European Journal of Pharmaceutics and Biopharmaceutics 85 (2013) 20–25

Contents lists available at SciVerse ScienceDirect

European Journal of Pharmaceutics and Biopharmaceutics

journal homepage: www.elsevier.com/locate/ejpb

Research paper

The role of CDX2 in Caco-2 cell differentiation

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ARTICLE INFO

Dedicated to Hans Peter Merkle on the occasion of his 70th birthday

Keywords:
Caco-2 cells
Intestine
CDX2
Differentiation
Wnt pathway
Genomic profiling

ABSTRACT

Background: CDX2 plays a key part in the differentiation of Caco-2 cells, a colon carcinoma derived cell line that undergoes spontaneous differentiation. The effect of CDX2 expression in Caco-2 cells over time in culture has not been studied yet on a genome-wide level.
Methods: The impact of CDX2 expression on the genomic profile of Caco-2 cells was studied by transducing cells with CDX2 targeting shRNAs. Knockdown efficiency was assessed on mRNA level and protein level by RTPCR, microarrays, and Western blots. Gene set enrichment analysis was performed to assess regulation of specific gene sets.
Results: CDX2 expression had an inhibitory effect on the transcriptional activity of β-catenin/TCF at early stages of culturing, while at later stages, its role in the trans-activation of target genes specific for small intestinal enterocytes seemed more dominant.
Conclusions: The unique induction of a small intestinal signature upon differentiation in Caco-2 cells seems to be at least partially under the control of CDX2.

1. Introduction

The proliferation and differentiation of intestinal epithelial cells is highly organized in a spatial and sequential manner. Several signaling pathways are involved to maintain homeostasis. A major player is the Wnt pathway in maintaining stem cell population and appropriately regulated differentiation [1–3]. The expression of intestine-specific genes and their resulting protein products is tightly regulated by activity of transcription factors including CDX2, CDX1, HNF1A HNF1B, HNF4A, and GATA [1]. CDX2 has recently been shown to be essential for the intestinal differentiation and to act as a dominant factor of intestinal cell fate [4,5]. Recent studies indicate that CDX2 is not just involved in the trans-activation of target genes, but also inhibition of the transcriptional activity of β-catenin/TCF by a different mechanism [6,7].

In contrast to other colon carcinoma (CRC) derived cell lines, Caco-2 cells undergo spontaneous differentiation expressing genes specific for small intestinal enterocytes [3,8]. Various studies have analyzed the changes in genomic and proteomic expression profiles during differentiation in Caco-2 cells or subclones [3,9–11]. In line with the differentiation process along the crypt-villi axis in vivo, genes associated with DNA synthesis, cell cycle, protein translation, and the Wnt/β-catenin are repressed, while genes involved in transport and metabolism, including phase II drug metabolizing enzymes, are induced. And genome-wide analysis of CDX2 binding in Caco-2 cells suggests that CDX2 plays indeed a key role in the differentiation of Caco-2 cells [1,5].

In this study, we show for the first time the effect of CDX2 on a genome-wide level in the spontaneous differentiation of Caco-2 cells illustrating its dual role as inhibitor of Wnt signaling at early stage and its role of direct trans-activator of intestinal genes at late stage.

2. Methods

2.1. Cell culture

Cell culture media and culture conditions were as described previously [8]. Briefly, cells were cultured at 37 °C and 5% CO_2 in Dulbecco's modified Eagle essential medium (DMEM) Glutamax, 10% FCS, 100 U/mL penicillin, and 100 μg/mL streptomycin. RNA

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http://dx.doi.org/10.1016/j.ejpb.2013.03.018

was extracted using Nucleo-Spin RNA-extraction kit from Machery-Nagel (Oensingen, Switzerland).

2.2. Real-time PCR

Real-time PCR amplification was performed using SYBR(r)-Green PCR assay on an ABI 7900 machine (Applied Biosystems, Foster City, USA) with the following thermal cycling conditions: 2 min at 50 °C, 10 min at 95 °C, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min for denaturation, annealing, and elongation. Primer probes were purchased from Sigma–Aldrich (Buchs, Switzerland). The primer sequences are listed in Supplementary files (see Supplementary Table S1). All biological samples were measured in triplicates, and the resulting data were normalized to HMBS and/or H3F3A.

2.3. Production of lentiviral stocks in HEK293FT cells and transduction of Caco-2 cells

Short hairpin RNAs (shRNA) targeting CXD2 were individually cloned into pLKO.1-puromycin vector purchased from Open Biosystem (SHGLY-NM_001265, Sigma–Aldrich, Switzerland). Lentiviral stocks were prepared using HEK293FT as packaging cells. Nearly confluent cells were transfected by the standard lipofectamine method with plasmids mix (5 μg of pLP1, 2.5 μg pLP2, 3 μg pLP/VSVG plasmids, and 10 μg of each pLKO.1-containing CDX2 shRNA inserts or pLentiV5-GFP). HEK293FT cells were grown in DMEM media supplemented with 10% FBS, nonessential amino acids, glutamine, and Na-pyruvate. For transfection, plasmid mix was diluted in 400 μL OPTIMEM; at the same time, 45 μL lipofectamine 2000 was diluted in 400 μL OPTIMEM. Following 5 min incubation at room temperature, the two solutions were combined and the mixture was added to the cells after 20 min of incubation at room temperature. The day after, the transfection mix was removed and fresh medium was added. After 48 h, the medium containing lentivirus was collected, stored at −80 °C (48 h virus medium), and new fresh medium was added to the HEK293FT cells. After 24 h, the medium containing lentivirus was collected and stored at −80 °C (72 h virus medium). One million of growing Caco-2 cells were harvested with trypsin solution, resuspended in 1 mL of 48 h virus medium plus 4 μL polybrene and seeded on p32 tissue culture dish. One hour after seeding, 1 mL of complete medium (DMEM w/o antibiotics) with 4 μL polybrene was added. Cells were cultured for 2 days in w/o changing the medium. Two days after infection, cells were harvested and seeded on p90 tissue culture dishes. In the following shCaco-2, cells were maintained in presence of puromycin (2 μg/mL).

2.4. Western blot

shRNA-transfected Caco-2 cells were seeded on PET filters at the density of 3×10^5 cells/cm^2 and collected at day 5 and 21 after seeding. Cells were then incubated in urea buffer (8 M urea, 100 mM NaH_2PO_4, and 10 mM Tris pH 8), for 5 min RT, scraped, and then briefly sonicated. Proteins were subjected to SDS–polyacrylamide gels electrophoresis. The resolved proteins were blotted overnight to nitrocellulose membranes, which then were blocked in PBS containing 5% nonfat milk for at least 1 h. Blots were incubated with the following primary antihuman antibodies: goat anti-Sucrase Isomaltase (Santa Cruz Biotechnology, USA), mouse anti CDX-2 clone CDX-88 (Biogenex, Fremont, CA, USA), and mouse anti-Hsp 72–73 clone Ab-1 (Oncogene, Cambridge, MA, USA). The membranes were then incubated for 45 min with the relevant secondary antibody (anti-mouse or anti-goat IgG (H+L)) conjugated with Alexa fluor 680 (Invitrogen, Carlsbad, CA, USA) or IRDye 800 (LI-COR Biosciences, Lincoln, NE, USA) and analyzed with the Licor Odyssey® Infrared Image System at 700 or 800 nm.

2.5. Migration and wound healing assays

Chemotaxis was assayed in 48-well Boyden microchambers (Neuro Probe, Cabin John, MD, USA). Cells were cultured in serum-free media overnight. For the migration studies, chemotaxis buffer containing 30% serum was placed in the lower wells, and 10^4 cells, suspended in chemotaxis buffer (serum-free culture media with 1% BSA), in the upper wells. A PVPF membrane (Poretics 25 × 80 mm) with 8 μm pores and coated with rat collagen type I was used to separate the two chambers. After 15 h of incubation, the membrane was removed, washed on the upper side with PBS, fixed, and stained with Diff-Quick staining kit. Migrated cells were counted at 1000-fold magnification in five randomly selected fields. Values were collected as average cell count of five high-powered fields (5 HPF). For wound healing assays, 2×10^5 cells were seeded in 12-well plates in triplicates. After 24 h, the monolayer was scratched with a collagen coated pipet tip. Scratches were monitored over 4 days and pictures were taken using a 10× objective.

2.6. BrdU incorporation

Cell cycle analysis was performed on growing cells by pulse-chase bromodeoxyuridine incorporation (BrdU, Sigma, St. Louis, MO, USA) and propidium iodide DNA staining, as previously described [12]. Briefly, for the BrdU incorporation, a pulse of 20 μM BrdU was given to the cell culture 30 min before starting the experiment. Afterward, BrdU-free medium was added to the cell culture, and samples were taken every 2 h for further 24 h. Mouse anti-BrdU (Becton–Dickinson cat# 347580, Franklin Lakes, NJ, USA) and FITC-conjugated F(Ab′)2 rabbit anti-mouse IgG (DAKO cat# F-0313, Glostrup, DK) antibodies were used to detect the BrdU incorporation. Twenty thousand events per sample were acquired by using a FACScan cytofluorimeter.

2.7. Immunofluorescence staining

Caco-2 cells were seeded on PET filter inserts and cultured for 21 days in complete medium. Localization of ZO-1 and claudin 4 was assessed by confocal laser scanning microscopy (Leica Confocal Microsystem TCS SP5, Wetzlar, DE). ZO-1 was detected using the polyclonal anti-ZO-1 antibody purchased by Zymed (cat #61-7300), while claudin-4 was detected using the polyclonal anti-Cla4 antibody purchased by Santa Cruz (cat #58898, USA). The secondary antibodies were Alexa Fluor 488 Goat antirabbit F(ab′)2 fragment antibodies; double-labeling of the nuclei was performed with 1 μg/mL DAPI in PBS. Cells were mounted with ProLong Gold anti-Fade Reagent (Molecular Probes, USA). Each image represented the maximal projection of 40-frames z-stacks; images were processed using Leica Application Suite 6000. Brightness and contrast were adjusted.

2.8. Data analysis

As described earlier [8], robust multi-array averaging (RMA) and quantile normalization were used to quantify gene expression. Significant differences were identified applying a Bayesian approach using the limma package (R 2.12.0, Bioconductor 2.7). A threshold of an adjusted P value $\leqslant 0.05$ was used to identify significant changes if not indicated otherwise. Gene set enrichment analysis (GSEA) was carried out according to Subramanian et al. [13], and P values were computed using a bootstrap distribution created by resampling gene sets of the same cardinality. Gene sets