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Research Article

Characterization of a beta-catenin nuclear localization defect in MCF-7 breast cancer cells

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ABSTRACT

Beta-catenin plays a key role in transducing Wnt signals from the plasma membrane to the nucleus. Here we characterize an unusual subcellular distribution of beta-catenin in MCF-7 breast cancer cells, wherein beta-catenin localizes to the cytoplasm and membrane but atypically did not relocate to the nucleus after Wnt treatment. The inability of Wnt or the Wnt agonist LiCl to induce nuclear localization of beta-catenin was not due to defective nuclear transport, as the transport machinery was intact and ectopic GFP-beta-catenin displayed rapid nuclear entry in living cells. The mislocalization is explained by a shift in the retention of beta-catenin from nucleus to cytoplasm. The reduced nuclear retention is caused by unusually low expression of lymphoid enhancer factor/T-cell factor (LEF/TCF) transcription factors. The reconstitution of LEF-1 or TCF4 expression rescued nuclear localization of beta-catenin in Wnt treated cells. In the cytoplasm, beta-catenin accumulated in recycling endosomes, golgi and beta-COP-positive coatomer complexes. The peripheral association with endosomes diminished after Wnt treatment, potentially releasing β-catenin into the cytoplasm for nuclear entry. We propose that in MCF-7 and perhaps other breast cancer cells, beta-catenin may contribute to cytoplasmic functions such as ER-golgi transport, in addition to its transactivation role in the nucleus.

1. Introduction

The aberrant activation of Wnt signaling in many cancers is linked to elevated levels of cytosolic and nuclear β-catenin leading to hyper-activation of lymphoid enhancer factor/T-cell factor (LEF/TCF)-dependent genes. This generally occurs through mutations in components of the β-catenin destruction complex, such as APC and Axin or mutations in β-catenin itself, that prevent it from being targeted for proteasomal degradation [1,2]. A prime example is colorectal cancer, wherein nearly 90% of cases harbor mutations in these key components leading to aberrant activation of the pathway and nuclear translocation of β-catenin [1–5]. However, in breast cancer, such mutations are rare. A screening of 227 breast carcinomas identified only a single case of APC truncation while a study of 24 breast cancer cell lines identified one other example [6,7]. No mutations have been detected in Axin [8,9] nor have N-terminal mutations of β-catenin been reported in breast carcinomas [6,10,11]. Despite the lack of mutations, β-catenin-TCF regulated target genes such as cyclin D1 and c-myc can in some instances be up-regulated in breast cancer cells [12].

In breast cancer, excessive Wnt activation can occur at the level of Wnt ligand-receptor interaction, through the up-regulation of ligands and receptors and the down-regulation of secreted inhibitors [13–16]. For example, multiple Wnt ligands and Fzd receptors are overexpressed in both human breast tumors and in breast cancer cell lines [13,14,17]. In ~80% of breast cancers there is loss of expression of frizzled related protein 1 (sFRP1), a soluble Wnt antagonist, which is predictably associated with poor prognosis [18,19]. Dishevelled (Dvl) overexpression has also been reported in 50% of ductal breast cancer cells and was linked to pathway activation [20]. Together, these reports suggest that modulators of Wnt signaling, both at the extracellular (sFRP1) and intracellular level (Dvl), rather than mutations in key downstream players of the Wnt/β-catenin cascade may have a critical role in the biological outcome.

Abbreviations: APC, adenomatous polyposis coli; Beta-COP, coatomer protein beta-subunit; COP, Coat protein complex; LEF-1, Lymphoid enhancer factor 1; TCF, T-cell factor

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The nuclear targeting of β-catenin is critical for Wnt signaling and for development of colorectal cancer, but there are relatively few studies analysing β-catenin subcellular location in breast cancer cells. To address this, β-catenin localization was investigated in different breast epithelial cell lines and found to be excluded from the nucleus in MCF-7 cells, even after Wnt stimulation. After 15 years of studying the nuclear localization of β-catenin this is the first instance we found of such clear nuclear exclusion. MCF-7 cells have quite high levels of β-catenin which is mainly sequestered in the cytoplasm and at the membrane. We now show that the block to nuclear localization was partly caused by depleted levels of the β-catenin-binding transcription factors LEF-1, TCF1 and TCF4 and identify an accumulation of β-catenin in the cytoplasm at specific structures including the golgi.

2. Materials and methods

2.1. Cell culture and transfection

The mouse fibroblastic cell line NIH 3T3, human embryonic kidney cell line HEK293T, human osteosarcoma cell line U2OS, human cervical adenocarcinoma cell line HeLa, human colon cancer cell line SW480 and the human breast cancer cell lines MCF-7 (adenocarcinoma) and T47D (ductal carcinoma) were cultured in supplemented Dulbecco's modified Eagles media (DMEM). The non-tumorigenic breast cell line MCF-10A was cultured in supplemented DMEM/F12 media (Sigma). For transfection, NIH 3T3 and MCF-7 cells were seeded into 6 well trays (Nunc) 24 h prior to transfection with 500 ng to 3 μg DNA using Lipofectamine 2000 (Invitrogen). Cells were all mycoplasma negative.

2.2. Drug treatments

When cells were at ~70% confluence, media was removed and cells were incubated with 1 ml of drug-media solution. For Wnt treatment, cells were treated with Wnt3a conditioned medium or LiCl (Wnt agonist) was made fresh for each experiment. To prepare the Wnt-3a-conditioned medium, L-cells (Ctrl and Wnt3a) were allowed to grow for 4 days until they reached 80–90% confluency. The medium was removed, filtered through a 0.22 μm membrane and stored at 4 °C. Fresh medium was added to the cells and cultured for an additional 3 days. The medium was taken off, filtered through a 0.22 μm membrane, combined with the first batch of media and stored at −80 °C. The proteasome inhibitor MG132 was stored in aliquots at −20 °C to avoid freeze-thaw cycles and was diluted in media immediately prior to administration (used at 20 μM). For the Rev assay (Supplementary Fig. S1) cells were treated with 10 ng/ml leptomycin B at 2 h prior to fixation.

2.3. Plasmids

The plasmids pLEF-1-HA and pTCF4-myc were kind gifts from Dr Behrens [21] and Dr Marian Waterman [22], respectively. Organelle markers pFusionRed-ER (endoplasmic reticulum), -golgi and -endo (endosomes) are commercially available and were purchased from Evrogen (Moscow, Russia).

2.4. Immunofluorescence microscopy

Cells were fixed using methanol acetone or formalin and immunostaining using the following antibodies: mouse monoclonal β-catenin (1:100, BD), mouse monoclonal Nup358 (1:100), mouse monoclonal mAb414 (1:400), mouse monoclonal Nup62 (1:500), mouse monoclonal Nup153 (1:200), rabbit polyclonal α-HA (1:150), rabbit polyclonal α-myc (1:300), rabbit polyclonal Rab7 (1:100, Cell Signaling), rabbit polyclonal β-COP (1:1000, Abcam) and Hoechst dye as a nuclear marker (1:100). Slides were processed using an upright Olympus Fluorescent BX40 system with a SPOT digital camera or Olympus FV1000 confocal microscope. For cellular distribution scoring, at least 100 cells per slide were scored for nuclear (nuclear (N) > cytoplasmic (C)), nuclear-cytoplasmic (N=C) or cytoplasmic (C > N) localization of β-catenin. An average score was obtained from 2–3 independent experiments. To quantify the cellular levels/intensities of β-catenin, acquired fluorescence microscopy images taken under exactly the same conditions were analysed using Scion imaging (Scion Corporation) or Fluoview 1.7b (Olympus confocal) software. In addition to confocal microscopy, cytoplasmic analysis of β-catenin was performed in cells with an Olympus IX71 microscope with DeltaVision deconvolution system (GE Healthcare Life Sciences) equipped with a CoolSNAP HQ^2 camera for image capture. Images collected using the DeltaVision system were further resolved using Softworx deconvolution software. Data from scoring experiments was collected from at least 100 cells, acquired from at least two independent experiments.

2.5. Fluorescence recovery after photobleaching (FRAP) assay

FRAP was used to measure nuclear import rate of β-catenin-GFP in live MCF-7 cells using an Olympus FV1000 confocal microscope. Cells with clear expression of nuclear β-catenin-GFP were selected for the assay. Briefly, three pre-bleach images of each cell were acquired, then nuclear fluorescence was bleached (~50 frames). Post-bleach imaging was in two stages: 30 frames at the fastest interval then 30 frames at 10 s intervals. Fluorescence intensities for cytoplasm, nucleus, and background were acquired using Olympus Fluoview software and exported to a Microsoft Excel file. *Data Analysis* — background values were subtracted from cytoplasmic and nuclear fluorescence intensities which were then expressed as a nuclear/cytoplasmic ratio. For each cell data set, the pre-bleach ratio was set to 100%, the time for the first post-bleach image was set to 0 s. The average of the data from two experiments (n=6–8) was plotted. Initial nuclear import rates for the first 30 s was analyzed in GraphPad Prism 5.0 using linear regression analysis.

2.6. CSK detergent extraction assay

MCF-7 cells were grown in Nunc 6-well trays on poly-L-lysine coated coverslips. After drug treatments, cells were washed with PBS and incubated on ice for 10 min in 1 ml of chilled CSK (10 mM Pipes, pH 6.8; 300 mM Sucrose; 5 mM $MgCl_2$; 50 mM NaCl; 0.01–0.1% Triton X-100) buffer. The % Triton was optimized depending on the application. Cells were then fixed with 3.7% formalin-PBS and processed for immunostaining as above.

2.7. Western blot analysis

Cells were harvested and lysed in RIPA buffer as described previously [23]. Protein quantification was performed using the Bradford Assay (Bio-Rad). Equivalent portions of each sample (50 μg of total extract) were separated by 7.5% SDS-PAGE and transferred to a nitrocellulose membrane (Millipore). The membrane was hybridized with the following antibodies: mouse monoclonal β-catenin (1:3000, BD Transduction labs, USA), mouse monoclonal β-tubulin (1:2000, Sigma), rabbit monoclonal TCF1 (1:1000, Cell Signaling), rabbit monoclonal TCF3 (1:1000, Cell Signaling), rabbit monoclonal TCF4 (1:1000, Cell Signaling), rabbit monoclonal LEF-1 (1:1000, Cell Signaling), anti-rabbit-HRP (1:5000, Sigma) and anti-mouse-HRP (1:5000, Sigma). β-tubulin was used in conjunction with Ponceau S (Sigma) staining as