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Transgenic Mouse Reporter to Study Muc5b *In Vivo*

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Competing interests

The authors declare that they have no competing interests.

Running title: Transgenic reporter mice to study Muc5b *in vivo*

Abstract
Dysregulation of gel-forming mucins is associated with many airway diseases. Better knowledge of the pathophysiological mechanisms linking mucins and respiratory diseases will advance the understanding of their pathogenesis and should provide opportunities to develop new therapeutic compounds for treatment. MUC5B and MUC5AC are the two main gel-forming mucins in the respiratory tract. The organization in domains and the expression profile of mouse Muc5b are very similar to those in humans, which makes the mouse a relevant model for studies of the translational activities of human mucins. To assess the *in vivo* biological functions of Muc5b, a mouse reporter tagged in frame with the green fluorescent protein marker has been engineered by homologous recombination. The proof of concept that this reporter model may be informative for translational studies was confirmed by the finding that interleukin 13 administration in living mice upregulated Muc5b production.

Keywords: airways; mucus; mucins; Muc5b–GFP; interleukin 13; GFP live imaging

The mucus gel that covers airway tissues is a highly heterogeneous mixture made mainly of water (>90%) and gel-forming mucins. These mucins are large and complex molecules, and are encoded in humans by five genes: *MUC2*, *MUC5AC*, *MUC5B*, *MUC6*, and *MUC19*. These genes are highly conserved in the mouse and are designated as *Muc2*, *Muc5ac*, *Muc5b*, *Muc6*, and *Muc19*, respectively. The four genes *MUC2*, *MUC5AC*, *MUC5B*, and *MUC6* belong to a cluster of genes on human chromosome 11 band p15.5 (1). The genomic order *MUC6/2/5AC/5B* and arrangement of the four mucin genes are conserved on mouse chromosome 7 band F5 (2). *Muc6* is transcribed from the telomere to centromere and with its orientation opposite to that of the three other mucin genes. A fifth gel-forming mucin, *MUC19*, is located on human chromosome 12 band q12, and its mouse orthologue is located on chromosome 15 band F1 (3).

These mucins are produced by specialized cells, such as goblet cells, and are stored intracellularly before being released as long polymers, which form more complex structures once in contact with water at the surface of secretory epithelial cells (4). Gel-forming mucins are multidomain proteins that are highly *O*-glycosylated. Their organization in domains has been

deduced from cDNA nucleotide sequences. Human *MUC5B* cDNA and genomic sequences have been fully determined (5–8). Errors in detecting nucleotide sequences and gaps in knowledge about some mucin nucleic sequences may give rise to fallacious interpretations of biological studies.

Many diseases of the secretory epithelium are associated with goblet cell hyperplasia and metaplasia, and an increased production of mucins. However, a decrease in goblet cell density can also be a hallmark of some pathological situations such as dry eye disease for which the goblet cell loss in the eye conjunctiva is reflected by the reduced tear levels of the gel-forming mucin MUC5AC (For review, see Bron et al. (9)). MUC5AC and MUC5B are the two main gel-forming mucins in the human airway. This review focuses on the mouse Muc5b gel-forming mucin and particularly on the recent development and use of a mouse reporter Muc5b–green fluorescent protein (GFP).

Human MUC5B *vs* Mouse Muc5b

The organization in domains of the human and mouse orthologous gel-forming mucins MUC5B/Muc5b is very similar (Figure 1). The amino- and carboxy-terminal regions are globular and are necessary for dimerization and oligomerization of the mucin (10). These regions are made of domains found in von Willebrand factor and flank the large central part, which is highly *O*-glycosylated. The amino- and carboxy-regions are 1324 and 808 amino acids (aa) in length in the human and 1330 aa and 798 aa long in the mouse, respectively. The C-terminal cystine knot domain mediates dimerization of MUC5B subunits in the endoplasmic reticulum, and the N-terminal von Willebrand D domain catalyzes disulfide binding in the Golgi apparatus, which creates multimers.
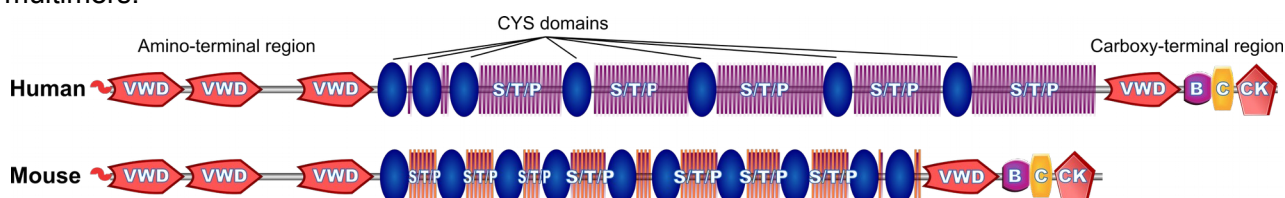
Figure 1. Schematic representation of the human MUC5B and mouse Muc5b peptide showing a similar domain organization. The amino- and carboxy-terminal regions are made of von Willebrand D (VWD), B, C, and cystine knot (CK) domains. The central part is made of CYS domains (blue ovals) interspersed with regions enriched in Ser, Thr, and Pro (S/T/P) that carry the numerous *O*-glycosylated chains characteristic of mucins. These S/T/P sequences differ in size and sequence between the two organisms.

The *O*-glycosylated region is made mainly of stretches of peptides enriched in Ser/Thr/Pro (S/T/P regions). The high content of Ser and Thr results in a dense *O*-glycosylation, which gives the characteristic bottle-brush structure of mucins (11). In humans, S/T/P regions of mucins are made of tandemly repeated (TR) sequences that are different in length and sequence between mucins. Notably, there is no obvious conservation of the S/T/P sequences between human mucin and their mouse or rat orthologues. In addition, the TR consensus sequence is less conserved in the mouse or rat than in the human, which is believed to be the sign of a faster evolutionary process in organisms with a shorter generation time (2,12,13).

TR sequences of mucins exhibit a polymorphism in terms of their number (variable number TRs or VNTRs) between individuals and between the two alleles within the same individual. However, for an unknown reason, this is not the case for MUC5B (14). VNTR polymorphism has not been investigated in inbred and outbred mouse strains, but the size of the central region of mucins might greatly influence the phenotype in mouse disease models. The central region of MUC5B is 3570 aa long encompassing five S/T/P regions (~2550 aa). The mouse Muc5b central part is shorter and has a predicted size of ~2670 aa.

The main structural difference between the mouse and human MUC5B orthologous peptides concerns the presence of seven copies in the human and 10 copies in the mouse of a domain named the CYS domain, which is linked with or interrupts stretches of S/T/P (Figure 1). This domain is ~110 aa long and seems to have been highly conserved during evolution. It has been found in two and nine copies in the two other mucins MUC2 and MUC5AC, respectively (15).

Data suggest that this domain may be crucial for establishing noncovalent interaction between mucins (for recent review, see (4)).

At the gene level, the two orthologous *MUC5B*/*Muc5b* genes are highly similar and are made of 49 exons; exon #31 is the largest (10713 bp in the human) and encodes the CYS domains and the S/T/P regions. The gene extends over 39.1 kb and 34 kb in the human and mouse, respectively. Human and murine promoter sequences seem conserved for only the first 157 nucleotides, which share 67.5% similarity (16).

Analysis of the *MUC5B*/*Muc5b* promoter using reporter gene analysis is sparse, especially in the mouse. The basal promoter activity of human *MUC5B* seems to reside within its 4.2 kb DNA fragment upstream of its transcriptional start site (17). Sp1 *cis* elements within the human *MUC5B* promoter sequence and within intron #1 have been shown to be important for *MUC5B* regulation in epithelial cell lines (8,17–20). An uncharacterized 42-kDa protein-binding SP1 site that localizes in a VNTR sequence and is not conserved in the mouse and overlaps one exon and the following intron in the 3' region of *MUC5B* (21) has been suggested to regulate *MUC5B* expression or *MUC5B* mRNA stability in the HT-29 MTX colon cancer cell line (22). The activating transcription factor 1 (ATF-1) binds the *MUC5B* promoter to stimulate *MUC5B* expression in the AGS and KATO-III gastric adenocarcinoma cell lines (19). E2 sex hormone has been reported to stimulate *MUC5B* gene overexpression directly in NCI-H292 cells (23).

Promoter methylation modulates *MUC5B* expression in cell lines (19,24). MUC5B hypermethylation has been studied in lung tissue from patients with idiopathic pulmonary fibrosis and has been recently associated with the regulation of *MUC5B* expression and as a risk factor for the development of lung disease (25). In the latter study, a forkhead box protein A2 (FOXA2) site located 3 kb upstream of the *MUC5B* transcriptional start site that is conserved in mammals, including rodents, was found to be essential for regulating *MUC5B* expression. Using quantitative chromosome conformation capture, Gosalia *et al*. found that CCCTC-binding factor regulates *MUC5B* expression in a cell type-specific manner (26). Vals and collaborators have shown in differentiated mouse middle ear epithelial cells that *Staphylococcus epidermidis* lysate stimulates human *MUC5B* expression and nontypable *Haemophilus influenzae* lysate can induce mouse *Muc5b* upregulation via proinflammatory cytokines, such as Cxcl2 (27,28). The same team has also reported that cigarette smoke condensate increases Muc5b mRNA expression in part via AP-1 NF-KB *cis* sites within the mouse *Muc5b* promoter sequence (29).

In the mouse, *Muc5b* promoter activity has been reported to be completely inhibited by thyroid transcription factor-1 factor but activated by GATA family factors (16). No study has attempted to show that transcriptional factors that regulate the mucin in one species are conserved in its orthologous counterpart, and the current knowledge comes mainly from investigations using cell lines.

Creation of the Mouse Muc5b–GFP Reporter

Human MUC5B has been shown to be expressed at the mRNA and protein levels in different adult tissues: salivary glands, ear, trachea, lungs, gallbladder, and cervix. Less was known of its expression in the mouse.

To understand the biological role of Muc5b in normal and pathological situations, a mouse Muc5b reporter has been engineered by homologous recombination using a synthetic sequence encoding a monomeric enhanced GFP in frame with Muc5b in place of the unique Stop codon located in exon #49 (30). The targeting strategy is shown in Figure 2. A Gly–Ser–Ile–Ala–Thr linker was placed between the last amino acid in Muc5b and the GFP sequence. This strategy is thought to allow the monitoring of Muc5b trafficking in mice, derived cells, and explants, and the model was designed to provide access to a new conditional knockout model complementary to that created by Evans *et al*. who targeted the 5' end of Muc5b by homologous recombination (31). To this end, the exons #48 and #49 were flanked by LoxP sites in the GFP reporter model.

The transgenic mouse was obtained by standard procedures. G418-resistant colonies were selected based on cell growth and morphology, and were screened by polymerase chain reaction (PCR) analysis. Positive embryonic stem (ES) cell clones were amplified and confirmed by Southern blot analysis using internal and external probes to the targeting vector. One positive ES cell clone was expanded and injected into C57BL/6J blastocysts reimplanted into OF1 pseudo-

pregnant females. Male chimeras were obtained and were bred with C57BL/6 ubiquitous FLP-recombinase-expressing deleter mice to allow germline excision of the FRT-flanked neomycin selection cassette located just downstream of *Muc5b*. Successful removal of the cassette was confirmed by PCR and Southern blot analyses. The 'clean' Muc5b-GFP knockin model has been backcrossed for at least 10 generations in the C57BL/6 genetic background and is routinely genotyped by PCR analysis.
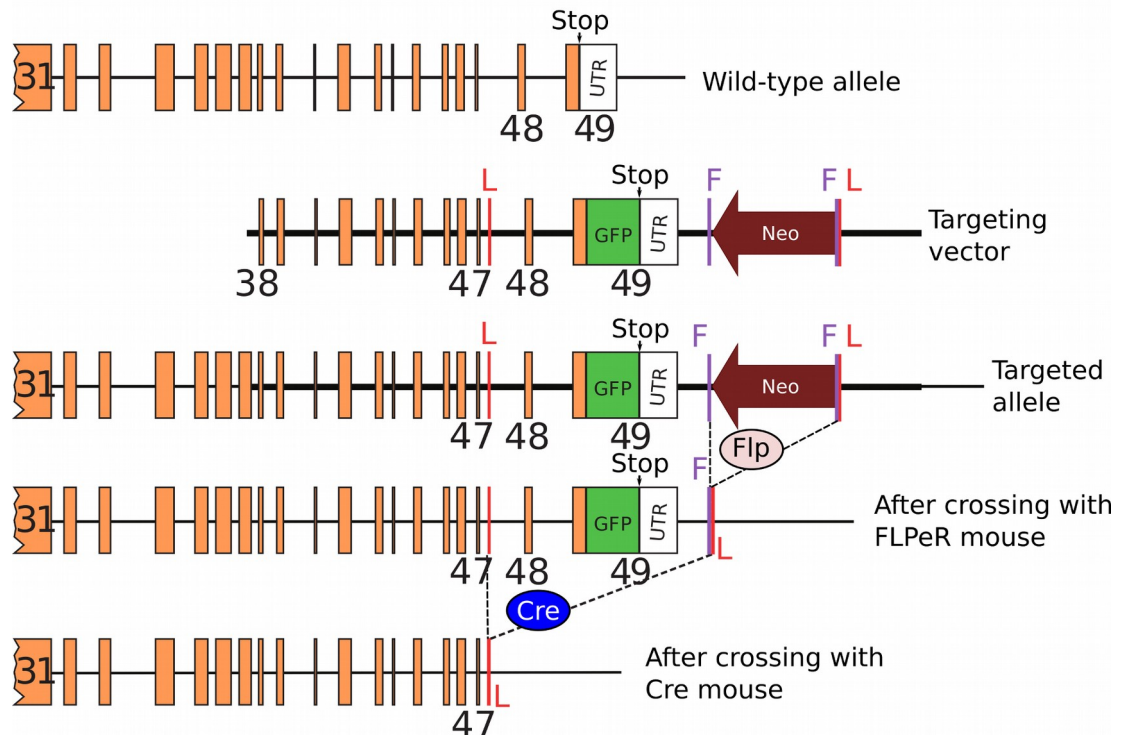
Figure 2. Strategy to target the Muc5b genomic locus. Schematic representation of the 3' end of the wild-type allele, targeted construct (targeted vector), and the 3' end of the targeting allele before and after FLP- and Cre-mediated excision. Some exons are numbered (exon #31 is the large central exon that encodes the CYS domains and S/T/P regions). A synthetic GFP sequence was inserted in frame of Muc5b in place of the unique Stop codon. A LoxP–FRT–neomycin (Neo)–FRT cassette was inserted downstream of *Muc5b*. A second intronic LoxP site was inserted in intron #47. Chimeric mice were bred with FLPeR mice to remove the neomycin cassette used for ES cell selection. The resulting Muc5b–GFP mice can be further crossed with Cre recombinase-expressing mice to generate the all-tissue or tissue-specific null Muc5b allele by excision of the two last exons containing the unique 3' untranslated region (UTR) with its unique Stop codon.

Muc5b Expression Pattern

Immunohistochemistry (IHC) studies were performed using Muc5b–GFP and wild-type mice with a commercial antibody directed against the GFP tag and an in-house antibody directed against a short peptide belonging to a CYS domain of Muc5b. These studies revealed that Muc5b is produced in the trachea, lung, ear, nose, gallbladder, and cervix (30). GFP fluorescence was also clearly observed in fresh tissues from the middle ear, cervix, trachea, nasal cavity, and eye conjunctiva. These findings showed that the expression/production profile of Muc5b is very similar to its human counterpart.

Because Muc5b seemed to be produced by many tissues and cavities accessible by endomicroscopy, the fluorescence of Muc5b–GFP was assessed by probe-based confocal laser endomicroscopy in anesthetized mice using a Cellvizio apparatus. The ultra-thin flexible fibered probe used is made of thousands of microfibers grouped in bundles. Each microfiber excites the GFP at 488 nm and records the live fluorescent activity as a movie. This method confirmed production of the mucin in the trachea, in the eye conjunctiva, nose, and vagina; in the vagina, the greatest fluorescence was observed just before ovulation, similarly to what has been described in women (32) and in pregnant mice (30). These results suggest that Muc5b is hormonally regulated.

Monitoring of Muc5b–GFP Production After Pharmacological Treatment

Interleukin 13 (IL-13) stimulates goblet cell differentiation. In cellular models of the airways or eye conjunctiva and, less often, explant cultures, several independent laboratories have reported that IL-13 upregulates MUC5AC. The few studies that have investigated MUC5B have produced conflicting results. More recently, our study using GFP-tagged mouse Muc5b demonstrated that Muc5b production is produced earlier than though before (30,33) and was stimulated by a single administration of IL-13 to whole lungs explant culture obtained at day embryonic age 12.5, as measured by fluorescence activity and confirmed by IHC (30). In that study, no modification of Muc5ac was observed by IHC (unpublished observations). In another report where decreased goblet cell density in the eye conjunctiva was chemically induced to mimic dry eye, we found that topical administration of IL-13 upregulated Muc5b and restored the Muc5b-positive goblet cell density (34).

Conclusions and Future Directions

MUC5B is a major gel-forming mucin in the airways. The gene and its expression pattern and domain architecture are highly conserved in the mouse, which makes the mouse a suitable organism model for assessing the role of the mucin in normal and pathological situations such as asthma, pulmonary fibrosis, airways infection, cystic fibrosis, ear effusion, and rhinitis. The recent development of the mouse GFP-tagged Muc5b reporter offers the opportunity to understand further the biological role in living mice of gel-forming mucin and abnormal Muc5b regulation in respiratory diseases. The next step is to develop immortalized GFP-positive cell lines and organoids to allow *in vitro* studies, in parallel with *in vivo* studies, to identify the molecules that modulate goblet cell differentiation and Muc5b expression, production, and release. The monomeric GFP-tag was added at the C-terminus of Muc5b. Homozygous mice are viable and fertile and do not show any abnormality but the correct dimerization of Muc5b-GFP via its C-terminal CK domain remains to be examined. Proteolytic degradation of the MUC5B polypeptide backbone by bacteria has been shown to occur naturally in saliva (35) and extracellular proteolytic degradation of MUC5B has been reported in sputum of cystic fibrosis subjects (10). The macro-molecule organization of MUC5B polymers is now better understood (10,36–38). However, the maturation process of the mouse Muc5b counterpart and Muc5b-GFP is unknown and studies are now needed to examine if the GFP-tag may alter the Muc5b maturation and the mucus rheology.

Because Muc5ac is the other main gel-forming mucin in the airways, engineering of a dual mouse Muc5b–Muc5ac reporter model should be attempted by genetically inserting in frame a non-GFP tag at the carboxy-terminal end of Muc5ac. Genome editing using the CRISPR/Cas9 system might be useful for generating a new reporter using Muc5b–GFP embryos. This method would allow the insertion of a LoxP site at the 3' end of *Muc5ac* to obtain a double Muc5ac–Muc5b null allele by Cre-mediated excision of the 3' end of *Muc5ac* and full *Muc5b*.

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