E2f4 is required for normal development of the airway epithelium

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Abstract

The airway epithelium is comprised of specialized cell types that play key roles in protecting the lungs from environmental insults. The cellular composition of the murine respiratory epithelium is established during development and different cell types populate specific regions along the airway. Here we show that *E2f4*-deficiency leads to an absence of ciliated cells from the entire airway epithelium and the epithelium of the submucosal glands in the paranasal sinuses. This defect is particularly striking in the nasal epithelium of *E2f4−/−* mice where ciliated cells are replaced by columnar secretory cells that produce mucin-like substances. In addition, in the proximal lung, *E2f4* loss causes a reduction in Clara cell marker expression indicating that Clara cell development is also affected. These defects arise during embryogenesis and, in the nasal epithelium, appear to be independent of any changes in cell proliferation, the principal process regulated by members of the E2f family of transcription factors. We therefore conclude that *E2f4* is required to determine the appropriate development of the airway epithelium. Importantly, the combination of no ciliated cells and excess mucous cells can account for the chronic rhinitis and increased susceptibility to opportunistic infections that causes the postnatal lethality of *E2f4* mutant mice.
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Introduction

The E2f family of transcription factors has primarily been implicated in the regulation of genes required for proliferation and passage through the cell cycle (Attwooll et al., 2004; Dimova and Dyson, 2005). To date, nine members of the E2f family have been identified. These can be divided into a number of subgroups based on differences in both their regulation and transcriptional activity. Notably, individual E2f subgroups appear to be predominantly involved in either the repression or activation of E2f-responsive genes. E2f4 and 5 appear to function primarily as transcriptional repressors and their nuclear localization seems to be dependent upon their association with members of the pocket protein family that includes the retinoblastoma protein (pRb) tumor suppressor, p107 and p130

(Apostolova et al., 2002; Gaubatz et al., 2001; Rayman et al., 2002; Verona et al., 1997). E2f5 specifically associates with p130 (Hijmans et al., 1995; Sardet et al., 1995), while E2f4 binds to all three of the pocket proteins *in vivo* (Moberg et al., 1996). The resultant E2f/pocket protein complexes can recruit histone deacetylases (HDACS) to the promoters of E2f-responsive genes to enforce their active repression (Giangrande et al., 2004; Rayman et al., 2002; Takahashi et al., 2000; Wells et al., 2000). In response to mitogenic signaling, the pocket proteins are phosphorylated by the cyclin dependent kinases (Dynlacht et al., 1994; Knudsen and Wang, 1997; Rubin et al., 2005; Xiao et al., 1996). This causes the repressive E2f/pocket protein complexes to dissociate and E2f4 and 5 are exported to the cytoplasm.

E2f4 is the most abundant E2f family member *in vivo*, so its loss was expected to cause de-repression of E2f-responsive genes. However, E2f4-deficient MEFs have no detectable defects in either target gene or cell cycle regulation (Humbert et al.,

2000; Landsberg et al., 2003; Rempel et al., 2000). Since the combined loss of E2f4 and 5 can impair these processes, this likely reflects compensation by E2f5 (Gaubatz et al., 2000). Notably, *E2f4* mutant mice do exhibit several defects including embryonic anemia, defects in red blood cell maturation, altered craniofacial morphology, reduced fertility, chronic rhinitis and poor postnatal survival (Humbert et al., 2000; Kinross et al., 2006; Rempel et al., 2000). The neonatal lethality of the *E2f4* mutants is caused by the chronic rhinitis and associated opportunistic bacterial infections (Humbert et al., 2000). Adoptive transfer experiments indicated that this was not due to a defect in the immune system of *E2f4* mutant mice (Humbert et al., 2000). The chronic rhinitis was thought to result from reduced or blocked drainage of mucus from the nasal cavity but the underlying cause of this was unclear.

To understand the cause of the chronic rhinitis in the *E2f4* mutant mice, we have investigated the development of the nasal passages and, in particular, the epithelium that normally plays key roles in preventing bacterial infections of the upper respiratory tract. Most studies of respiratory epithelia growth and development have looked at the trachea and more distal areas of the lung (Perl and Whitsett, 1999; Rawlins and Hogan, 2006). Fewer studies have examined development of the nasal respiratory epithelium and these studies largely address the response of the epithelium to environmental insults (Harkema et al., 2006). The nasal respiratory epithelium is predominantly comprised of ciliated columnar cells, goblet cells and basal cells. Coordinated beating of cilia moves mucus towards the esophagus removing trapped or absorbed particulate matter, allergens, toxic chemicals and pathogens from the air prior to its passage into the lungs. Goblet cells produce mucins, a component of mucus, both constitutively and in an inducible manner following various environmental stimuli (Perez-Vilar et al., 2003; Rogers, 1994). This induction can occur at both the level of secretion and the cellular level, i.e. an increase in the population of mucin secreting cells. It is unclear what the precursor cells for ciliated or goblet cells are: basal cells were proposed to fulfill this role; however, recent studies challenge this model (Evans et al., 2001; Rawlins and Hogan, 2006). Here we show that mutation of *E2f4* results in airway epithelia that lack cilia and this can occur independently from any detectable defect in cell proliferation indicating that *E2f4* plays a key role in determining the cellular composition of the airway epithelium.

Materials and methods

Animal maintenance and histological analyses

The generation of the *E2f4* mutant mouse strain and genotyping protocols have been described previously (Humbert et al., 2000). In brief, the first five exons (apart from the first four codons) encoding the DNA binding and dimerization domains were deleted and replaced with stop codons in all three reading frames and a *neo* resistance cassette by homologous recombination in embryonic stem cells. This resulted in a recessive null mutation in *E2f4*. No phenotypes have been detected in mice heterozygous for this mutant allele in comparison with wild-type mice. For this study, mice were maintained on a mixed C57BL/6×129Sv background in a conventional facility. All animal procedures followed protocols approved by the Institute's Committee on Animal Care. Gestation was dated by detection of a vaginal plug. For analyses of cell

proliferation, pregnant mice were injected with 10 µl/gm body weight of 6 mg/ml 5-Bromo-2′-deoxyuridine (BrdU) in phosphate buffered saline (PBS) 2 h prior to tissue collection. Collected embryo tissues were fixed overnight at room temperature in either Bouin's Fixative, 3.7% formaldehyde in PBS or at 4 °C overnight in PBS containing 4% paraformaldehyde as appropriate and then dehydrated via an ethanol series prior to embedding in paraffin for sectioning. For each embryo, at least three different levels along the proximal-distal or dorsal-ventral axis were examined. From each level, one section was stained with hematoxylin and eosin (HNE) using standard procedures and adjacent sections (all 5 µm) stained as described below.

Histochemistry and immunohistochemistry

For all procedures, slides were re-hydrated through an ethanol series following de-waxing in xylenes and rinsed in water or PBS as required. For the periodic acid, Schiff (PAS) reaction slides were incubated in 0.5% periodic acid for 5 min, rinsed in water, incubated in Schiff reagent (Poly Scientific) for 15 min, washed in tap water for 10 min, counterstained with Harris hematoxylin and mounted using standard protocols. For the Alcian blue pH 2.5 staining, slides were incubated in 3% acetic acid for 3 min then in Alcian blue solution (Alcian Blue 8GK 1% w/v, 3% acetic acid v/v, pH 2.5) for 45 min at room temperature, washed in tap water for 5 min, counterstained with Harris hematoxylin and mounted using standard protocols.

Immunohistochemistry was performed using the following mouse monoclonal antibodies: acetylated α-tubulin (1:8000 T6793, Sigma), BrdU and Ki67 (1:50 347580 and 550609, BD biosciences), PCNA (1:2000 sc56, Santa Cruz), cytokeratin 8 (1:100 PRO61038, RDI), Foxa1 and Foxj1 (1:1000 WMAB2F83 and 319, Seven Hills Bioreagents), p63 (1:500 sc8431, Santa Cruz), E2f4 (1:1 LLF4.2 Moberg et al., 1996), MUC5AC (1:100, MS-145, LabVision), CC10 (1:100 goat polyclonal sc9772, Santa Cruz), SP-C (1:100 rabbit polyclonal sc13979 and goat polyclonal sc7706, Santa Cruz) and T1α (1:500 hamster monoclonal #8.1.1, Developmental Studies Hybridoma Bank, University of Iowa). Slides were washed in PBS 0.15% Triton X-100 followed by inactivation of endogenous peroxidases by incubation with 3% H_2O_2 in PBS or 0.5% H_2O_2 in methanol (Ki67). Antigen retrieval was performed either by heating for 20 min in 10 mM sodium citrate, 0.05% Tween 20, pH 6.0 in a boiling water bath or by heating in an 800 W microwave for 6.5 min at full power followed by three rounds of 5 min at 60% power using a solution 8.2 mM sodium citrate, 1.8 mM citric acid, pH 6.0 (Ki67, p63, E2f4 and Foxj1). Slides were blocked with PBS containing 5% of the appropriate serum and incubated overnight with the primary antibody diluted in PBS 0.15% Triton X-100 or this buffer alone or a non-specific antiserum as controls. Secondary antibodies (Vectastain ABC kits, Vector laboratories) were diluted 1:200 in PBS containing 0.4% of the appropriate blocking serum and detected using a DAB substrate following the manufacturers instructions (Vector Laboratories). A MOM kit (Vector Laboratories) was used according to the manufacturers instructions for PCNA and MUC5AC staining and an UltraVision LP Detection System (Lab Vision Corporation) for Foxj1 and E2f4 staining. BrdU immunohistochemistry was performed as described previously (Tsai et al., 2002). Following the detection reaction, slides were counterstained with Harris hematoxylin and mounted using standard protocols. In some cases slides were stained with Alcian Blue pH 2.5 prior to counter staining. For each marker analyzed, unless stated otherwise, a minimum of four pairs of control (wild-type or *E2f4*+/−) and *E2f4*−/− littermate embryo sections matched for their position along the proximal-distal axis were stained and, unless stated otherwise, all scored with the described phenotype.

To assess the levels of proliferation, control and *E2f4*−/− sections from littermates matched for their level along the proximal-distal axis were selected and the percentage of positive nuclei was determined by counting 100–600 (depending on the stage of development) nuclei within the respiratory epithelium per section. For each of the indicated gestational stages, four to eight pairs of embryos were analyzed for BrdU, Ki67 and PCNA. Images were captured on a Nikon Eclipse E600 using a SPOT RTdigital camera.

Transmission electron microscopy

Tissue samples were fixed directly in a solution 2.5% gluteraldehyde, 2.5% formaldehyde, 0.1 M cacodylic acid pH 7.2 for 60–90 min at 4 °C. Samples were then postfixed with 2% osmium tetroxide for 60 min, dehydrated via an