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Betacellulin transgenic mice develop urothelial hyperplasia and show sex-dependent reduction in urinary major urinary protein content

Helene Schulz [a,b], Maik Dahlhoff [c], Aleksandra Glogowska [a], Lin Zhang [a], Georg J. Arnold [d], Thomas Fröhlich [d], Marlon R. Schneider [c], Thomas Klonisch [a,e,f,*]

[a] Dept. of Human Anatomy and Cell Science, University of Manitoba, Faculty of Health Sciences, College of Medicine, Winnipeg, MB R3E 0J9, Canada
[b] National Microbiology Laboratory, Winnipeg, Manitoba, Canada
[c] Institute of Molecular Animal Breeding and Biotechnology, Gene Center, LMU Munich, Feodor-Lynen-Str. 25, 81377 Munich, Germany
[d] Laboratory for Functional Genome Analysis LAFUGA, Gene Center, Ludwig-Maximilians-University, Munich, Germany
[e] Dept. of Medical Microbiology & Infectious Diseases, University of Manitoba, Faculty of Health Sciences, College of Medicine, Winnipeg, MB R3E 0J9, Canada
[f] Dept. of Surgery, University of Manitoba, Faculty of Health Sciences, College of Medicine, Winnipeg, MB R3E 0J9, Canada

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ABSTRACT

The epidermal growth factor (EGF)-like ligands and their cognate ERBB1–4 receptors represent important signaling pathways that regulate tissue and cell proliferation, differentiation and regeneration in a wide variety of tissues, including the urogenital tract. Betacellulin (BTC) can activate all four ERBB tyrosine kinase receptors and is a multifunctional EGF-like ligand with diverse roles in β cell differentiation, bone maturation, formation of functional epithelial linings and vascular permeability in different organs. Using transgenic BTC mice, we have studied the effect of constitutive systemic BTC over-expression on the urinary bladder. BTC was detected in microvascular structures of the stromal bladder compartment and in umbrella cells representing the protective apical lining of the uroepithelium. ERBB1 and ERBB4 receptors were co-localized in the urothelium. Mice transgenic for BTC and double transgenic for both BTC and the dominant kinase-dead mutant of EGFR (Waved 5) developed hyperplasia of the uroepithelium at 5 months of age, suggesting that urothelial hyperplasia was not exclusively dependent on ERBB1/EGFR. Mass spectrometric analysis of urine revealed a significant down-regulation of major urinary proteins in female BTC transgenic mice, suggesting a novel role for systemic BTC in odor-based signaling in female transgenic BTC mice.

1. Introduction

Epidermal growth factor (EGF) ligands bind to epidermal growth factor receptors 1 to 4 (ERBB1–4) and include epidermal growth factor, transforming growth factor alpha (TGFA), betacellulin (BTC), heparin binding EGF-like growth factor (HBEGF), amphiregulin (AREG), epiregulin (EREG), epigen (EPGN), cripto, and neuregulins 1–4 (NRG1–4) (Harris et al., 2003; Yarden and Sliwkowski, 2001; Schneider and Wolf, 2009). Initially expressed as transmembrane precursors, EGF-like ligands are proteolytically cleaved to liberate the extracellular region (Sunnarborg et al., 2002; Hinkle et al., 2004; Sahin et al., 2004). In a membrane-bound or soluble form, EGF-like ligands can bind to and activate homo- and heterodimers of the four currently known membrane-anchored tyrosine kinase receptors ERBB1 (EGFR, HER1), ERBB2 (Neu antigen, HER2), ERBB3 (HER3), and ERBB4 (HER4) (Jorissen et al., 2003). The main signal cascades activated by EGF-like ligand and EGFRs include the MAPK, AKT and JNK pathways which constitute key components in the regulation of cell proliferation, differentiation, survival, embryonic development, homeostasis, and tumorigenesis (Yarden and Sliwkowski, 2001; Waterman and Yarden, 2001; Schneider, 2014).

Betacellulin was first isolated from conditioned media of a pancreatic beta cell tumor cell line (Sasada et al., 1993). BTC is expressed in a variety of mesenchymal and epithelial cell lines and in many tissues (Seno et al., 1996; Kallincos et al., 2000). We previously described a transgenic mouse model with systemic over-expression of BTC under the control of a chicken beta actin promoter (Schneider et al., 2005). These mice present with serious pathological features including deformations of the skull, pulmonary hemorrhage syndrome and complex eye pathology (Schneider et al., 2005). We demonstrated intestinal and gastric epithelial hyperplasia in BTC over-expressing mice (Dahlhoff et al., 2008; Dahlhoff et al., 2012), identified BTC as an important new factor in bone formation in mice (Schneider et al., 2009) and provided evidence for a novel role of BTC in the regulation of epidermal homeostasis, hair follicle morphogenesis and cycling, and wound angiogenesis (Schneider et al., 2008).

* Corresponding author at: Dept. of Human Anatomy and Cell Science, Faculty of Health Sciences, College of Medicine, University of Manitoba, 130 Basic Medical Sciences, 730 William Ave., Winnipeg, MB R3E 0J9, Canada.
E-mail address: thomas.klonisch@umanitoba.ca (T. Klonisch).

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ERBB1–4 receptors and their EGF-like ligands are present in the uroepithelium of the normal urinary bladder and urothelial cancer. Several studies have linked enhanced expression of ERBB1/EGFR with high tumor stage, fast progression, and poor clinical outcome in bladder carcinoma (Chow et al., 1997; Rotterud et al., 2005; Kramer et al., 2007; Kim et al., 2005; el-Marjou et al., 2000; Naik et al., 2011). Notably, upregulation of BTC was reported in chemically induced mouse bladder cancer (el-Marjou et al., 2000).

In the present study, we assessed the expression of BTC and its receptors EGFR/ERBB1 and ERBB4 in the urinary bladder of BTC transgenic mice (Schneider et al., 2005), mice carrying the antimorphic Wa5 allele (Lee et al., 2004), and in BTC transgenic/Wa5 mouse models (Schneider et al., 2009). BTC was detected in stromal microvascular structures and in the apical umbrella cell lining of the uroepithelium. At 5 months of age, BTC and BTC/Wa5 mice showed urothelial hyperplasia suggesting that BTC signaling was not solely dependent on ERBB1/EGFR signaling. BTC was not detected in urine. However, systemic BTC over-expression coincided with significantly lower urinary content of major urinary proteins (MUPs) exclusively in female BTC transgenic mice.

2. Materials and methods

2.1. Generation and genotyping of BTC transgenic mice

BTC transgenic (tg) mice were maintained in the FVB/N background and genotyped as described previously (Schneider et al., 2005). Waved5 (Wa5) mice expressing a dominant kinase-dead EGFR mutant were provided by the Medical Research Council (Oxfordshire, UK) via Dr. David Threadgill (University of North Carolina) (Lee et al., 2004). All animal experiments were approved by the institutional animal care committee and carried out in accordance with the German Animal Welfare Act.

2.2. Tissue preparation and immunohistochemistry

Mouse urinary bladders were fixed in 4% buffered formalin and paraffin-embedded. For immunohistochemistry, tissue sections (5 μm thick) were deparaffinized by immersion in xylene for twice 10 min followed by a descending ethanol series and equilibration in tris-buffered saline (TBS) at pH 7.6. Epitope retrieval was performed by boiling in 10 mM citrate buffer at pH 6.0 for 30 min and activity of endogenous peroxidase was quenched with 3% peroxide in methanol for 20 min, with three TBS washings after each step. For the detection of BTC, tissue sections were incubated with 1:200 dilution of polyclonal goat anti-mouse BTC antibodies (AF1025; R&D Systems, Minneapolis, MN, USA) in TBS with 0.1% Tween 20 (TBST) in a humid chamber at 4 °C overnight. After three washing steps, a 1:200 dilution of biotinylated rabbit anti-goat IgG antibodies (PK-6105; Vector Laboratories, Burlingame, CA, USA) was applied for 60 min at room temperature (RT). For immunodetection of EGFR/ERBB1, ERBB4, and CD44v6, a 1:100 dilution of polyclonal rabbit antisera to EGFR (2232; Cell Signaling Technology, USA), ERBB4 (SC-283; Santa Cruz Biotechnologies, CA, USA), and CD44v6 (AB2080; Millipore, Temecula, CA, USA) were used with biotinylated goat anti-rabbit IgG secondary antibodies (BA-1000; Vector Laboratories). For the detection of Ki67 proliferation marker, a 1:100 dilution of monoclonal rat anti-Ki67 antibody (M7249; DakoCytomation, Glostrup, Denmark) was used with biotinylated rabbit anti-goat IgG secondary antibodies (PK-6105; Vector Laboratories). Sections were incubated for 30 min in avidin–biotin complex (Vector Laboratories,) and 2× DAB substrate (Thermo Scientific, Waltham, MA USA) for specific immunodetection of the antibodies used. Nonspecific binding sites were blocked for 1 h at RT with 5% normal rabbit or goat serum (Sigma, Saint Louis, MO, USA) of the same species as the secondary antibody and non-immune IgG at the same concentration as the primary antiserum was used as a negative control. Specific immunostaining was developed using the DAB kit (Pierce, IL, USA). Tissue sections were counterstained with hematoxylin and embedded prior to bright field imaging with a Zeiss M2 microscope (Zeiss, Jena, Germany). Images were captured and processed with an AxioCam camera and Zeiss Axiovision software, respectively.

2.3. Morphometric analysis

Morphometry was performed on images at ×200 magnification of H&E stained and ERBB4 immunostained urinary bladders using the AxioVision and Zen software system (Zeiss). Mice with the four genotypes were divided into three age groups: 2–4, 5–6, and 8–12 months (Table 1). The height of the uroepithelium was determined in sections located approximately medially in non-distended urinary bladders as determined by microscopic inspection. We measured the shortest distance from the basal membrane to the lumen along the grain of the tissue and excluded non-perpendicular regions. The average height of the urinary bladder epithelium was calculated as a mean of at least 500 individual measurements and up to 2150 measurements for each animal; exceptions included bladder sections of 2, 5, and 12 month old animals where only 251, 136, and 386 measurements could be obtained, respectively. The weighted mean and standard deviation for each age group was calculated and results are presented as dot plots.

2.4. Protein preparation from mouse urine samples

To determine if the increased BTC expression in BTC transgenic mice resulted in detectable BTC levels and changes of urinary proteins in urine, we collected urine from three wild type and three tg BTC males at 2 months of age and four 8 month old female mice of the same genotypes for protein analysis. For mass spectrometry, urine samples (10 μl) were diluted with 12.5 μl 1× Laemmli buffer and filled up with 2.5 μl 5× Laemmli buffer to a final volume of 15 μl. The diluted urine samples were separated by a 12% SDS-PAGE and stained with Coomassie-Brilliant-Blue-R (Sigma). Every protein lane of the gel was cut into several slices for trypsin digestion. Gel slices were equilibrated twice with 50 mM NH_4HCO_3 for 10 min and reduced with 45 mM dithiothreitol for 30 min at 55 °C. Cysteine residues were blocked with 100 mM iodoacetamide for 30 min at RT, washed twice for 15 min in 50 mM NH_4HCO_3, minced and subjected to overnight digestion at 37 °C with 1 μg porcine trypsin (Promega, Madison, WI, USA) per slice. The supernatant was preserved and peptides were further extracted by additional washes with 50 mM NH_4HCO_3 and 80% acetonitrile (ACN). The ACN supernatant and the NH_4HCO_3 fractions were combined and concentrated in a SpeedVac concentrator (Bachofer, Reutlingen, Germany) (Table 2).

2.5. Mass spectrometry (MS) analysis

LC–MS/MS analyses were performed with a nano-liquid chromatography system (Ettan MDLC; GE Healthcare, Munich, Germany) coupled to a linear ion trap mass spectrometer (LTQ, Thermo Fisher Scientific, MA, USA). Tryptic peptide solutions were reconstituted in 0.1% formic acid, injected onto a C18 trap column (C18 PepMap100, 5 μm particle size, 100 Å, 300 μm × 5 mm column size; LC Packings Dionex, Sunnyvale, CA, USA) and subsequently separated by RP chromatography using an analytical column (ReproSil-Pur C18 AQ, 3 μm; 150 mm × 75 μm, Dr. Maisch, Ammerbuch-Entringen, Germany). Solvent A consisted of

Table 1
Age, gender and phenotype of animals included in this study.

Age (month)	Gender	Number of animals	Genotype			
			wt	BTC	Wa5	BTC/Wa5
2–4	Female = 4; male = 5	9	5 (f = 2; m = 3)	4 (f = 2; m = 2)	0	0
5–6	Female	17	3	7	3	4
8–12	Male	17	4	7	3	3