Wnt/β-Catenin and Estrogen Signaling Converge in Vivo*

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Wnt and estrogen signaling represent important regulatory pathways, each controlling a wide range of biological processes. While an increasing number of observations suggest potential convergence between these pathways, no direct evidence of their functional interaction has been reported. Using human colon and breast cancer cells, we found that estrogen receptor (ER) α- and β-catenin precipitated within the same immunocomplexes, reciprocally enhanced the transactivation of cognate reporter genes, and were reciprocally recruited to cognate response elements in the promoters of endogenous target genes. Using transgenic Drosophila that ectopically expressed human ERα alone or together with metabolically stable β-catenin/Armadillo mutants, we demonstrated genetic interaction between these signal transducers in vivo. Thus, we present here the first direct evidence of cross-talk between Wnt and estrogen signaling pathways via functional interaction between β-catenin and ERα.

Estrogens regulate a plethora of physiological functions in the developing and adult organism and act predominantly via the activation of ERα and ERβ. Liganded ER dimers bind to promoter estrogen response elements (EREs) and regulate the transcription of target genes. This ER-mediated regulation requires the recruitment of different co-factor complexes and is associated with rearrangement of chromatin structure at EREs within target gene promoters (1, 2). ER can also act as a co-factor at non-ERE sites via interaction with other DNA-bound transcriptional factor complexes, such as c-Jun/c-Fos on the AP-1 site (3) or c-Jun/NFκB on the tumor necrosis factor response element (4). The physiological significance of ERs is demonstrated by the severe abnormalities in development and function of major organs and tissues in mice with ablated ERα and/or ERβ (5). Also, both positive and negative impacts of estrogens in different types of cancer have been well documented (6).

Wnt signaling plays a critical role in numerous processes of development and in adult tissues and appears to be conserved across all animal taxa. β-Catenin is an intracellular transducer of canonical Wnt or Wnt/β-catenin signaling and, thus, has a dual function: as a transcriptional factor and, in a cadherin-bound form, as a regulator of cell adhesion and migration. Cytoplasmic or signaling β-catenin is unstable and rapidly targeted to phosphorylation-ubiquitination-coupled proteasomal degradation. Wnt signaling inhibits this degradation, resulting in the accumulation of β-catenin in the nucleus and its association with members of the T cell factor/lymphoid enhancer factor (TCF/LEF) family of transcriptional factors that leads to the activation of Wnt target genes. Mutations that increase the stability of cytoplasmic β-catenin have been implicated in numerous malignant transformations and represent a leading cause of colorectal tumorigenesis (7–9).

Consistent with the concept of morphogen gradients (10) β-catenin exerts different biological effects, such as induction of cell proliferation and apoptosis or stimulation and repression of the same target genes, in a threshold-dependent manner (11, 12). Thus, slight modulation of β-catenin signaling through cross-talk with other pathways may trigger serious physiological consequences. Potential cross-talk between Wnt/β-catenin and estrogen signaling in vivo has been implicated in physiological studies on tissues as different as brain (13) and uterus (14). Furthermore, although males and females develop colorectal cancer with approximately the same frequency, its incidence rate is significantly lower in women undergoing hormone replacement therapy (15, 16). While these and other observations suggested the possibility of functional interaction between ER and β-catenin, previous attempts failed to detect such an interaction (13, 17, 18), and no direct evidence of Wnt and estrogen signaling pathway convergence has been reported.

Compared with vertebrates, Wnt signaling has been far better characterized in Drosophila, in which it is not obscured by involvement of other, evolutionary more recent multiple pathways. Thus, Drosophila provides a powerful experimental system for analysis of functional interaction in vivo between Wnt signaling and other regulatory pathways, including those merged at the later stages of evolution. Therefore, in addition to mammalian cells, to detect functional interaction between Wnt/β-catenin and estrogen signaling in vivo we used transgenic Drosophila that ectopically expressed human ERα coupled to an ERE-dependent green fluorescent protein (GFP) reporter gene alone or together with constitutively active mutants of Armadillo, a Drosophila homologue of β-catenin. Using different approaches, we obtained in this study the first evidence of physical association and transcriptional and genetic interaction in vivo between ERα and β-catenin.

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‡ The abbreviations used are: ER, estrogen receptor; ERE, estrogen response element; TCF, T cell factor; LEF, lymphoid enhancer factor; TBE, TCF/LEF binding element; CSFCS, charcoal-stripped fetal calf serum; ChIP, chromatin immunoprecipitation; TUNEL, terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling; LBD, ligand binding domain; wt, wild-type.

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EXPERIMENTAL PROCEDURES

Immunoprecipitation and Immunoblotting—Cells grown in the presence of charcoal-stripped fetal calf serum (CSFCS) were transfected with FLAG-hERα expression vector and harvested 28–30 h post-transfection, after treatment for 3 h with vehicle (ethanol) or 10^{-8} M 17β-estradiol (Sigma), tamoxifen (Sigma), or ICI 182,780 (Tocris). Anti-β-catenin E-5 or H-102 antibodies (Santa Cruz Biotechnology) or preimmune rabbit serum IgG (as a negative control) were used for immunoprecipitation. Western blots were visualized with anti-FLAG M2 (Sigma) or anti-ERα HC-20 (Santa Cruz Biotechnology) antibodies.

Transfection and Reporter Assay—Cells grown in Opti-MEM, 5% CSFCS were transfected with 250 ng of reporter (ERE-tk-luc or tk-luc for MCF7 cells and TOPFLASH or FOPFLASH for colon cancer cells) and 1 ng of pRi (Promega) plasmid (control for transfection efficiency) together with 100 ng of empty (control) or cDNA (β-catenin S33Y for MCF7 cells and ERα for colon cancer cells) expression vector and treated for 16–20 h with vehicle or 10^{-8} M ligand, as indicated. To nullify nonspecific effects on basal promoters, TOPFLASH and ER/EK-luc reporter activities were normalized against FOPFLASH and tk-Luc reporter activities, respectively, from parallel experiments.

Chromatin Immunoprecipitation (ChIP) Assay—Association of ERα and β-catenin with ERE in the pS2 gene promoter (19) and TCF/LEF binding element (TBE) in the Axin2 gene promoter (20) was analyzed using the Chromatin Immunoprecipitation Kit (Upstate Biotechnology) and HC-20 or E-5 antibody, respectively. As a control for nonspecific chromatin precipitation with these antibodies, a set of primers was used to amplify a pS2 gene DNA segment that does not have ERE or TBE sequences. In addition, IgG from normal preimmune rabbit serum was used as a negative control.

Histology and Immunostaining—All techniques were performed as described previously (21, 22). Expression of ERα and GFP in Drosophila eye discs were detected using Zeiss Confocal Laser Scanning System 510 and quantified by calculation of pixels of the corresponding signals using Adobe Photoshop 7 software facilities. TUNEL labeling was performed using the TACS2 TdT-Fluor In Situ Apoptosis Detection Kit ( Trevigen).

Drosophila Lines and Stocks—The UAS-ΔArm and UAS-ArmS10 mutants were obtained from the Bloomington Drosophila Stock Center. Generation and characterization of the used UAS-ERα, ERE-GFP transgenic Drosophila lines were described in Ref. 23. Briefly, cDNA encoding full-length human ERα, ligand binding domain (LBD) deletion mutant ERα)(1–302), or GFP reporter under control an ER containing promoter were recloned into the pCaSpeR vector. Transgene constructs together with p25.7wc transposase were microinjected into w^{1118} embryos using a micromanipulator (Leica). Several independent transmartant lines have been generated. To target ERα expression into the eye disc, transgenic Drosophila were crossed with flies of a GMp-GAL4 line expressing GAL4 driver in the retina under control of the tissue-specific glass multimer gene promoter.

RESULTS

Physical Association of ERα and β-Catenin—Human colon cancer HCT116 cells express metabolically stable β-catenin due to mutation at its putative phosphorylation site. These cells, however, do not express detectable ER. HCT116 cells were transfected with a FLAG-tagged human ERα expression plasmid, and endogenous β-catenin was immunoprecipitated from cell lysates following 3-h preincubation with estrogen or vehicle. IgG from normal rabbit serum was used as a control for nonspecific immunoprecipitation. Obtained immunocomplexes were subjected to Western blotting and analyzed by immunostaining with antibodies against FLAG-tag and ERα.

ERα co-immunoprecipitated with β-catenin even in the absence of ligand; however, ERα-β-catenin association was markedly stimulated by estrogen (Fig. 1A). Similar results (data not shown) were obtained using SW480 human colon cancer cells, in which non-mutant β-catenin was stabilized by a loss-of-function mutation in the gene of tumor suppressor Adenomatous polyposis coli, an essential component of the β-catenin degradation machinery. Brief exposure to ligand did not affect FLAG-ERα expression in this (Fig. 1A) or further experiments.

As anti-β-catenin antibodies co-precipitated a C-terminally truncated FLAG-ERα(1–396) (Fig. 1B), it appeared that an intact LBD was not essential for the ER interaction with β-catenin. Predictably, C-terminal truncation of ERα abolished the ligand sensitivity of the interaction.

We then analyzed whether ligands that inhibited the transcriptional activity of ERα would also affect its interaction with β-catenin. Immunoprecipitation of ERα with antibodies against β-catenin was significantly stimulated by the ERα partial, tamoxifen, and complete, ICI 182,780, antagonists (Fig. 1C).

Transcriptional Interaction between ERα and β-Catenin—Next, we investigated whether the apparent physical association between ERα and β-catenin was consequential for transcriptional function of the proteins. Transactivation of an ERE-dependent reporter by endogenous ERα was studied in human breast cancer MCF7 cells, in which the Wnt pathway is practically silent. Expression of stabilized β-catenin S33Y in these cells enhanced ligand-dependent expression of the reporter without affecting its basal activity in the absence of ligand (Fig. 2A). Expression of ERα in human colon cancer SW480 (Fig. 2B) and HCT116 (data not shown) cells enhanced the activation of the Wnt-responsive TOPFLASH reporter by endogenous β-catenin in the absence of ligand. Treatment with estrogen resulted in further moderate activation of reporter expression, while ER antagonists appeared not to affect reporter gene activity (Fig. 2B).

The reciprocal activation of cognate reporters in the transfection experiments suggested that ERα and β-catenin might reciprocally recruit each other to their corresponding response elements in endogenous target gene promoters. Indeed, antibody against β-catenin precipitated ERα of the pS2 gene promoter from chromatin of β-catenin S33Y expressing MCF7 cells in an estrogen-dependent manner (Fig. 2C). Conversely, anti-ERα antibody precipitated in a ligand-dependent manner Axin2 gene promoter putative TBE from chromatin of SW480 cells transfected with an ERα expression construct, while recruitment of β-catenin to the TBE was not sensitive to the presence of estrogen (Fig. 2D). The used antibodies did not display nonspecific chromatin precipitation (Fig. 2E).

Consistent with the results obtained using MCF7 cells, ERα transactivation was markedly enhanced in vivo by the stabilized Armadillo mutants ΔArm (24) (Fig. 2F) or ArmS10 (25) (data not shown) when ectopically co-expressed in the Drosophila eye disc.

Genetic Interaction between ERα and β-Catenin—Constitutive activation of Armadillo in the Drosophila eye disc has been shown to induce apoptosis and consequent degeneration in the adult eye (26, 27). Potentiation of β-catenin transcriptional activity by ERα in SW480 cells (Fig. 2B) and functional interaction between ERα and Armadillo (Fig. 2F) would predict activation of endogenous Armadillo by the ectopic ERα expression in
the Drosophila eye disc leading to development of a phenotype characteristic of abnormal Wnt/β-catenin activation.

We performed TUNEL staining of the third instar larval eye discs with ectopic expression of ERα alone or together with the constitutively active Armadillo mutant ΔArm. When expressed singly, ERα and ΔArm both induced a slight increase in apoptosis compared with wild-type (wt) eye discs from Drosophila of the parental line. Co-expression of ERα and ΔArm resulted in a marked increase in apoptotic cell number. Importantly, while estrogen had no discernible effect on apoptosis in wt eye discs and those expressing either ERα or ΔArm alone (data not shown), treatment with estradiol significantly increased apoptosis rates when ERα and ΔArm (ERα,ΔArm+E2) were co-expressed (Fig. 3A). Activated Armadillo has a mild apoptotic effect in the third instar larva eye disc, reportedly due to the protective counteraction at this stage by the EGFR/MAPK signaling (27). This allowed us to detect differences in apoptosis patterns in transgenic fly eye discs at this developmental stage that would otherwise be difficult to distinguish due to the onset of massive cell death at the later stages.

We compared adult eye phenotypes of flies from these transgenic lines and the wt (Fig. 3B). The normal Drosophila eye is composed by regularly spaced ommatidia with regularly oriented interommatidial bristles. Expression of ERα in the eye disc leads to development of phenotypes similar to those caused by expression of ΔArm: rough eye appearance and disorientation or loss of interommatidial bristles. Co-expression of ΔArm and ERα synergistically enhanced this abnormal eye development. Again, while estradiol appeared not to affect the separate ΔArm or ERα expression phenotypes (data not shown), treatment with estrogen, however, further aggravated the severity of eye abnormalities in the ERα and ΔArm co-expression lines with different chromosomal localization of the ERα transgene.

**DISCUSSION**

We found that β-catenin associated with ERα even in the absence of ligand and that estrogens further enhanced this interaction. While it is possible that the ligand-independent association was due, at least in part, to the overexpression of one of the interacting proteins, the association between β-catenin and C-terminally truncated ERα suggested that the ligand binding was not essential but might rather induce a more favorable conformation for ERα to interact with β-catenin. This may be of functional significance at physiological concentrations of the interacting proteins. Interestingly, β-catenin recruitment to EREs and ERα recruitment to TBEs in the promoters of endogenous target genes were both highly ligand-dependent. The apparently equal stimulation of ERα-β-catenin interaction by ER agonists and antagonists may have important implications for the design of novel therapeutic strategies.

Our most significant finding was that ERα functionally interacted with β-catenin/Armadillo in vivo in transgenic Drosophila. The ligand-dependent transactivation function of ERα was significantly enhanced by the co-expression of stabilized Armadillo mutants. Abnormalities in the eye development in-
duced by targeted expression of activated Armadillo and ERα were of a similar nature. Co-expression of both proteins synergistically enhanced the abnormal phenotype that was further aggravated by treatment with estradiol. Importantly, in mammals, estradiol is shown to have a prominent neuroprotective activity thought to be mediated by ER (28).

Physical and transcriptional interaction between β-catenin and androgen receptor has been observed previously (17, 18). However, in experiments presented in these reports no interaction between β-catenin and other nuclear hormone receptors, including ER, has been detected.

Thus, we have shown that Wnt and estrogen signaling pathways cross-talk in vivo through functional interaction between ERα and β-catenin. This interaction may underlie mechanisms of estrogen effects in pathological conditions and processes in which abnormalities of Wnt/β-catenin signaling have been implicated, such as in colorectal cancer. In addition, we have established a novel experimental system in which to identify factors conserved between humans and Drosophila that may be involved in regulation of cross-talk between Wnt and estrogen signaling and for the screening of novel compounds able to interfere with this cross-talk.

Although other mechanisms may be involved (e.g. intranuclear sequestration), transcriptional modulation appears to be the major mechanism of functional ERα-β-catenin interaction. The genomic function of nuclear receptors is dependent on the recruitment of different coactivator and chromatin remodeling complexes (1, 2, 29, 30). β-Catenin has been shown to recruit coactivators, such as the p300/CBP complex (31), and components of the mammalian SWI/SNF and RSC chromatin remodeling complexes (32) that are also known to interact with ERα. Recruitment of additional co-activator and chromatin remodeling complexes may account for the transcriptional outcome of ERα-β-catenin interaction. The physiological consequences of this interaction may also depend on cell and tissue specificity in composition of the recruited regulatory complexes. Further experiments to identify all ERα-β-catenin complex components are required to determine whether the ERα-β-catenin interaction results only in quantitative changes in the composition of the recruited regulatory proteins or if factors specific to ERα-β-catenin protein complexes are involved.

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