript in a patient with schizophrenia[28]. The inserted exon, which was named exon i45c by Hattori et al.[11], is localized between exons 4 and 5. Ishunina and Swaab[27] identified numerous ERα variants in the human brain and reported one exon-skipping ERα variant with a novel 49-bp insert (ERα Δ4 + 49 bp) located between exons 7 and 8. Furthermore, our recent cloning experiments[9,11] revealed that multiple cryptic exons (exons i34, 4_L, i45a-c, i56, and i67) are present in the intronic regions between exons 3 and 7 in the human ERα gene. We also identified C-terminally-truncated ERα isoform transcripts (ERαi34, CTERP-1, ERαi45a$_L$, ERαi45a$_S$, ERαi45b$_L$, ERαi45b$_S$, ERαi45c, ERαi56, and ERαi67) that include the intronic sequences. The C-terminally-truncated ERα proteins encoded by exon 5-lacking transcripts (ERαi34, CTERP-1, ERαi45a$_L$, ERαi45a$_S$, ERαi45b$_L$, ERαi45b$_S$, and ERαi45c) exhibited

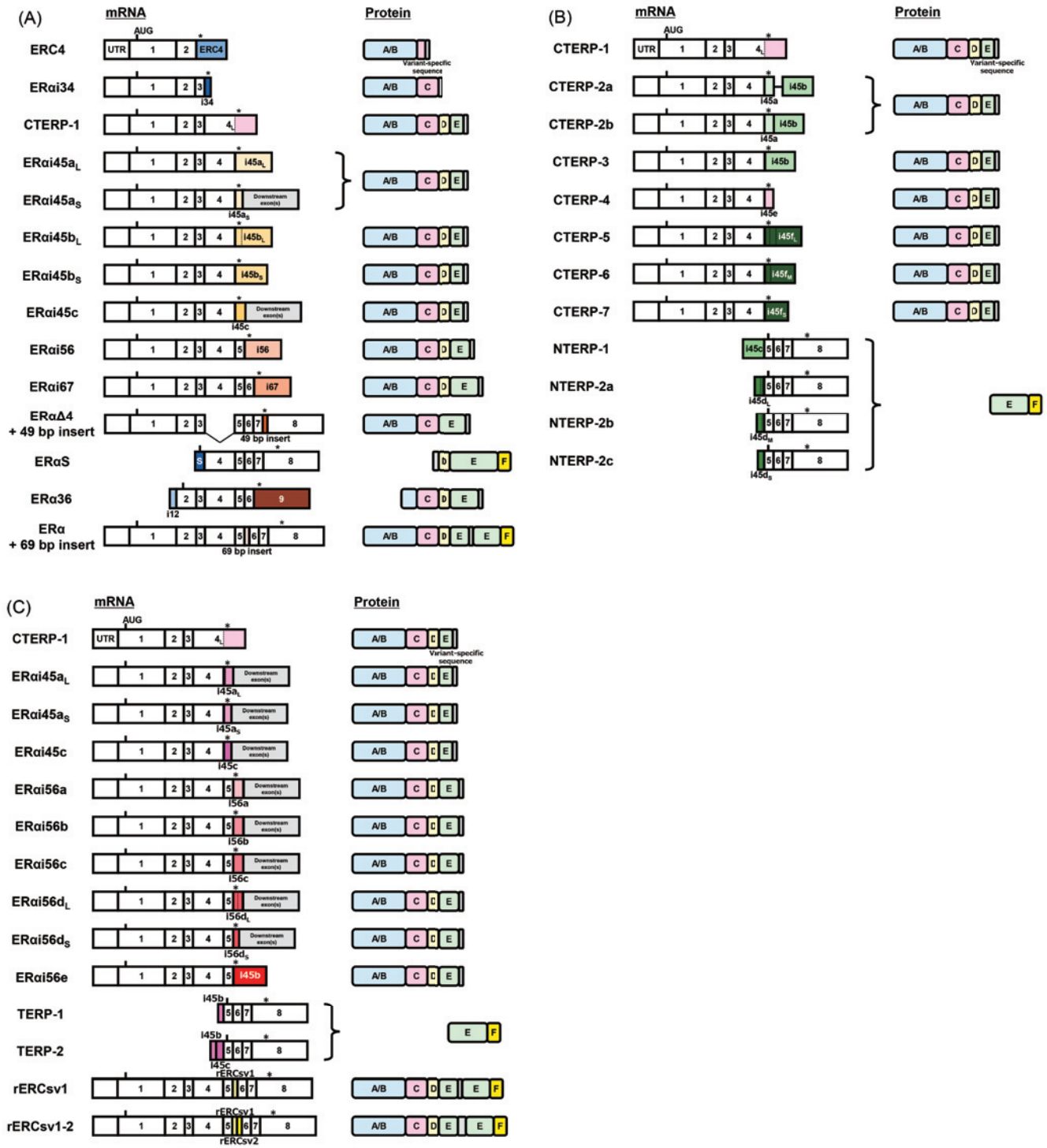

Fig. 3 Structures of ERα isoform mRNAs and proteins

mRNA (left) and protein (right) structures of human (A), mouse (B), and rat (C) ERα isoforms are represented schematically. The images are not to scale.

weak to strong constitutive transactivation. These variants except ERαi34 are localized predominantly in the nuclei. Since the NLS of ERαi34 is truncated, the variant was distributed in both the cytoplasmic and nuclear regions. By contrast, isoform proteins encoded by exon 5-containing transcripts (ERαi56 and ERαi67) were located mainly in the nuclei, but a faint or weak extranuclear distribution was also observed. The exon 5-containing isoforms did not display transactivation.

For comparative analysis, the existence and alternative use of cryptic exons in the rat ERα gene were examined[10]. The rat ERα gene contains multiple cryptic exons (exons 4$_L$, i45a-c, and i56a-e) between exons 4 and 6, and the alternative use of these exons generates C-terminally-

truncated ERα isoform transcripts (CTERP-1, ERαi45a$_L$, ERαi45a$_S$, ERαi45c, ERαi56a, ERαi56b, ERαi56c, ERαi56d$_L$, ERαi56d$_S$, and ERαi56e). Although C-terminally-truncated isoforms are generated from human, mouse, and rat ERα genes, the nucleotide sequences of the cryptic exons are poorly conserved.

N-terminally-truncated ERα Isoforms

The transcripts for the human ERα S isoform (ERαS), mouse N-terminally-truncated estrogen receptor α products (NTERPs), and rat truncated estrogen receptor products (TERPs) are transcribed by initiation from the promoters located in intronic regions[10,12,30,33]. Mouse NTERP-1 and NTERP-2 transcripts are generated by alternative splicing from mouse leader exons i45c and i45d to exon 5, respectively. Mouse exon i45d contains three alternative splice donor sites, and the alternative use of these sites yields NTERP-2a-c variants. Rat TERP-1 mRNAs are produced by splicing from the rat leader exon i45b to exon 5. Rat internal exon i45c is inserted between exons i 45b and 5 to yield TERP-2 mRNA. Mouse NTERP and rat TERP proteins are translated from the start codons in exon 5, and the truncated isoforms retain the C-terminal regions of the LBDs. NTERP and TERP isoforms lack NLSs; therefore, their extranuclear distribution was observed in transfected cells[10,12]. Furthermore, they exhibited neither transcriptional transactivation nor modulated ERα66-mediated transactivation. Although a human ERαS isoform has not yet been functionally analyzed, the isoform contains the whole LBD and is presumed to have a ligand-binding ability.

ERα Isoform with Both N- and C-terminal Truncations

The transcriptional activation of an i12-corresponding promoter and alternative termination to exon 9 yields an mRNA (i12-2-3-4-5-6-9) encoding an ERα protein of 36 kDa (ERα36), which lacks both N- and C-termini. Wang et al.[29] were the first to identify this variant sequence (BX 640939) in the full-ORF resource of the German cDNA Consortium[35]. ERα36 did not exhibit transactivation of an ERE promoter in transfected cells but was predominantly located at the plasma membrane and transduced non-genomic mitogenic signaling in an estrogen-dependent manner[36]. Furthermore, wide spectra of ER antagonists activated ERα36-mediated kinase signaling pathways, indicating involvement of ERα36 in the non-genomic effects of estrogens and the acquisition of chemotherapy resistance.

ERα Isoforms with Insertions in the LBD

Murphy et al.[32] identified a human ERα isoform with a splice insert between exons 5 and 6. The insert is in-frame (69 bp) and lacks premature stop codons; therefore, the variant includes additional 23-amino acids in the LBD. Recently, Kundu et al.[26] reported the presence of two internal exons (exons "rERCsv1" and "rERCsv2") between exons 5 and 6 in the rat ERα gene and the generation of two ERα isoforms (ERCsv1 and ERCsv1-2) with insertions in their LBDs. The structures and splicing patterns of the human and rat insertion-containing ERα isoforms are comparable to those of mouse and rat ERβ2[37,38], although the inserted sequences are not homologous with each other or those of rodent ERβ2.

Constitutive Transactivation Properties of C-terminally-truncated ERα Isoforms

C-terminal truncation of ERα isoforms is a mechanism for gain of constitutive transactivation. Constructs without the whole LBD constitutively transactivate ERE-containing promoters. However, previous studies have reported conflicting results regarding the functions of ERα variants with partial LBD truncation. Fuqua et al.[17] were the first to clone an exon-skipping ERα variant (ERαΔ5) that encoded a C-terminally-truncated ERα protein. Initial studies of ERαΔ5 reported its constitutively active functions[16,17], whereas several subsequent studies described its dominant-negative properties[14,15]. Moreover, several C-terminally-truncated ERβ variants have been identified[39–41], and they displayed non-functional or dominant-negative activities. Thus, most ER researchers believed that ER variants with partially truncated LBDs were non-functional or dominant-negative forms. Recently, we identified the critical determinant for constitutive transactivation in C-terminally-truncated ERα isoforms and characterized their transactivation properties[10,11]. LBD truncation from the C-terminus to H5 is the critical determinant for constitutive transactivation. The remaining parts of the LBD and variant-specific sequences in the C-terminally-truncated isoforms influence transactivation efficiencies. The transcripts for full-length and C-terminally-truncated ERα isoforms are transcribed by initiation of the same promoters and are therefore presumed to colocalize in the same cells. Thus, examination of the modulatory effects of C-terminally-truncated ERα isoforms on ERα66- or native ER-mediated transactivation is essential in understanding their biological roles. C-terminally-truncated ERα isoforms suppressed ligand-dependent activation of ERα66 in transfected HEK293

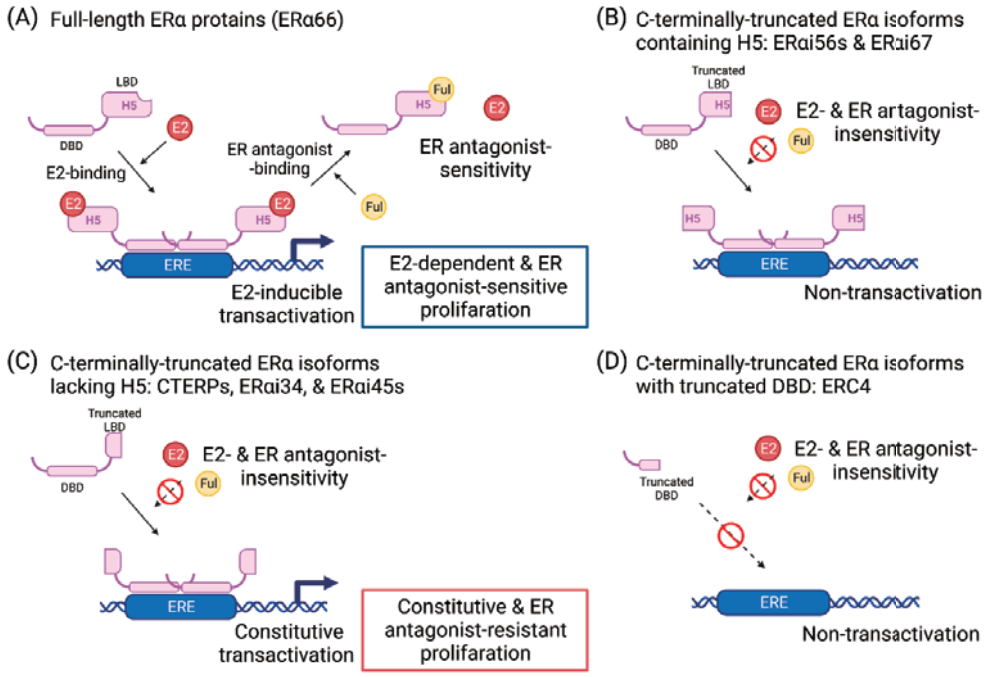

Fig. 4 Transactivation profiles of C-terminally-truncated ERα isoforms

Transactivation profiles of full-length ERα proteins (A), C-terminally-truncated ERα isoforms containing (B) and lacking (C) H5, and C-terminally-truncated ERα isoforms with truncated DBD (D) are summarized schematically.

E2, 17β-estradiol; Ful, fulvestrant; H5, helix 5 in the LBD. This figure was created with Biorender.com.

cells, whereas the isoforms exerted additive effects on native ER-mediated transactivation in transfected MCF-7 cells. Furthermore, transactivation by C-terminally-truncated ERα isoforms was insensitive to ER antagonists.

ERα66 proteins exhibit nuclear localization and relatively high basal activity in the absence of ligands. Constitutive functions of the C-terminally-truncated variants are not a mere enhancement of basal activity. The co-transfection of a p300 co-activator with constitutively active isoforms augmented their transactivation abilities, although the basal activity of ERα66 was not enhanced[9–11]. Therefore, the truncation from the C-terminus to H5 transforms repressive ERα proteins to their active forms.

Analysis of constructs with further C-terminal truncation revealed that proteins lacking the HD are localized to both the cytoplasmic and nuclear regions and retained constitutive transactivation. Deletion of the DBD impaired transcriptional transactivation of an ERE-driven promoter. Therefore, constitutive transactivation of C-terminally-truncated ERα isoforms requires at least the N-terminal transactivation domain and the DBD. These domains are encoded in exons 1-3; therefore, ERα isoforms encoded in exons 1-3 or 1-4 plus additional nucleotide sequences can exhibit constitutive transactivation.

Transactivation profiles of C-terminally-truncated ERα isoforms are summarized in **Figure 4**.

Future Perspectives

The critical determinant for gain-of-function of C-terminally-truncated ERα isoforms has been identified structurally. Therefore, potential functions of LBD-truncated ERα variants can be deduced from their structures. Although we have mainly discussed C-terminally-truncated ERα isoforms generated by the alternative use of cryptic exons, several other mechanisms that yield LBD-impaired ERα variants were recently reported. Recurrent rearrangements of the ERα gene can promote development of ER-positive breast and endometrial cancers to more aggressive forms. Recurrent rearrangements between the ERα gene and its neighboring genes generate LBD-impaired fusion proteins, in which the C-terminal regions of ERα are replaced with those of the counterpart proteins[42]. An ERα and *YAP1* fusion gene lacking exons 5-8 of ERα was identified in breast cancer-derived xenografts, and the encoded LBD-impaired protein enhanced the growth of stably transfected breast cancer

cells in an estrogen- and fulvestrant-insensitive fashion[43,44]. Moreover, recurrent amplifications of exons 1-3 and 1-4 of the ERα gene were implicated in acquired chemotherapy resistance of primary endometrium cancers[45].

The constitutive transactivation by ERα isoforms did not compete with native ERα-mediated transactivation in breast cancer-derived cells. Therefore, constitutively active isoforms are candidate molecules for estrogen insensitivity in malignant breast cancers. Furthermore, LBD-impaired ERα variants display ER antagonist resistance. Therefore, constitutively active ERα isoforms can be involved in endocrine resistance and chemotherapy resistance of estrogen-dependent cancers. Future studies should evaluate C-terminally-truncated ERα isoforms as potential biomarkers for estrogen insensitivity and chemotherapy resistance, and their elevated expression in malignant breast and endometrium cancers should be examined.

Conflict of Interest: The authors declare no conflicts of interest.

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