Essential role for complex *N*-glycans in forming an organized layer of bronchial epithelium

(glycosyltransferase mutation/DNA *in situ* hybridization/chimera/mouse lung)

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ABSTRACT Mice lacking the complex subset of *N*-glycans due to inactivation of the *Mgat1* gene die at mid-gestation, making it difficult to identify specific biological functions for this class of cell surface carbohydrates. To circumvent this embryonic lethality and to uncover tissue-specific functions for complex *N*-glycans, WW6 embryonic stem cells with inactivated *Mgat1* alleles were tracked in chimeric embryos. The *Mgat1* gene encodes *N*-acetylglucosaminyltransferase I (GlcNAc-TI; EC 2.4.1.101), the transferase that initiates the synthesis of complex *N*-glycans. WW6 cells carry an inert β -globin transgene that allows their identification in chimeras by DNA–DNA *in situ* hybridization. Independent *Mgat1*^{−/−} and *Mgat1*^{+/-} mutant WW6 isolates contributed like parent WW6 cells to the tissues of embryonic day (E) 10.5 to E16.5 chimeras. However, a cell type-specific difference was observed in lung. Homozygous null *Mgat1*^{−/−} WW6 cells did not contribute to the epithelial layer in more than 99% bronchi. This deficiency was corrected by transfection of a *Mgat1* transgene. Interestingly, heterozygous *Mgat1*^{+/-} WW6 cells were also deficient in populating the layer of bronchial epithelium. Furthermore, examination of lung bud in E9.5 *Mgat1*^{−/−} mutant embryos showed complete absence of an organized epithelial cell layer in the bronchus. Thus, complex *N*-glycans are required to form a morphologically recognizable bronchial epithelium, revealing an *in vivo*, cell type-specific function for this class of *N*-glycans.

Glycan units attached to protein or lipid are arrayed at the surface of mammalian cells and contain sugar sequences that may function as recognition determinants (1). However, it is not clear to what extent the enormous variation in glycan structures has biological relevance. Since the action of specific glycosyltransferases generates the observed variety, questions of glycan function can be addressed by inactivating genes that code for these enzymes (2–6). Interestingly, glycosylation-defective cell mutants with a highly simplified spectrum of surface glycans grow normally in culture (7–10). Thus, Chinese hamster ovary (CHO) cells lacking *N*-acetylglucosaminyltransferase I (GlcNAc-TI; EC 2.4.1.101) and synthesizing only oligomannosyl carbohydrates (Man_{5–9}GlcNAc₂Asn) at *N*-glycan sites, suffer no apparent consequences (8, 9). By stark contrast, mice lacking GlcNAc-TI die at mid-gestation (2, 3). Therefore, the complex *N*-glycans whose synthesis is prohibited in *Mgat1*^{−/−} mice are essential for development beyond embryonic day (E) 9.5–10.5.

There is no single tissue or cell type that appears to be responsible for the demise of *Mgat1*^{−/−} embryos (2, 3, 11). Mutant embryos are underdeveloped and show cellular disorganization in many tissues (2, 3). Thus, specific functions for *N*-glycans are not readily apparent from the mutant phenotype. Strategies for circumventing embryonic lethality so as to identify specific consequences of a null mutation include

tracking of mutant embryonic stem (ES) cells in chimeras (12–15), characterizing the differentiation properties of mutant ES cells (16) or the properties of mutant mouse embryo fibroblasts (17), and replacing endogenous genes with a “floxex” homologue that can be inactivated in a particular tissue (18). The former approach involves minimal assumptions and tests the widest range of possible functions. In fact, WW6 ES cells were developed specifically for this purpose (19). They can be tracked to all nucleated cell types in chimeras by virtue of their inert β -globin transgene which is detected by DNA–DNA *in situ* hybridization (19). In this paper we show that *Mgat1*^{−/−} null mutant WW6 cells do not contribute to the epithelial cell layer in >99% of bronchi in E10.5 to E16.5 chimeric embryos and that E9.5 *Mgat1*^{−/−} embryos have no morphologically recognizable layer of bronchial epithelium, revealing a cell type specific function for complex *N*-glycans.

MATERIALS AND METHODS

Isolation and Characterization of Mutant WW6 Cells. Independent WW6 ES cell lines with two disrupted *Mgat1* alleles were selected from $\approx 10^7$ WW6.15 *Mgat1*^{+/-} WW6 cells (2) using medium containing 1.5 mg/ml of G418. After 12 days, 66 surviving colonies were plated into single wells of a 48-well tray containing SNL-2 feeder layer cells (2). After 4 days at 37°C, cells from each well were replated onto two 35-mm dishes containing a feeder layer and expanded for freezing and characterization. All cell lines were grown for two passages without feeder layer cells and in the presence of leukemia inhibitory factor before characterization. Extraction of DNA and RNA, Southern and Northern blot analyses, assay of GlcNAc-TI activity, and detection of complex *N*-glycans by staining with fluoresceinated *Phaseolus vulgaris* leukoagglutinin (L-PHA) were performed as described (2). Differentiation to cystic bodies induced by retinoic acid treatment of WW6 cells in culture and tumor formation in nude mice were also performed as described previously (19).

Chimera Production and *in Situ* DNA–DNA Hybridization. WW6 ES cells and mutant derivatives were injected into 3.5 days postcoitum (dpc) C57BL/6J blastocysts as described (2). At E10.5–16.5, embryos were recovered and chimeras that possessed the β -globin transgene in yolk sac DNA were fixed as described (19). Sagittal sections (5–7 μ m) were hybridized to a biotinylated β -globin gene probe and counterstained with hematoxylin and eosin Y as described (19). Quantitation of the degree of chimerism was determined by digitized imaging of adjacent sections that were hybridized but not stained with hematoxylin and eosin. Collection of images was performed with an Ektron 1412 digital Ccd camera (Ektron Applied

Abbreviations: ES, embryonic stem; dpc, day(s) postcoitum; GlcNAc-TI, *N*-acetylglucosaminyltransferase I; E, embryonic day; L-PHA, *Phaseolus vulgaris* leukoagglutinin.

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FIG. 1. Isolation and characterization of *Mgat1*^{-/-} WW6 cell lines. (Upper) *Mgat1* alleles in WW6 cells and mutant lines selected in G418 are shown in the diagram: the open reading frame (hatched box), coding exon (bold line), intron sequence (thin line), probes A and B, and the recognition sites of restriction enzymes *Sac*I and *Xma*III are identified. End, endogenous allele; HR, allele homologously recombined. (Middle) Southern blot analysis of *Bam*HI-digested genomic DNA from parental and *Mgat1* mutant WW6 ES cells hybridized to probe B. *Bam*HI does not cut in the endogenous genomic fragment shown but does cut once within the *neo* gene (19). Fragments diagnostic for the End *Mgat1* allele (≈ 10 kb) and the disrupted HR allele (≈ 3.8 kb) are indicated (2, 19). The β -globin transgene (TG; ≈ 5.7 kb), present at ≈ 1000 copies per cell, is detected by the *Mgat1* probe, presumably due to the presence of plasmid DNA sequences (2,

Imaging, Bedford, MA) with 8-bit grayscale plug-in software written for Adobe Photoshop. Illumination was provided by a Gordon planar lightbox (Gordon Instruments, Orchard Park, NY). The density of hybridization signals was estimated using a Macintosh Quadra 950 computer using the public domain NIH IMAGE 1.55 program (written by Wayne Rasband, National Institutes of Health, and available from the Internet by anonymous ftp from zipy.nimh.nih.gov or floppy disk from the National Technical Information Service, Springfield, VA, part no. PB93-504868). Histograms were loaded into Microsoft EXCEL and plotted on a log curve. Each chimera was represented by one curve representing average values from scans of three to four hybridized sections and three to four nonhybridized sections from the same embryo. The area under the curves was calculated as number of pixels and plotted as a histogram that represents the density of *in situ* hybridization signal over sagittal sections of the entire embryo.

Correction of the GlcNAc-TI Deficiency in *Mgat1*^{-/-} WW6 Cells with a *Mgat1* Transgene. WW6.10 *Mgat1*^{-/-} cells (1.2×10^7) were coelectroporated with 5 μ g *Nde*I-linearized plasmid DNA from a plasmid containing a puromycin resistance gene under control of the *pgk* promoter (20), and either 25 μ g DNA from a *Bam*HI-linearized *Mgat1* cDNA in the plasmid pCDM7 (21) or 25 μ g DNA from a *Hind*III-linearized λ H32S3A subclone that carries a 3.8-kb *Sac*I genomic DNA fragment of the mouse *Mgat1* gene in pGEM-7Zf(+) (2, 21). Electroporation was performed as described (2) in 0.6 ml of phosphate-buffered saline and cells were grown overnight in nonselective medium. Medium containing 2 μ M puromycin and 1000 units of leukemia inhibitory factor per ml was added on the next day and replaced every 1–2 days. After 12 days, surviving clones were picked, expanded, and analyzed by staining with L-PHA-fluorescein isothiocyanate as described (2) to identify transfectants expressing complex *N*-glycans. Cells from three stable clones were injected into 3.5 dpc blastocysts, and embryos were collected at E16.5, sectioned, and subjected to DNA–DNA *in situ* hybridization with the β -globin probe as described (19).

RESULTS

Properties of WW6 Cells Lacking Complex *N*-Glycans.

WW6 ES cell lines doubly targeted at the *Mgat1* locus were isolated from WW6.15 *Mgat1*^{+/-} WW6 cells (2) by selection with G418 at high concentration. Resistant cells are presumed to have undergone an interchromosomal recombination event at the *Mgat1* locus, resulting in duplication of the mutant allele that carries the *neo* gene insertion (22). Two independent colonies, termed WW6.10 and WW6.20, were shown to carry a disruption at both *Mgat1* alleles (Fig. 1). *Mgat1* RNA expression was not detected in either isolate (Fig. 1) and neither had GlcNAc-TI activity (≤ 0.1 nmol/hr/mg protein), though both had normal levels of Golgi β (1, 4)galactosyltransferase activity (≥ 28.3 nmol/hr/mg protein). The independent *Mgat1*^{+/-} lines, WW6.15 and WW6.220, had 1.4–1.7 nmol/hr/mg protein GlcNAc-TI activity compared with 2.4 nmol/hr/mg protein in WW6 cells. No complex *N*-glycans were detected at the surface of WW6.10 or WW6.20 cells by staining of cells with fluoresceinated L-PHA that specifically recognizes complex *N*-linked carbohydrates (24, 25), whereas *Mgat1*^{+/-} and WW6 cells strongly bound L-PHA–fluorescein isothiocyanate. Following differentiation with retinoic acid in culture, both independent *Mgat1*^{-/-} lines formed cystic bodies with properties indistinguishable from cystic bodies derived from WW6 cells (19). Both homozygous and heterozygous

19). (Lower) Northern blot analysis of total RNA from parental and *Mgat1* mutant WW6 ES cells hybridized to probe A or a human cardiac actin probe (21, 23). The position of *Mgat1* mRNA is indicated, and the *Mgat1* genotype of each line is given under each lane.

mutants also formed teratocarcinomas with a spectrum of differentiated cell types similar to those generated from WW6 cells in nude mice (19). Functions for *N*-glycans in organ development were therefore investigated by tracking *Mgat1* mutant cells to the tissues of chimeric embryos.

Virtual Failure of *Mgat1*^{-/-} ES Cells to Contribute to the Bronchial Epithelial Cell Layer in Chimeric Embryos. At E16.5, when morphogenesis has been largely completed, the three strongest chimeras formed with *Mgat1*^{-/-} cells, were small and anemic and their hearts were not beating, indicating that chimeras with a large mutant cell component could not survive. However, nine other *Mgat1*^{-/-} chimeras appeared indistinguishable from chimeras formed with WW6 cells. The extent of chimerism was crudely assessed by Southern blot detection of the WW6 β -globin transgene in yolk sac DNA

(19), and more precisely quantitated by digitized imaging of sagittal sections subjected to *in situ* hybridization (Fig. 2 *Lower*). Though the contribution of injected cells to chimeras varied somewhat, both *Mgat1*^{+/-} and *Mgat1*^{-/-} WW6 cells gave a good degree of overall chimerism. These singly and doubly targeted WW6 cells were previously shown to populate mid-brain and intestine equivalently to WW6 cells (19) and they also contribute similarly to WW6 cells to all other tissues of the embryo (data not shown). However, a close examination of cell types in each tissue, revealed a dramatic deficiency in their ability to populate bronchial epithelium (Fig. 2 *Upper*, top and bottom rows). In contrast to WW6 cells, *Mgat1*^{-/-} null mutants were not found in >99% of bronchi (Fig. 2; Table 1). In the remaining 0.6% of bronchi, only one or two *Mgat1*^{-/-} derived cells were present per bronchus. Importantly, heterozygous

FIG. 2. (*Upper*) Absence of *Mgat1*^{-/-} cells from bronchial epithelium of chimeras: rescue with a *Mgat1* transgene. Sections through the lung of E16.5 chimeric embryos generated by injecting *Mgat1*^{+/+} (+/+), *Mgat1*^{+/-} (+/-), *Mgat1*^{-/-} (-/-) WW6 cells, or *Mgat1*^{-/-} WW6 cells carrying a *Mgat1* transgene (Rescue), into 3.5 dpc blastocysts. The β -globin transgene that is present in all nucleated cells derived from injected WW6 cells was detected by *in situ* DNA-DNA hybridization and the sections were stained briefly with hematoxylin and eosin. All cells without a hybridization signal derived from the normal blastocyst. [Bar = 25 μ m (top row); bar = 10 μ m (bottom row).] (*Lower*) The histograms show quantitation of the extent of chimerism in four embryos of each group obtained by injecting the cells designated (+/+), (+/-) or (-/-), respectively. The histograms were generated by digitized computer imaging of four sections per embryo that had been subjected to *in situ* hybridization but not subsequently stained with hematoxylin and eosin. Integrated densities were determined after subtraction of nonhybridized controls and are plotted as number of pixels under the averaged curve for each embryo.

Table 1. *Mgat1*^{-/-} WW6 ES cells contribute to the layer of bronchial epithelium in <1% bronchi: rescue with a *Mgat1* transgene

WW6 cell line	Passage no.	Chimeras analyzed	Stage of development	Bronchi scored	Positive bronchi	% positive
<i>Mgat1</i> ^{+/+}						
WW6	10	1	E16.5	379	378	100
WW6	22	2	E16.5	1574	1383	88
WW6	12	3	E14.5	1733	1517	88
WW6	12	2	E12.5	47	44	94
<i>Mgat1</i> ^{+/-}						
WW6.15	17	4	E16.5	1856	728	39
WW6.220	17	3	E16.5	470	183	39
<i>Mgat1</i> ^{-/-}						
WW6.10	22	4	E16.5	954	6	0.63
WW6.20	25	3	E16.5	482	3	0.62
WW6.10	23	1	E12.5	38	0	0
WW6.20	26	2	E12.5	63	0	0
WW6.10	22	4	E10.5	19	0	0
WW6.20	25	4	E10.5	29	0	0
<i>Mgat1</i> ^{-/-} rescue*						
WW6.10λ4	25	1	E16.5	440	125	28
WW6.10λ4	25	1	E16.5	589	116	20
WW6.10λ4	25	1	E16.5	825	799	97
WW6.10.E15	25	1	E16.5	479	0	0

Sagittal sections through chimeric embryos were subjected to *in situ* hybridization and bronchi scored for cells containing the β -globin transgene. The number of sections scored was 60–100 per E14.5 and E16.5 chimera, 40 per E12.5 chimera, and 30 per E10.5 chimera. The number of bronchi per section was approximately equal for each chimera at a particular developmental stage, and therefore total bronchi examined are presented for simplicity. A bronchus was considered positive if at least one cell of the epithelial layer contained a hybridization signal. The number of positive cells in each bronchus was 13% to $\geq 50\%$ for *Mgat1*^{+/+}, 1–15% for *Mgat1*^{+/-}, and one or two per bronchus for *Mgat1*^{-/-} chimeras. To rescue *Mgat1*^{-/-} cells with a *Mgat1* transgene, WW6.10 cells were transfected with a genomic DNA fragment (λ 4) containing the coding region for GlcNAc-T1 or with a *Mgat1* cDNA (E15) under the control of a cytomegalovirus promoter/enhancer. Three chimeras were obtained from the transfectant WW6.10λ4, but the results are not grouped because they differed significantly, presumably due to variation in the overall degree of embryonic chimerism.

*Transfected with a *Mgat1* transgene.

Mgat1^{+/-} ES cells were also markedly under-represented in bronchial epithelium (Table 1). However, each cell line contributed, in proportion to its degree of chimerism, to lung mesenchyme (Fig. 2), and to thyroid and pancreas (data not shown), which share their origins with lung.

Rescue of *Mgat1*^{-/-} Mutant Phenotype in Chimeras by a *Mgat1* Transgene. To prove that the *Mgat1* gene product was solely responsible for the markedly reduced ability of *Mgat1*^{-/-} WW6 cells to contribute to the layer of bronchial epithelium, rescue was attempted by transfection of a *Mgat1* cDNA (21) under the control of the human cytomegalovirus promoter/enhancer, or a 3.8-kb *SacI* genomic DNA fragment that contains the *Mgat1* coding region (Fig. 1) and no exogenous promoter (2). Cotransfection with a plasmid containing a puromycin-resistance gene (20) allowed selection of transfectants. Survivors were characterized for complex *N*-glycan synthesis by L-PHA staining. Those exhibiting stable expression of a *Mgat1* transgene were injected into 3.5 dpc blastocysts and tracked in E16.5 chimeric embryos by *in situ* hybridization.

In a chimera obtained from a *Mgat1* cDNA transfectant (WW6.10E15), all tissues were extensively populated by transfectant descendants, but there was no contribution to lung epithelium (Table 1), showing that rescue had not occurred with this construct. However, in three chimeras obtained from a genomic DNA transfectant (WW6.10.λ4), bronchial epithelium was populated in accord with the overall degree of chimerism of the embryo (Table 1 and Fig. 2). Thus, an active *Mgat1* transgene was sufficient to rescue the ability of *Mgat1*^{-/-} cells to populate the bronchial epithelial cell layer. One of the chimeras obtained from WW6.10.λ4 cells, exhibited essentially complete rescue of the *Mgat1*^{-/-} phenotype (Table 1).

Absence of a Defined Bronchial Epithelium in *Mgat1*^{-/-} Embryos. *Mgat1*^{-/-} WW6 mutant cells were similarly deficient in contributing to bronchial epithelium at developmental stages earlier than E16.5, including E14.5, E12.5, and E10.5 (Table 1). The fact that the *Mgat1*^{-/-} cell defect was evident as early in development as E10.5, predicted that *Mgat1*^{-/-} embryos containing exclusively *Mgat1*^{-/-} cells, might completely lack a bronchial epithelium cell layer. Examination of sagittal sections of embryos at E9.5 revealed the lung bud with main bronchus lined by differentiated epithelial cells in both wild-type and *Mgat1*^{+/+} embryos (Fig. 3). By contrast, their *Mgat1*^{-/-} littermates had only a space of the appropriate contour for a bronchus in the lung bud, but no morphologically distinct epithelial cell layer was discernible (Fig. 3). Therefore, complex *N*-glycans are essential for either the formation, differentiation, or maintenance of the organized epithelial cell layer that lines the bronchus.

DISCUSSION

The strategy of tracking WW6 ES cells that possess one or two inactivated *Mgat1* alleles in chimeras has identified a cell autonomous function for complex *N*-glycans in the development of a morphologically recognizable bronchial epithelium. Homozygous null *Mgat1*^{-/-} ES cells essentially fail to contribute to the epithelial layer lining bronchi (Fig. 2 and Table 1). They are not present at the earliest stage examined, E10.5 chimeras (Table 1). As might be expected from these findings, the bronchus in E9.5 *Mgat1*^{-/-} mutant embryos lacks a morphologically distinct epithelial cell layer (Fig. 3). The combined data suggest that, in *Mgat1*^{-/-} embryos, development of precursor cells to this epithelial cell type may fail to occur, or

FIG. 3. Bronchial epithelium in *Mgat1*^{-/-} and *Mgat1*^{+/+} embryos. Embryos from a timed mating between *Mgat1*^{+/+} mice were collected at E9.5. *Mgat1*^{+/+} and *Mgat1*^{-/-} embryos were identified by Southern analysis of yolk sac DNA. Sagittal sections were fixed and stained with hematoxylin and eosin and lung buds were examined for overall morphology and for the presence of an organized epithelial cell layer lining the main bronchus. [Bar = 25 μ m (Top); bar = 10 μ m (Lower).]

that differentiated lung epithelial cells may fail to migrate to their characteristic position in the bronchus. Alternatively, epithelial cells may be formed but fail to organize, or to attach or remain attached, to the basement membrane. It seems perhaps likely, given the altered array of cell surface *N*-glycans on *Mgat1*^{-/-} WW6 cells, that the defect stems from altered cell-cell recognition. The molecular basis for the phenotype may be difficult to investigate in chimeric embryos. But it might be tractable in adult *Mgat1*^{+/+} heterozygotes.

The finding that independent *Mgat1*^{+/+} heterozygous cells contribute markedly less well than parent WW6 ES cells to bronchial epithelium (Table 1), was unexpected. Though heterozygotes have only half the GlcNAc-TI activity of WW6 cells, this need not necessarily translate into a corresponding reduction in complex *N*-glycan content in differentiated lung cells. Since, however, the *Mgat1*^{+/+} WW6 cells exhibit a distinct phenotype in chimeras, it may be that *Mgat1*^{+/+} adult mice have a lung phenotype. Preliminary investigations of lung tissue from adult *Mgat1*^{-/-} mice have revealed no significant morphological abnormalities in histological sections (A. Borczuk, Y.L., and P.S., unpublished observations). However, these animals have been continuously housed in a barrier facility. If they were exposed to normal environmental pathogens or subjected to conditions that stress lung function, a specific pathology may emerge. Such experiments are important as they may point the way to identifying a disease state in humans that arises from inactivation of a single *Mgat1* allele.

Tracking of mutant WW6 cells in chimeras provides a useful strategy for investigating the function of other embryonic lethal mouse mutations. While these experiments presumably do not shed light on the cause of death in *Mgat1*^{-/-} embryos, since E9.5 mice should not require functional lungs for sur-

vival, the experiments pin-point a cellular defect that would have been extremely difficult to identify in any other way. The general cellular disorganization in E9.5 *Mgat1*^{-/-} embryos precluded drawing specific functional conclusions from the fact that the bronchus in these embryos has no recognizable epithelial layer (Fig. 3). Thus, tracking of WW6 mutant ES cells was critical to uncovering a specific requirement for complex *N*-glycans in forming or maintaining bronchial epithelium and should be useful in characterizing other mutations in the mouse.

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