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Induction of epithelial-mesenchymal transition with O-glycosylated oncofetal fibronectin

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ABSTRACT

Epithelial-mesenchymal transition (EMT) has been shown to play a key role in embryogenesis and cancer progression. We previously found that fibronectin (FN) carrying *O*-GalNAc at a specific site is selectively expressed in cancer and fetal cells/tissues, and termed oncofetal FN (onfFN). Here, we show that (i) a newly-established monoclonal antibody against FN lacking the *O*-GalNAc, termed normalFN (norFN), is useful for isolation of onfFN, (ii) onfFN, but not norFN, can induce EMT in human lung carcinoma cells, (iii) onfFN has a synergistic effect with transforming growth factor (TGF)β1 in EMT induction.

1. Introduction

Numerous studies have demonstrated that carbohydrates expressed as glycosphingolipids (GSLs) or N- or O-linked glycans of glycoproteins are correlated with cell phenotypes and are involved in various cell functions such as cell adhesion, signal transduction, growth, motility, and invasiveness [1–5]. In our previous studies, we demonstrated the functional role of certain carbohydrate structures in epithelial-mesenchymal transition (EMT) [6–8].

The EMT process, was initially observed during early embryogenesis and organ formation [9,10], and was demonstrated to play an essential role in disease development such as cancer progression [11–14] and fibrosis [15]. During the EMT process in cancer progression, transformed epithelial cells lose their apical-basal polarity and change to an elongated fibroblastic morphology. The cells also display reduced expression of epithelial cell markers such as E-cadherin (Ecad), concomitant with enhanced expression of mesenchymal cell markers such as vimentin, N-cadherin (Ncad), fibronectin (FN), and enhanced cell motility [11–15]. FN is known to exist in multiple forms resulting from alternative splicing and different glycosylation. The role of different FN isoforms in the EMT process remains largely unknown, although the up-regulation of FN has been widely used for the assessment of EMT induction. We previously established mAb FDC6, using FN isolated from a human cell line, HUH7 [16] as the immunogen. FDC6 reacts with FN in fetal and cancer tissue and cells, but not with FN in normal adult tissue and cells. FDC6-positive FN was therefore termed "oncofetal FN" (onfFN) and FDC6-negative FN as "normal FN" (norFN) [16,17]. Subsequent studies revealed that the epitope of FDC6 is based on the addition of an O-glycan (GalNAcα1-*O*-Ser/Thr or Galβ1-3GalNAcα1-*O*-Ser/Thr) to the Thr residue of the peptide sequence -Val-Thr-His-Pro-Gly-Tyr-, which is located at the type III homology connective segment (IIICS) domain of FN [17,18]. Our recent study [6] on the role of onfFN in EMT process demonstrated that (i) the expression of onfFN is strongly up-regulated during transforming growth factor TGFβ1-induced EMT process in human prostate cell lines and (ii) the knock-down of UDP-*N*-acetylgalactosamine: polypeptide *N*-acetylgalactosaminyltransferase (GalNAc-T)3 and GalNAc-T6 with siRNA targeted to them, abolishes the enhanced expression of onfFN without any change in total FN (tFN), and also inhibits EMT induced with TGFβ1. These results indicate the functional role of onfFN in EMT induction.

In this study, we isolated onfFN and norFN using mAb FDC6 and a newly established mAb against norFN, and examined their

Abbreviations: FN, fibronectin; EMT, epithelial-mesenchymal transition; TGF, transforming growth factor; Ecad, E-cadherin; onfFN, oncofetal FN; norFN, normal FN

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activities to induce EMT and their synergistic effects with TGFβ1 in EMT induction using human non-small cell lung carcinoma (NSCLC) cells.

2. Materials and methods

2.1. Cell culture

NSCLC cell lines, A549 and NCI-H358, were obtained from the American Type Culture Collection (ATCC, Rockville, MD), and cultured in RPMI 1640. Benign human hepatoma HUH7 cells [16], which were donated by Dr. J. Sato (Okayama Univ., Japan), and HUH7/T6 [6] were grown in DMEM. These media were supplemented with 10% FBS, penicillin (100 IU/ml) and streptomycin (100 μg/ml) unless described otherwise. All cells were cultured in a humidified chamber at 37 °C in 5% CO_2/95% air.

2.2. Reagents and antibodies

Antibodies used: anti-Ecad (IgG1; BD Biosciences, San Jose, CA), anti-vimentin (IgM; Sigma, St. Louis, MO), anti-GAPDH (IgG1; Sigma, St. Louis, MO), anti-total FN (EP5, IgG1; Santa Cruz Biotechnology, Santa Cruz, CA), mAb FDC6 (IgG1) was established in our laboratory [16], and mAb 2E11 (IgG1) for human GalNAc-T6 was kindly donated by Drs. U. Mandel and H. Clausen (University of Copenhagen, Denmark) [19]. Horseradish peroxidase (HRP)-labeled goat anti-mouse IgG and -mouse IgM were from Southern Biotech (Birmingham, AL). Human plasma FN was from Sigma and TGFβ1 was from BD Biosciences. Other reagents were from Sigma, unless described otherwise.

2.3. Production of mAb against norFN

The peptide, KTPFVTHPGYDTGNTCQC, consisting of 17 amino acid sequence within the IIICS domain of FN and an additional cysteine residue (C), was purchased from GenScript, (Piscataway, NJ). The peptide was chemically linked with the imject maleimide-activated mcKLH (Thermo, Rockford, IL) through the C, according to the manufacturer's instructions. Balb/c (6–8 weeks old) mice were intraperitoneally injected with 40 μg of the KLH-conjugated peptide emulsified in TiterMax Gold adjuvant (Titermax, Norcross, GA) twice at 2-week intervals. Seven days after the second injection, the antibody tire in serum was determined by ELISA, using the peptide-conjugated with bovine serum albumin (BSA). The mice that showed high antibody titer were given the last boost with 40 μg of FDC6-negative FN without adjuvant. Three days later, splenocytes were collected and fused with SP2/0 mouse myeloma cells and hybridomas were selected, as previously described [20]. Antibody production was screened by ELISA using total FN. After cloning by limiting-dilution, YKH1 clone was established. Its isotype was determined with IsoStrips (Boehringer Mannheim, Indianapolis, IN). All procedures with mice were approved by the Institutional Animal Care and Use Committee (IACUC).

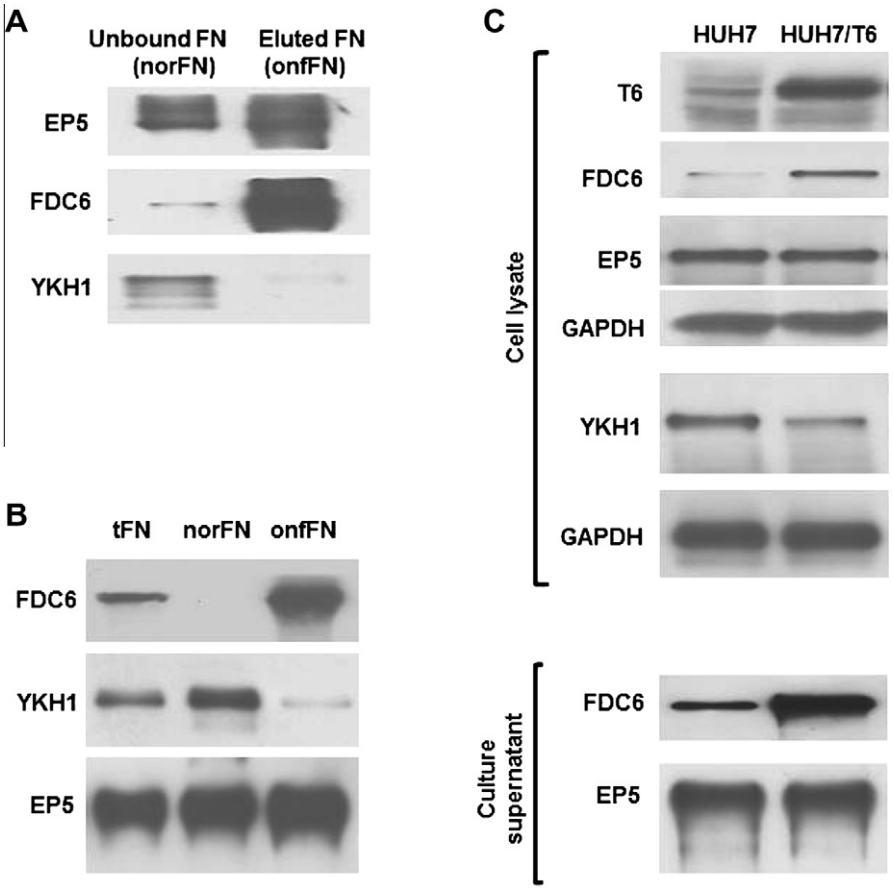

Fig. 1. Production of mouse mAb specific for norFN and preparation of onfFN and norFN using the mAb. (A) Specificity of mAb YKH1 was determined by Western blot using the unbound fraction (norFN), and the bound and eluted fraction (onfFN) from FDC6-column. Aliquots (0.1 μg protein) of each FN were analyzed with mAbs EP5, FDC6, and YKH1. (B) Separation of norFN and onfFN by immunoaffinity absorption. Aliquots (0.1 μg protein) of tFN, norFN (unbound to FDC6) and onfFN (unbound to YKH1) were analyzed by Western blot using EP5, YKH1 and FDC6. (C) Production and secretion of onfFN in HUH7 cells overexpressing GalNAc-T6 (HUH7/T6). Expression of GalNAc-T6, total FN, norFN and onfFN were detected with mAbs 2E11, EP5, YKH1 and FDC6, respectively, using cell lysates and culture supernatants.