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Disruption of Paneth and goblet cell homeostasis and increased endoplasmic reticulum stress in *Agr2*−/− mice

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ABSTRACT

Anterior Gradient 2 (AGR2) is a protein disulfide isomerase that plays important roles in diverse processes in multiple cell lineages as a developmental regulator, survival factor and susceptibility gene for inflammatory bowel disease. Here, we show using germline and inducible *Agr2*−/− mice that *Agr2* plays important roles in intestinal homeostasis. *Agr2*−/− intestine has decreased goblet cell Mucin 2, dramatic expansion of the Paneth cell compartment, abnormal Paneth cell localization, elevated endoplasmic reticulum (ER) stress, severe terminal ileitis and colitis. Cell culture experiments show that *Agr2* expression is induced by ER stress, and that siRNA knockdown of *Agr2* increases ER stress response. These studies implicate *Agr2* in intestinal homeostasis and ER stress and suggest a role in the etiology of inflammatory bowel disease.

Introduction

Anterior Gradient 2 (*AGR2*) is a developmental regulator and survival factor with multiple functions that, although highly conserved in evolution, remain poorly characterized. Originally characterized in *Xenopus* laevis, heterologous *AGR2* expression induces ectopic cement gland differentiation and alters neuronal cell fate (Aberger et al., 1998). In multiple mammalian cells, *AGR2* stimulates cell proliferation, cell adhesion, motility and inhibits apoptosis (Fritzsche et al., 2006; Innes et al., 2006; Wang et al., 2008; Zhang et al., 2007). *AGR2* mRNA and protein occur at high levels in several metastatic adenocarcinomas, e.g., colorectal, esophagus, prostate, and breast, where it stimulates proliferation and inhibits apoptosis (Fritzsche et al., 2007; Fritzsche et al., 2006; Innes et al., 2006; Smirnov et al., 2005; Valladares-Ayerbes et al., 2008; Wang et al., 2008; Zhang et al., 2007). In contrast, human *AGR2* genetic variants that decrease its expression are associated with increased risk of both Crohn's disease and ulcerative colitis (Zheng et al., 2006).

Structurally, *AGR2* is a member of the endoplasmic reticulum (ER) protein disulfide isomerase (PDI) gene family (Park et al., 2009; Persson et al., 2005). PDIs facilitate the isomerization of specific client proteins into their bioactive conformations as they traffic through the ER for secretion or membrane association. When PDI substrates fail to isomerize, misfolded membrane-associated and secreted proteins accumulate in the ER, initiating ER stress, cell cycle arrest and apoptosis. For example, *AGR2* binds directly to Mucin 2 (Muc2), a major component of intestinal mucus enabling the large number of Muc2 Cys residues to pair correctly. Germline deletion of mouse *Agr2* disrupts Muc2 protein stability and decreases intestinal mucus production. However, *Agr2* deficiency is reported only to cause modest ER stress in the intestine and the impact of *Agr2* deficiency on the biology and functional state of additional intestinal cell lineages is poorly characterized (Park et al., 2009).

The lower gastrointestinal tract consists of the small intestine and colon. The lining epithelium of both organs is organized into crypts. Undifferentiated stem and progenitor cells populate the base of small intestine crypts and give rise to four major cell types: absorptive enterocytes, goblet cells, Paneth cells and enteroendocrine cells (Cheng and Leblond, 1974). Enterocytes absorb nutrients, secrete hydrolytic enzymes into the intestinal lumen and are the most

Abbreviations: AGR2, Anterior Gradient 2; ER, endoplasmic reticulum; PDI, protein disulfide isomerase; BrdU, bromodeoxyuridine.

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common intestinal cell type. Goblet cells produce mucous to protect the intestinal barrier from infiltration by luminal contents. Paneth cells protect against microbial infection and are located at the crypt bottom. Enteroendocrine cells secrete a variety of hormones and are relatively rare. Colonic crypts contain three major cell types, including a higher proportion of goblet cells, somewhat fewer absorptive cells, and scattered enteroendocrine cells.

In normal intestine, *AGR2* is expressed in several cell types. These include mature goblet, Paneth and enteroendocrine cells, as well as Musashi-1 (MSI1)-positive intestinal stem/early progenitor cells and proliferating secretory progenitors (Wang et al., 2008).

Intestinal epithelial cells are sensitive to ER stress. Targeted deletion in the mouse of the ER stress response transcription factor XBP1s, the intestine-specific ER stress response sensor IRE1β, or mice carrying missense mutations that impair Muc2 oligomerisation and protein stability of Muc2, cause intestinal cell ER stress, cell cycle arrest, apoptosis and chronic inflammation (Bertolotti et al., 2001; Heazlewood et al., 2008; Kaser et al., 2008). Therefore, based on its structure and expression pattern, it was anticipated that *AGR2* might play a role in the intestinal ER stress response and suppression of inflammation.

Here, we show using germline and inducible *Agr2* knockout mice and siRNA knock down in cell culture that *Agr2* plays important roles in intestinal goblet and Paneth cell homeostasis, Paneth cell positioning and intestinal ER stress response. These data expand the known roles played by *Agr2* in homeostasis of the intestine.

Materials and methods

Generation of Agr2−/− mice

The gene-targeting strategy is described in supplementary methods and summarized in Fig. 1. Mice were housed in a temperature (~21 °C) and humidity (~55%)-controlled room in an AALAC accredited vivarium with a 12 h light:12 h dark cycle. Entry into the vivarium was restricted to appropriate personnel, but no SPF precautions were available. Food pellets (Research Diets, New Jersey) and water was available *ad libitum*.

Histology, immunohistochemistry and immunofluorescence

Mouse intestines were removed and washed in cold phosphate-buffered saline (PBS), fixed by immersion in 4% paraformaldehyde, embedded in paraffin, and 5 μm sections were applied to Probe-on Plus™ slides (Fisher). For histology review, sections were stained with hematoxylin and eosin or Alcian blue and nuclear fast red as detailed in supplementary methods. For immunohistochemistry, primary antibodies and according biotinylated secondary antibodies were using. Staining was detected with Vectastain ABC reagent (PK-6100, Vector) and development with DAB chromogen (DakoCytomation). For immunofluorescence, primary antibodies and according Texas red or fluorescein labeled secondary antibody were applied, then slides were covered with Vectashield mounting medium with DAPI (H-1200, Vector). For details and sources of antibodies, please refer to supplementary methods.

BrdU labeling proliferation assay

To test proliferation, mice were injected 2 h before sacrifice with BrdU labeling reagent. Tissues were processed and the BrdU labeling cell proliferation assay were performed according to the manufacturer's protocol (BrdU labeling and Detection Kit I, Roche).

Epithelial RNA isolation and quantification

Sheets of small intestinal epithelium were separated from underlying lamina propria using 3 mM EDTA, 0.5 mM DTT in PBS (Whitehead et al., 1993). Briefly, mouse intestine was removed, opened and washed in PBS 3 changes. The linearized intestine was decontaminated by soaking in 0.04% (vol/vol) sodium hypochlorite in PBS for 20 min at room temperature, then incubated in 3mM EDTA plus 0.5mM DTT in PBS at 22 °C for 90 min with occasional gentle stirring. The digestion mixture of the intestine was resuspended in PBS and epithelium detached by vigorous shaking in a sealed container. Intestinal epithelium was harvested by centrifuge at 1000 rpm for 5 min. Total RNA isolated using Qiagen RNAeasy columns was reverse transcribed to cDNA by using cDNA Kit (Applied Biosystems). qRT-PCR was performed using an ABI 7900HT-SequenceDetection System with SYBR Green Master Mix. For quantitative analysis, all samples were normalized to *Gapdh* expression using the ΔΔCT value method. Gene-specific primers refer to supplementary methods.

Western blot

Samples were separated by SDS/PAGE and transferred to Immobilon-P PVDF (Millipore). Membranes were blocked with 5% nonfat dry milk and incubated overnight at 4 °C with primary antibodies. Band was detected by chemiluminesence using HRP-conjugated secondary antibody and ECL Western blotting reagents (GE Healthcare).

Purification and analysis of Cryptdin peptides

Cryptdin peptides were isolated using modified procedures described previously (Mastroianni and Ouellette, 2009; Selsted et al., 1992). Ilea were excised from seven inducible *Agr2−/−* mice (after 4 days of tamoxifen administration) and seven wild type mice (also received tamoxifen administration for 4 days). Protein extracts were prepared from "complete" ileum, consisting of tissue plus luminal contents. Details refer to supplementary methods.

XBP1 splicing assay

XBP1 splicing was measured by specific primers (Kaser et al., 2008) flanking the splicing site yielding PCR product sizes of 164 and 138 bp for human *XBP1u* and *XBP1s*, and 171 and 145 bp for mouse *Xbp1*. Products were resolved on 2% agarose gels and band intensity was determined densitometrically.

Cell line and small interfering RNA (siRNA) transient transfections

Pancreatic cancer cell line, Su.86.86 (American Type Culture Collection) and ON-TARGETplus SMARTpool Human siRNA-*AGR2* and Non-targeting Pool control scrambled siRNA (siRNA-Scr) (Dharmacon, Inc.) were used. Details are described in supplementary methods.

SuperArray screening

Epithelial RNA was converted to cDNA using RT² first strand kit (SABioscience, C-03). Real-time PCR was done using SuperArray RT² SYBR Green qPCR Master Mix (SABioscience, PA-012-12) and SuperArray RT² Profiler™ PCR Array (PAMM-077). Thermal cycling parameters were 95 °C for 10 min, followed by 40 cycles of amplifications at 95 °C for 15 s, 60 °C for 60 s. Data were analyzed by PCR Array Data Analysis Web Portal using the default set format.

In situ hybridization

Mouse ileum was fixed in 4% paraformaldehyde at 4 °C overnight, then transferred to 30% sucrose solution and incubated at 4 °C overnight, finally frozen in OCT compound and stored at -80 °C. *In situ*