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Intestinal lineage commitment of embryonic stem cells

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ABSTRACT

Generating lineage-committed intestinal stem cells from embryonic stem cells (ESCs) could provide a tractable experimental system for understanding intestinal differentiation pathways and may ultimately provide cells for regenerating damaged intestinal tissue. We tested a two-step differentiation procedure in which ESCs were first cultured with activin A to favor formation of definitive endoderm, and then treated with fibroblast-conditioned medium with or without Wnt3A. The definitive endoderm expressed a number of genes associated with gut-tube development through mouse embryonic day 8.5 (Sox17, Foxa2, and Gata4 expressed and Id2 silent). The intestinal stem cell marker Lgr5 gene was also activated in the endodermal cells, whereas the Msi1, Ephb2, and Dcamkl1 intestinal stem cell markers were not. Exposure of the endoderm to fibroblast-conditioned medium with Wnt3A resulted in the activation of Id2, the remaining intestinal stem cell markers and the later gut markers Cdx2, Fabp2, and Muc2. Interestingly, genes associated with distal gut-associated mesoderm (Foxf2, Hlx, and Hoxd8) were also simulated by Wnt3A. The two-step differentiation protocol generated gut bodies with crypt-like structures that included regions of Lgr5-expressing proliferating cells and regions of cell differentiation. These gut bodies also had a smooth muscle component and some underwent peristaltic movement. The ability of the definitive endoderm to differentiate into intestinal epithelium was supported by the vivo engraftment of these cells into mouse colonic mucosa. These findings demonstrate that definitive endoderm derived from ESCs can carry out intestinal cell differentiation pathways and may provide cells to restore damaged intestinal tissue.

1. Introduction

The intestinal epithelium is a highly dynamic tissue constituted of a number of specialized epithelial cells characterized by high rates of cell turnover (van der Flier and Clevers, 2009). In addition, the intestinal mucosa is highly active immunologically, with epithelial cells playing a critical barrier function and facilitating the movement of antigens, antibodies, and anti-microbial proteins into and out of the intestinal lumen (Brandtzaeg, 2009). There are many instances in which damage to the intestinal epithelium leads to a breakdown in its digestive, absorptive, and barrier functions. These include conditions such as ulcerative colitis, Crohn's disease, and celiac disease, as well as damage induced by radiation and chemotherapies. Developing new approaches to save or restore intestinal tissue could therefore find a broad range of applications.

During embryonic development the early gut tube is comprised of an endodermal cells surrounded by visceral mesoderm (Zorn and Wells, 2009). The endoderm differentiates into the epithelial lining of the gut, while mesodermal cells ultimately develop into smooth muscle and other cell types of the intestinal wall. Signaling between the mesoderm and endoderm in the developing gut through Hedghog, Wnt, FGF, and other pathways helps establish anterior/posterior patterning and the radial differentiation characteristics of the gut (van der Flier and Clevers, 2009; Ormestad et al., 2006; Beck et al., 2000; Haegebarth and Clevers, 2009; Pinto and Clevers, 2005). Within the adult intestine, epithelial cells are maintained through the presence of stem cells located within epithelial crypts (van der Flier and Clevers, 2009; Pinto and Clevers, 2005; Barker et al., 2008). The survival, proliferation and multipotency of adult intestinal stem cells are maintained in a niche region where trophic factors are provided by adjacent mesenchymal cells, notably subepithelial myofibroblasts (Yen and Wright, 2006; Brittan and Wright, 2002). The intestinal stem cell niche supports active production of Wnt ligands, which play a central role is maintaining intestinal epithelium. A better understanding of how the intestinal epithelium is generated and maintained could

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provide important insight into how this tissue might be effectively repaired and restored following damage induced by chronic inflammatory conditions or cancer therapy.

Cell turnover and lineage tracing analyses have identified stem cell populations within the intestinal crypts. These cells can be identified by their expression of a number of markers, including Lgr5. Lgr5 was first identified as a potential intestinal stem cell marker by virtue of its regulation by the Wnt/APC/β-catenin pathway (Morin et al., 1997). It was subsequently shown to be expressed in a limited number of columnar cells present at the crypt base, and lineage tracing supports the view that Lgr5 activation preceded cellular differentiation to all epithelial cell lineages (Barker et al., 2007). Moreover, isolated Lgr5-positive cells from the intestine can give rise to crypt-like structures in culture (Sato et al., 2009). Other markers that have been put forward as intestinal stem cell markers include Msi1, Ephb2, and Dcamkl1 (Samuel et al., 2009; Nishimura et al., 2003; Potten et al., 2003; Batlle et al., 2002; May et al., 2008, 2009). All of these proteins are expressed in cells near the base of the crypt. Interestingly, while Dcamkl1-positive cells have been shown to generate glandular epithelial structures in athymic mice, they are quiescent within the crypts whereas Lgr5 cells are proliferative. The usefulness of these markers for guiding the differentiation and lineage commitment of stem cells in culture is not entirely clear.

An important goal for regenerative biology is to understand signaling pathways involved in tissue development and maintenance, and then exploit this knowledge to generate cells for tissue and organ repair. Since gut epithelial cells are derived from embryonic endoderm, we were interested in determining whether directing embryonic stem cell (ESC) development to an endodermal lineage could provide a source of cells for generating intestinal tissue. Here we provide evidence that induction of endoderm differentiation results in activation of the intestinal stem cell marker Lgr5, but not Dcamlk1, Msi1, or Ephb2. Interestingly, exposure of these cells to fibroblast conditioned medium with Wnt3A promotes the expression of the other stem cell markers, and leads to the expression of a range of genes associated with early intestinal development. In addition, the endoderm cells we generate are capable of associating with mouse intestinal mucosa and forming crypt-like structures. These characteristics are consistent with intestinal lineage commitment and a potential reparative capacity of these cells. We propose that endoderm-committed cells are primed to generate mucosal tissue and are therefore well-suited to study this differentiation pathway. These cells may ultimately be useful for repairing damaged intestinal tissue resulting from various pathologies and treatments.

2. Materials and methods

2.1. ESC maintenance

C57BL6/N, GFP transgenic mouse ESCs (GlobalStem, Rockville, MD) were maintained on primary mouse embryo fibroblasts (MEFs) in DMEM media supplemented with 15% fetal bovine serum, 1% nucleosides, 1% Pen-Strep, 1% non-essential amino acids, 1% L-glutamine, 1% β-mercaptoethanol and 1000 units/mL ESGRO® (LIF) at 37 °C, 5% CO_2. All the reagents were Chemicon EmbryoMax ES Cell products and purchased from Millipore Corporation (Billerica, MA). H9 human ESC (WA09) were maintained on MEFs in DMEM-F12 media supplemented with 20% knockout serum replacer, 1 mM (0.5%) L-glutamine, 0.1 mM (1%) non-essential amino acids, 0.55 mM β-mercaptoethanol and 4 ng/mL bFGF (all from Invitrogen). Human ESCs were mechanically passaged using 1.0 mg/mL (1.0%) collagenase (Gibco) and washed 3 × with PBS prior to induction of differentiation.

2.2. Preparation of conditioned media

Mouse fibroblast L cells and L cells expressing Wnt3A were purchased from ATCC (Manassas, Virginia) and maintained in DMEM with or without 0.4 mg/mL G-418, respectively. Control conditioned medium and Wnt3A conditioned medium were prepared according to the ATCC protocol.

2.3. Activin A induced differentiation and conditioned media-induced differentiation

Media and reagents not otherwise specified were purchased from Invitrogen (Carlsbad, CA). Mouse embryoid bodies (EBs) were formed from undifferentiated ESCs in serum-free differentiation media (SFD) containing 75% IMEM, 25% Ham's F12 media supplemented with 0.5X N2 supplement, 0.5X B27 supplement, 1% Pen-Strep, 2 mM L-glutamine, 0.05% BSA (Sigma-Aldrich, St. Louis, MO), 0.5 mM ascorbic acid (Sigma-Aldrich), and 0.45 mM 1-thioglycerol (Sigma-Aldrich). Activin A (R&D systems, Minneapolis, MN) was supplemented in SFD to a final concentration of 50 ng/mL on day 2 and day 4 when changing the medium. Definitive endoderm EBs were either harvested on day 6 or subjected to further treatment with conditioned media. Definitive endoderm bodies from day 6 were collected and resuspended in SFD medium supplemented with 10% FBS and 30% control conditioned medium or Wnt3A conditioned medium. Media were changed every 2 days. The γ-secretase inhibitor, N-[N-3,5-Difluorophenacetyl]-L-alanyl-S-phenylglycine Methyl Ester (DAPM; Calbiochem, San Diego, CA) was used from day 12 to day 14 at a final concentration of 10 μM. Differentiation of H9 was initiated under serum-free induction conditions for 2 days in RPMI1640 media (Gibco) containing GlutaMAX (Invitrogen), 100 ng/mL activin A (R&D Systems), and 25 ng/mL Wnt3A (R&D Systems, Minneapolis, MN) to induce expression of mesendoderm markers as per D'Amour et al. (2005). Removal of Wnt3a and addition of 0.5% FBS and the PI3kinase inhibitor LY294002 (Biomol, Plymouth Meeting, PA) for 2 additional days induced definitive endoderm gene expression and repression of extra-embryonic endoderm markers as per McLean et al. (2007).

2.4. Real-time quantitative PCR

Total RNAs were extracted using TRIzol® Reagent (Invitrogen, Carlsbad, CA) following the manufacturer's instruction. cDNAs were synthesized with high capacity cDNA reverse transcription kit (Applied Biosystems, Foster city, CA) and stored at −20 °C until using. TaqMan gene expression assays were purchased from Applied Biosystems. A 10 μl reaction mixture containing Taqman 2X PCR master mixture, 80 ng cDNA, and 2 μl Taqman gene expression assays was prepared for real-time quantitative PCR. Reactions were performed for 40 cycles (50 °C 20 s, 95 °C 15 s, and 60 °C 1 min) using an Applied Biosystems 7300 Fast Real-time PCR system.

2.5. Animals

Male C57BL/6 mice were purchased form Taconic (Germantown, NY, USA). Mice were maintained in a temperature-controlled, light-cycled room, and allowed free access to drinking water and standard diet (LM-485, Harlan Teklad). Animals were weighed every day and mice were checked daily for signs of weight loss or lethargy indicating intestinal obstruction or anemia associated with tumors. Animal experiments were conducted with approval from the Center for Laboratory Animal Care committee, University of Connecticut Health Center.