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Fetal Bovine Serum impacts the observed N-glycosylation defects in TMEM165 KO HEK cells

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Abstract

TMEM165 is involved in a rare genetic human disease named TMEM165-CDG (Congenital Disorders of Glycosylation). It is Golgi localized, highly conserved through evolution and belongs to the uncharacterized protein family 0016 (UPF0016). The use of isogenic TMEM165 KO HEK cells was crucial in deciphering the function of TMEM165 in Golgi manganese homeostasis. Manganese is a major cofactor of many glycosylation enzymes. Severe Golgi glycosylation defects are observed in TMEM165 KO HEK cells and are rescued by exogenous manganese supplementation. Intriguingly, we demonstrate in this paper that the observed Golgi glycosylation defect mainly depends on fetal bovine serum (FBS), particularly its manganese level. Our results also demonstrate that iron and/or galactose can modulate the observed glycosylation defects in TMEM165 KO cells. While isogenic cultured cells are widely used to study the impact of gene defects on proteins' glycosylation patterns, these results emphasize the importance of the use of validated FBS in glycomics studies.

Keywords

FBS; manganese level; N-glycosylation defects; TMEM165

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Author contribution

D.V and M.H performed the cell biology experiments. A.G contributed to ICP-MS analysis. L.C. and V.L. generated the KO cell lines and took part in the writing. W.M performed and analyzed the mass spectrometry data. F.F designed the study and wrote the paper.

INTRODUCTION

Congenital Disorders of Glycosylation (CDG) are a rapidly growing and heterogeneous group of rare genetic diseases (Freeze 2007; Jaeken and Matthijs 2007; Schachter and Freeze 2009; Jaeken 2013; Scott *et al.* 2014). The deficiencies observed in CDG affect the biosynthesis of glycoproteins leading to macro and/or micro-heterogeneity of the protein glycosylation status. They show heterogeneous phenotypes comprising mostly neurological involvement and dysmorphism (Jaeken and Péanne 2017; Péanne *et al.* 2017). A new era in CDG is started with the identification of defects in genes not directly linked to glycosylation but involved in vesicular Golgi trafficking (Wu *et al.* 2004; Foulquier *et al.* 2006, 2007; Kranz *et al.* 2007; Foulquier 2009; Reynders *et al.* 2009; Paesold-Burda *et al. 2009)* and Golgi homeostasis (Kornak *et al.* 2008). In order to understand the molecular mechanisms that fine-tune the glycosylation machinery to physiological requirements, several cellular and animal models were created. Regarding CDG, isogenic cell lines represent an interesting toolset to better understand the molecular and cellular mechanisms of the glycosylation process itself. This was used to find out the function of TMEM165 in Golgi glycosylation. Indeed, in 2012, we identified *TMEM165* as a gene involved in a novel CDG-II, TMEM165-CDG (OMIM entry #614727) (Foulquier *et al.* 2012; Zeevaert *et al.* 2012). TMEM165 is a 324 amino-acids transmembrane Golgi protein belonging to the uncharacterized protein family 0016 (UPF0016; Pfam PF01169). The cellular and molecular functions of the UPF0016 family members remain controversial. Our previous results unambiguously demonstrated a link between TMEM165 and Golgi Mn^{2+} homeostasis (Potelle *et al.* 2016) through the rescue of Golgi glycosylation defects observed in TMEM165 KO HEK cells by $MnCl_2$ supplementation (Potelle *et al.* 2016; Houdou *et al.* 2019). Recently, we noticed that suppression of these glycosylation defects depends on cell culture conditions. In this paper, we investigate the effects of different fetal bovine sera (FBS) on Golgi glycosylation defects in TMEM165 KO HEK cells.

RESULTS

Serum impacts the observed Golgi glycosylation defects in TMEM165 KO HEK cells.

We previously reported that LAMP2 glycosylation defects found in TMEM165 KO HEK cells were totally suppressed by the addition of exogenous $MnCl_2$ in the culture medium. This was observed from 8h of incubation with 1 μM $MnCl_2$ (Potelle *et al.* 2016; Houdou *et al.* 2019). We recently observed that this suppression could appear without any supplementation of $MnCl_2$ probably due to cell culture conditions (data not shown). This urged us to investigate the effects of different sources of FBS on the appearance and/or rescue of the N-glycosylation defects in TMEM165 KO HEK cells. To investigate this, LAMP2 glycosylation profile was assessed by western blot in TMEM165 KO HEK cells grown in medium supplemented with 6 different FBS: four from animal origin (FBS 1, 2, 3 and 4 in this study) and two synthetic serum substitutes. After a few passages, HEK cells (controls and TMEM165 KO) did not survive when cultured with the synthetic serum substitutes (data not shown). Regarding the sera from animal origin, differential gel mobilities of LAMP2 can be easily seen after 9 days of culture (Fig 1). When cells were grown with FBS 3, LAMP2 gel mobility was less pronounced compared to cells cultured

with FBS 1 or FBS 2. Intriguingly, a very pronounced gel mobility arguing for a severe LAMP2 N-glycosylation defect was observed with FBS 4 (Fig 1). Similar results were observed with TMEM165 KO HeLa-GalT cells cultured with FBS 4 (supplementary figure 1). This suggests that the severity of the observed glycosylation defects depends on the source of the serum used for cell culture.

To confirm these results and pinpoint the potential differences in N-glycosylation that result from the use of each FBS, mass spectrometry analysis of total N-glycans was performed in the different cell culture conditions. For control cells, no significant changes in N-glycan structures were observed with any serum tested (Fig 2, panels A to D). Similar to our previously published analyses (Potelle *et al.* 2016; Morelle *et al.* 2017), TMEM165 KO HEK cells showed massive hypogalactosylation in all tested conditions with the accumulation of agalactosylated glycan structures detected at mass-per-charge (m/z) *ratios* of 1591, 1836, 2081 and 2326 (Fig 3, panels E to H). Interestingly, the proportion of these abnormal glycan structures found was totally dependent upon the FBS chosen for cell growth (see table 1). On the one hand, the proportion of these abnormal glycan structures was comparable, and found in rather low quantity to control cells when TMEM165 KO HEK cells were cultured with FBS 2 or 3 (table 1). On the other hand, they largely increased in abundance in TMEM165 KO HEK cells cultured with FBS 1 or 4 (24% general increase of abnormal glycan structures compared to FBS 2/3). The mass spectrometry analyses not only confirmed the results obtained from LAMP2 gel mobility, but also highlighted the crucial importance of the use of validated FBS in analysing the Golgi glycosylation defects and/or rescue observed in TMEM165 KO HEK cells.

Independently of the serum used for cell growth, Mn^{2+} supplementation suppresses the glycosylation defects in TMEM165 KO HEK cells

As we have previously shown that Mn^{2+} supplementation could rescue the observed N-glycosylation defects in TMEM165 KO HEK cells, we wondered whether Mn^{2+} supplementation would overcome the issues we have observed with regard to FBS. First, each culture medium was supplemented with 1 μM $MnCl_2$, and LAMP2 glycosylation was assessed by western blot. Such treatment partially suppressed the increased gel mobility observed with FBS 1, 2 and 3 (Fig 4). However, this was not the case for the condition with FBS 4 where only a slight decrease in LAMP2 gel mobility was seen (Fig 4). This last condition was retested with increased amounts of MnCl2, 5 and 10 μM, in addition to FBS 4. Very interestingly, a pronounced suppression of LAMP2 glycosylation defect could be seen for these two Mn^{2+} concentrations with a significant increase in the fully glycosylated forms of LAMP2 (Fig 5). Nevertheless, a significant fraction of underglycosylated LAMP2 remained after treatment. Therefore, 5 μM MnCl2 was applied to the cells cultured in FBS 4 for 72h with cells harvested at multiple time point (Fig 6). The results showed that fully glycosylated forms of LAMP2 started to appear after 8h of Mn^{2+} treatment and progressively increased until 72h, while underglycosylated LAMP2 forms decreased from 2 to 72h (Fig 6). These results demonstrated that the fraction of underglycosylated LAMP2 observed under Mn^{2+} supplementation depends on LAMP2 turnover, Mn^{2+} concentration and incubation time. Altogether, these results strongly suggest that the observed

glycosylation defect in TMEM165 KO HEK cells certainly depends on Mn^{2+} level in the different FBS used for cell culture.

To test this hypothesis, manganese levels of each FBS were quantified by Inductively Coupled Plasma-Mass Spectrometry (ICP-MS). As observed in Figure 7, manganese levels vary between sources of FBS. In synthetic serum substitutes, the manganese level was extremely low, between 0.03 and 0.05 μM. In the four other sera from animal origin, our results showed that the concentration is between 0.56 μM and 0.61 μM for FBS 1 and FBS 4 and 1.08 and 1.21 μM for FBS 2 and FBS 3 (Fig 7). This result confirms that the observed differences in severity of the glycosylation defects in TMEM165 KO HEK cells may be correlated with the manganese level present in the FBS used for cell culture.

Iron supplementation can also suppress the glycosylation defects in TMEM165 KO HEK cells.

While our results clearly support that the serum Mn^{2+} level is an important factor to take into consideration, it cannot by itself explain the observed differences in the suppression of the glycosylation defects. This suggests that additional factors within sera could also affect glycosylation in TMEM165 KO cells. To tackle this point, the culture medium of TMEM165 KO HEK cells grown in FBS 4 was supplemented with 100 μM of many different ions, and LAMP2 glycosylation was assessed by western blot (Supplementary Fig 2). Interestingly, we observed that in addition to Mn^{2+}, Fe^{3+} was also capable of rescuing the abnormal LAMP2 glycosylation profile. To assess the sensitivity of glycosylation to Fe^{3+}, a similar experiment was performed with a reduced Fe^{3+} concentration of 5 μM. In addition, Fe^{2+} was also tested. As shown in Figure 8, while fully glycosylated forms of LAMP2 appeared under Mn^{2+} supplementation, this was not the case under Fe^{2+} and Fe^{3+} supplementation where only partially LAMP2 glycosylated forms were observed. Altogether these results suggest that iron is also capable of rescuing Golgi glycosylation in a TMEM165 KO background but not in the same concentration range as manganese.

Galactose supplementation enhances the Mn^{2+} effect in TMEM165 KO HEK cells grown in FBS 4.

Our previous work showed that galactose supplementation could suppress some glycosylation defects of TMEM165-CDG (Morelle et al., 2017). We then wondered whether this suppression could also depend on the source of FBS. To tackle this point, TMEM165 KO HEK cells were cultured in two different FBS (FBS 2 or FBS 4) and supplemented with 1 μM Mn^{2+}, 1 mM galactose or 1 μM Mn^{2+} + 1mM galactose. Intriguingly, all treatments resulted in fully glycosylated forms of LAMP2 in TMEM165 KO HEK cells cultured in FBS 2 (Fig 9A). The result was completely different in cells grown in FBS 4 (Fig 9B). In the latter condition, 1 μM galactose or Mn^{2+} supplementation poorly rescued LAMP2 glycosylation (Fig 9B). However and very interestingly, a combination of these two factors rescued fully glycosylated forms of LAMP2 (Fig 9B). This result demonstrates that depending on the source of the FBS, Mn^{2+} can enhance the galactose effect (or vice-versa) on the suppression of LAMP2 glycosylation. These results reinforce the extreme importance of serum content on glycomics' results.

DISCUSSION

Isogenic TMEM165 KO HEK cells are a powerful model for studying the function of TMEM165 in Mn^{2+}-mediated regulation of the Golgi glycosylation process. TMEM165 KO HEK cells present strong Golgi glycosylation defects affecting different classes of Golgi glycosylation (N- and O-glycosylation and glycolipids glycosylation) (Potelle *et al.* 2016; Morelle *et al.* 2017). The hypothesis that TMEM165 is crucial in regulating Golgi homeostasis of manganese, a major cofactor of many glycosylation enzymes (Powell and Brew 1976; Ramakrishnan *et al.* 2006), came from the observation that low Mn^{2+} concentrations (100 nM-1 μM) in the culture medium were sufficient to suppress the observed Golgi glycosylation defects (Potelle *et al.* 2016; Houdou *et al.* 2019). In the present study, we examined the contribution of serum on these observed Golgi glycosylation defects. This came from the observation that Golgi glycosylation defects could be suppressed depending on culture conditions. In this paper, we demonstrate that the manganese content in serum is a crucial factor to take into account when analyzing Golgi glycosylation defects in TMEM165 KO cells. Although logical, this finding is quite unexpected as manganese is found in serum at a very low concentration between 0.56 μM and 1.21 μM. We calculate that the final manganese concentration in the culture media is between 56 and 121 nM. Our results suggest that slight Mn^{2+} variations in FBS can have huge impacts on the mature N-glycan structures. This result is also in accordance with the observation that only 100nM Mn^{2+} supplementation could rescue the observed LAMP2 glycosylation defects in TMEM165 KO HEK cells in our previous work (Potelle *et al.* 2016). While our results clearly support that serum Mn^{2+} levels are an important factor to take into consideration, we have demonstrated that galactose and/or iron can also affect the observed glycosylation defects in TMEM165 deficient cells. Since 5 μM Mn^{2+} was able to rescue a significant portion of LAMP2 glycosylation, we can guess that serum that is under/over supplemented with other ions could alter the efficiency of the glycosylation machinery.

At a fundamental level, and as isogenic cultured cells and patients' cells are widely used to study the impact-of gene defects on proteins' glycosylation patterns, our results point out that glycomics results, obtained with cultured cells, crucially depend on the level of Mn^{2+} and other factors in FBS. This paper emphasizes the importance of the use of validated FBS in glycosylation analysis.

MATERIAL AND METHODS

Antibodies and other reagents

Anti-LAMP2 antibody was purchased from Santa Cruz Biotechnology (Dallas, USA). Polyclonal goat anti-mouse immunoglobulins HRP conjugated were purchased from Dako (Glostrup, Denmark). D-(+)-Galactose, zinc chloride ($ZnCl_2$) and nickel sulfate hexahydrate ($NiSO_4$) were purchased from Sigma (Saint Louis, USA), manganese (II) chloride tetrahydrate ($MnCl_2$) was from Riedel-de-Haën (Seelze, Germany), calcium chloride ($CaCl_2$) and iron (III) chloride ($FeCl_3$) were from ACROS Organics (New Jersey, USA), copper (II) sulphate ($CuSO_4$) was from Prolabo (France), lithium chloride (LiCl) was from Bio Basic Canada (Canada), magnesium chloride ($MgCl_2$) was from Euromedex

(Souffelweyersheim, France) and iron (II) tetrahydrate ($FeCl_2$) was purchased from WVR Chemicals (Germany).

Cell culture, drug treatments and transfections.

Control and TMEM165 KO HEK / HeLa-GalT cells were generated as previously described in Potelle et al., 2017. Briefly, TMEM165 was knocked out using CRISPR/Cas9 mediated deletion with guide RNAs targeting the first exon (target sequence: TCCAGGGAACGGCCGCGCAT). Clones were first screened for lack of detection of TMEM165 protein with TMEM165 antibodies in western blot and immunofluorescence experiments and then by sequencing. Clones were analyzed by PCR using genomic DNA as a template and primers F7 (tggaggaagcagaagtgaa) and R4 (ctaattcctctgcgttcctaaag) producing an amplicon length around 1200 bp. Sequencing of KO clones showed a deletion of 347 bp. Control cells were from a clone that went through the screening process but was immunoreactive with TMEM165 antibodies and showed no mutations by sequencing. Cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) (Lonza, Basel, Switzerland) supplemented with either 10% fetal bovine serum from animal origin from Dutscher (France), Corning (France) or PAN Biotech (Germany), or 10% synthetic serum substitute (Panexin) from PAN Biotech (Germany) at 37°C in humidity-saturated 5% CO_2 atmosphere. To simplify both writing and reading, we named the different FBS from animal origin as follow: FBS 1 for FBS from Dutscher (old batch, lot n°S10536S1810), FBS 2 for FBS from Dutscher (new batch lot n°S15642S1810), FBS 3 for FBS from Corning (lot n °35079011) and FBS 4 for FBS from PAN (lot n°35079002). When used, the cells have been cultured for at least 9 days with the different sera.

Western Blotting

Cells were scraped in DPBS and then centrifuged at 6000 rpm , 4°C for 10 min. Supernatant was discarded and cells were then resuspended in RIPA buffer [Tris/HCl 50 mM pH 7.9, NaCl 120mM, NP40 0.5%, EDTA 1mM, Na_3VO_4 1mM, NaF 5mM] supplemented with a protease cocktail inhibitor (Roche Diagnostics, Penzberg, Germany). Cell lysis was done by passing the cells several times through a syringe with a 26G needle. Cells were centrifuged for 30 min, 4°C at 14 000 rpm. Protein concentration contained in the supernatant was estimated with the micro BCA Protein Assay Kit (Fisher Scientific, Waltham, USA). 10 µg of total protein lysate were mixed with NuPAGE LDS sample buffer (Fisher Scientific, Waltham USA) pH 8.4 supplemented with 4% β-mercaptoethanol (Sigma, USA). Samples were heated 10 min at 95°C then separated on 4%−12% Bis-Tris gels (Fisher Scientific, Waltham, USA) and transferred to nitrocellulose membrane Hybond ECL (GE Healthcare, Little Chalfont, UK). Membranes were blocked in blocking buffer (5% milk powder in TBS-T [1X TBS with 0.05% Tween20]) for 1h at room temperature, then incubated overnight with the primary antibody (used at a dilution of 1:2000) in blocking buffer and washed three times for 5 min in TBS-T. Membranes were then incubated with the peroxidase-conjugated secondary anti-mouse antibody (Dako; used at a dilution of 1:20 000) in blocking buffer for 1h at room temperature and later washed five times for 5 min in TBS-T. Signal was detected with chemiluminescence reagent (Super Signal West Pico PLUS chemiluminescent Substrate, Thermo Scientific) on imaging film (GE Healthcare, Little Chalfont, UK).

Glycan analysis by mass spectrometry

Cells were sonicated in extraction buffer (25 mM Tris, 150 mM NaCl, 5 mM EDTA and 1% CHAPS, pH 7.4) and then dialyzed in 6–8 kDa cut-off dialysis tubing in an ammonium bicarbonate solution (50 mM, pH 8.3) for 48h at 4°C and lyophilized. The proteins/glycoproteins were reduced and carboxyamidomethylated followed by sequential tryptic and peptide N-glycosidase F digestion and Sep-Pak purification. Permethylation of the freeze-dried glycans and MALDI-TOF-MS of permethylated glycans were performed as described elsewhere (Morelle and Michalski 2007)

Mn^{2}+ measurement by ICP-MS

Instrumentation and analysis—Serum samples were diluted 50 times with 1,5% (v/v) nitric acid (ultrapure quality 69,5%, Carlo Erba Reagents, Val de Reuil, France) solution in ultrapure water (Purelab Option-Q, Veolia Water, Antony, France) containing 0,1% TritonX-100 (Euromedex, Souffelweyersheim, France), 0,2% butan-1-ol (VWR Chemicals, Fontenay-sous-Bois, France), and 0,5 µg/L rhodium (Merk, Darmstadt, Germany). Assays were performed on an ICP-MS THERMO ICAP™ Q (Thermo Scientific, Courtaboeuf Cedex, France). The limit of quantification was 0,2µg/L.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Take-home message:

Manganese serum level is a crucial factor in glycomics experiments.

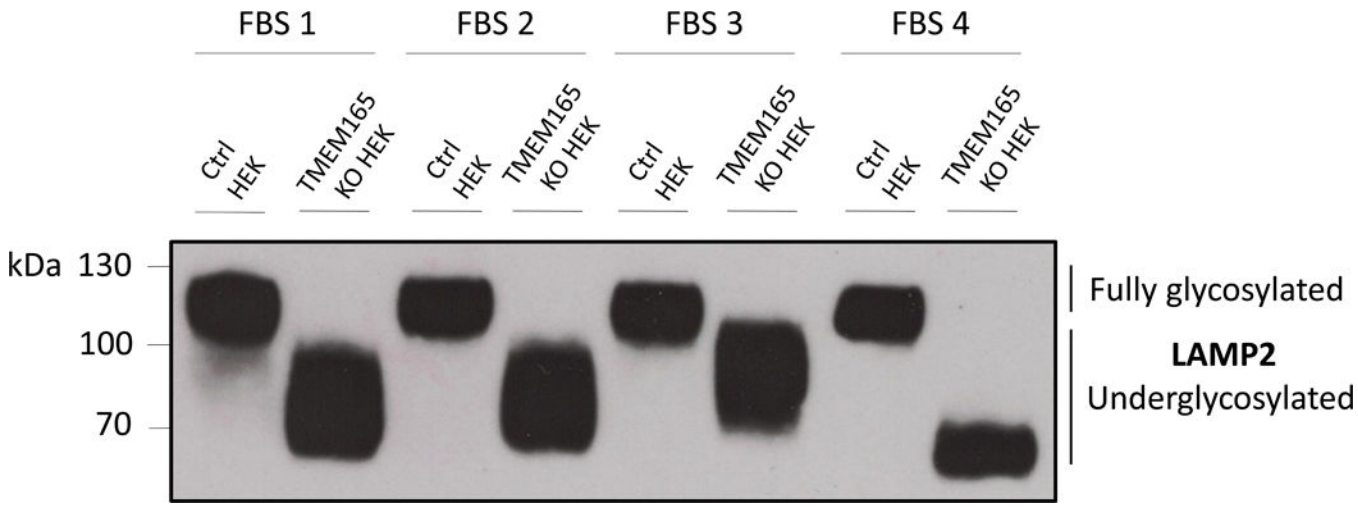


Figure 1: The appearance of LAMP2 glycosylation defects in TMEM165 KO HEK cells depends on the FBS used for cell culture.

Control and TMEM165 KO HEK cells were cultured in DMEM supplemented with 10% FBS 1, 2, 3 or 4. Total cell lysates were prepared, subjected to SDS-PAGE and western blot with the indicated antibody.

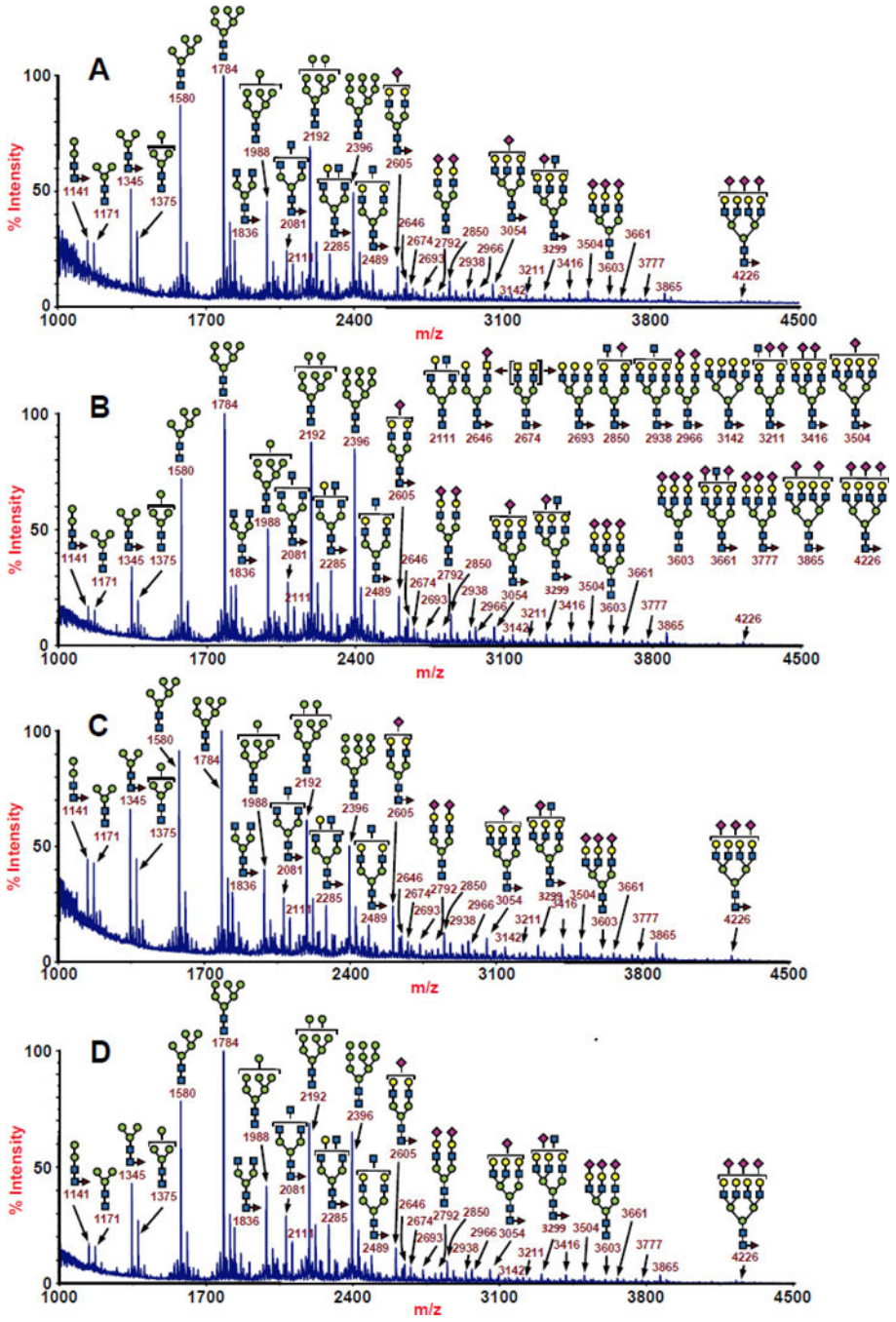


Figure 2: The *N*-glycosylation defects severity observed in TMEM165 KO HEK cells depends on the FBS used for cell culture.

MALDI-TOF-MS spectra of the permethylated *N*-glycans from control cells following different cell culture conditions. **A to D**. HEK control cells cultured in DMEM supplemented with 10% FBS 1 (A) or 2 (B) or 3 (C) or 4 (D). Symbols represent sugar residues as follow: blue square, *N*-acetylglucosamine; green circle, mannose; yellow circle, galactose; purple diamond, sialic acid; red triangle, fucose. Linkages between sugar residues have been removed for simplicity.

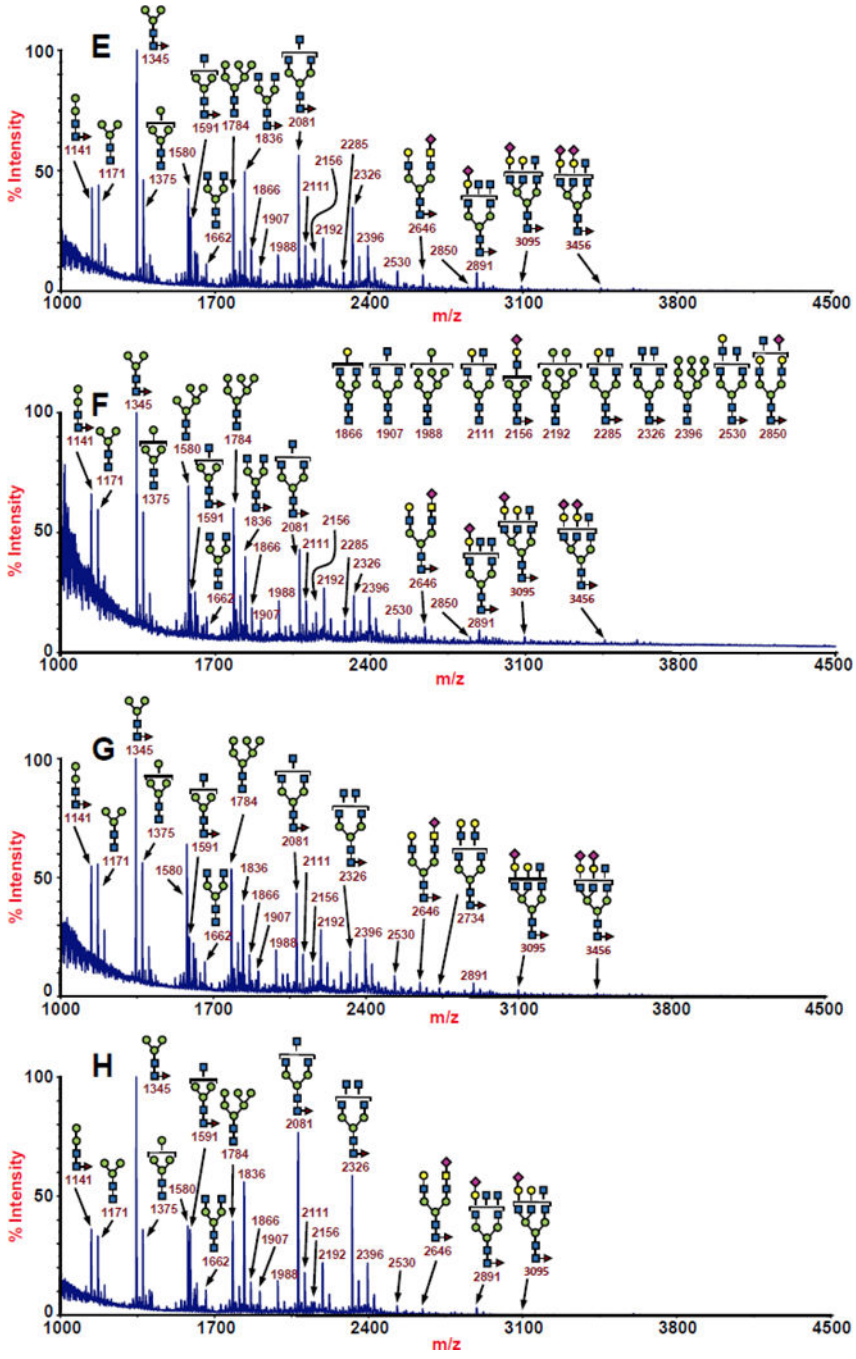


Figure 3: The *N*-glycosylation defects severity observed in TMEM165 KO HEK cells depends on the FBS used for cell culture.

MALDI-TOF-MS spectra of the permethylated *N*-glycans from TMEM165 KO HEK cells following different cell culture conditions. **E to H.** TMEM165 KO HEK cells cultured in DMEM supplemented with 10% FBS 1 (E) or 2 (F) or 3 (G) or 4 (H). Symbols represent sugar residues as follow: blue square, *N*-acetylglucosamine; green circle, mannose; yellow circle, galactose; purple diamond, sialic acid; red triangle, fucose. Linkages between sugar residues have been removed for simplicity.

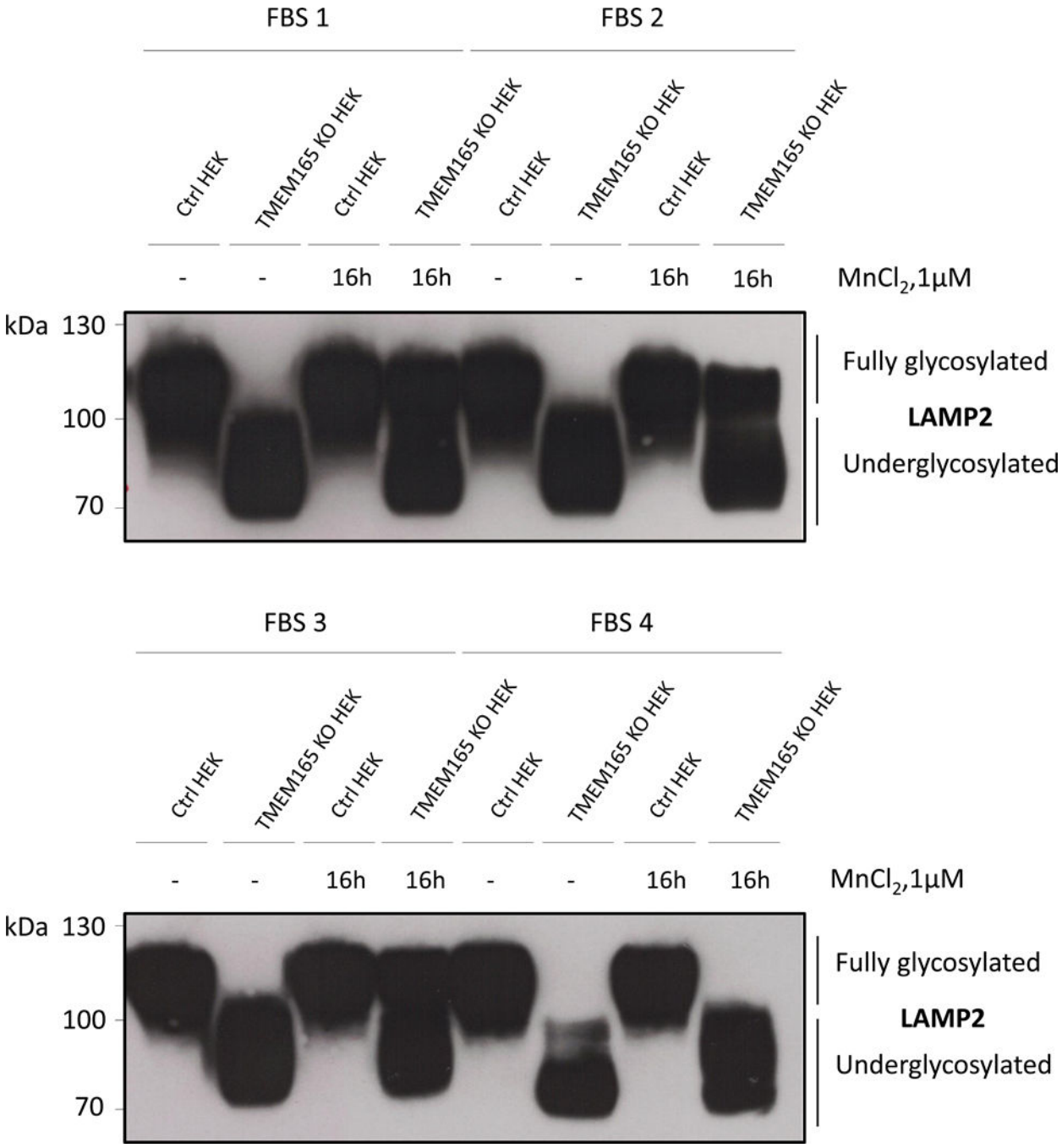


Figure 4: The suppression of LAMP2 glycosylation defect by Mn^{2+} supplementation in TMEM165 KO HEK cells depends on the FBS used for cell culture.

Control and TMEM165 KO HEK cells were cultured in DMEM supplemented with 10% FBS 1, 2, 3 or 4. Cells were incubated with or without 1 µM $MnCl_2$ for 16h. Total cell lysates were prepared, subjected to SDS-PAGE and western blot with the indicated antibody.

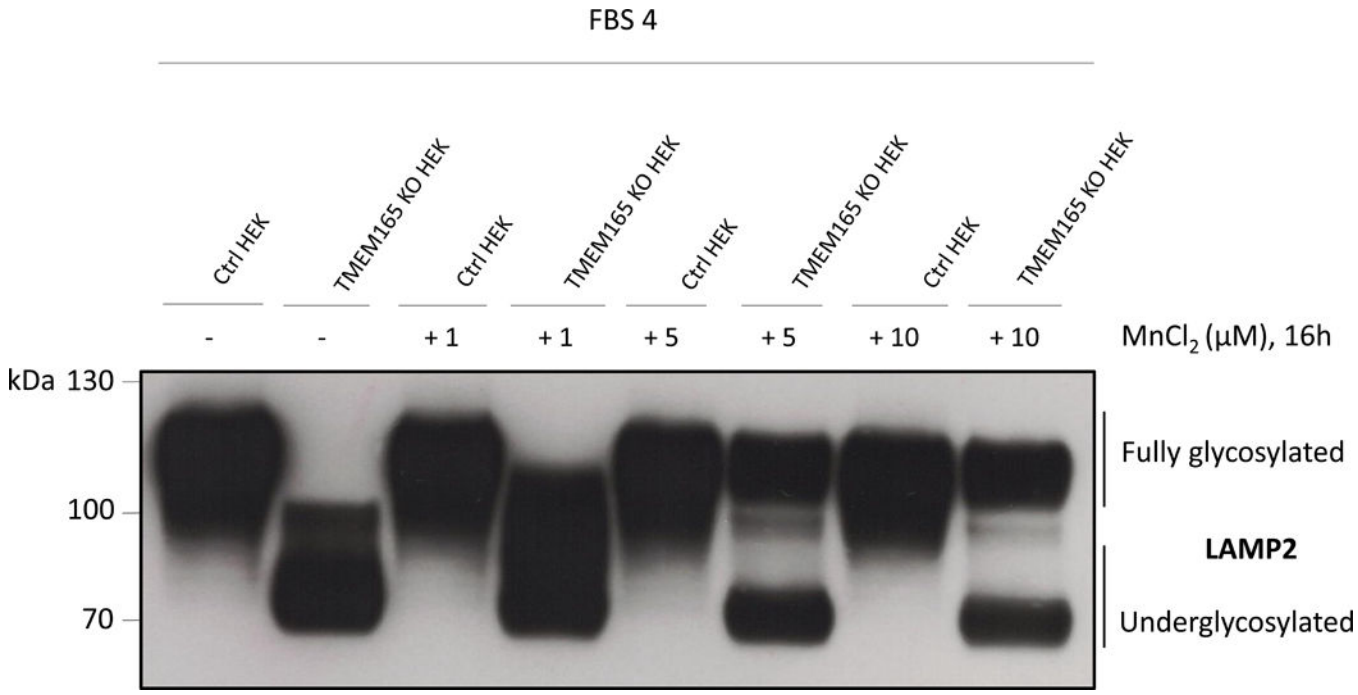


Figure 5: Increasing concentrations of $MnCl_2$ are required to rescue LAMP2 glycosylation defect in TMEM165 KO HEK cells cultured with FBS 4.

Control and TMEM165 KO HEK cells were cultured in DMEM supplemented with 10% FBS 4. Cells were incubated with either 1, 5 or 10 µM $MnCl_2$ for 16h. Total cell lysates were prepared, subjected to SDS-PAGE and western blot with the indicated antibody.

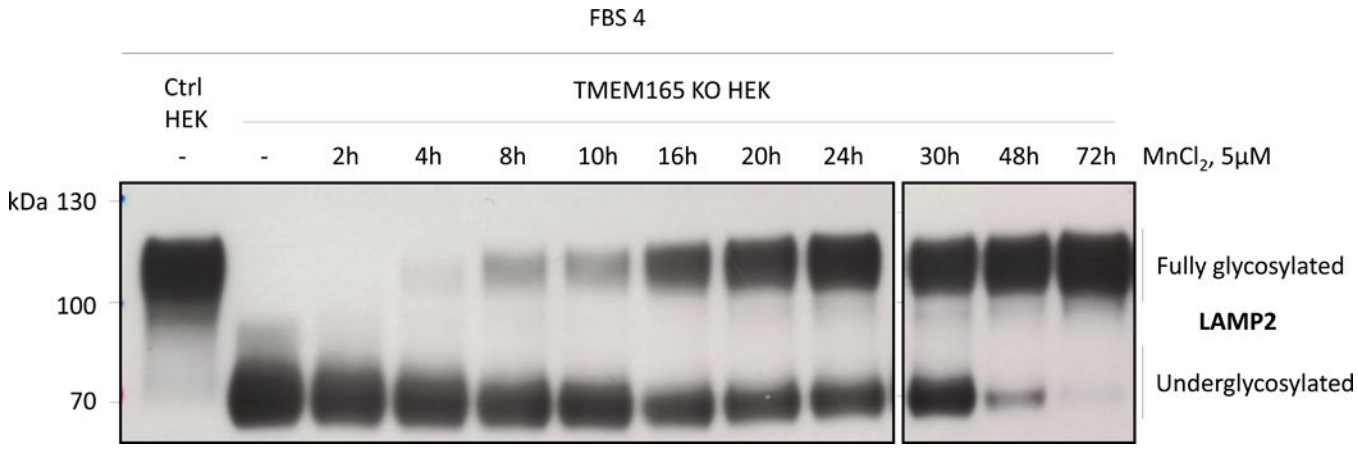


Figure 6: Time course of the Mn^{2+} induced LAMP2 glycosylation rescue in FBS 4.

Control and TMEM165 KO HEK cells were cultured in DMEM supplemented with 10% FBS 4. Cells were incubated with 5 µM $MnCl_2$ during indicated times. Cell culture medium was renewed every 16h to 24h. Total cell lysates were prepared, subjected to SDS-PAGE and western blot with the indicated antibody.

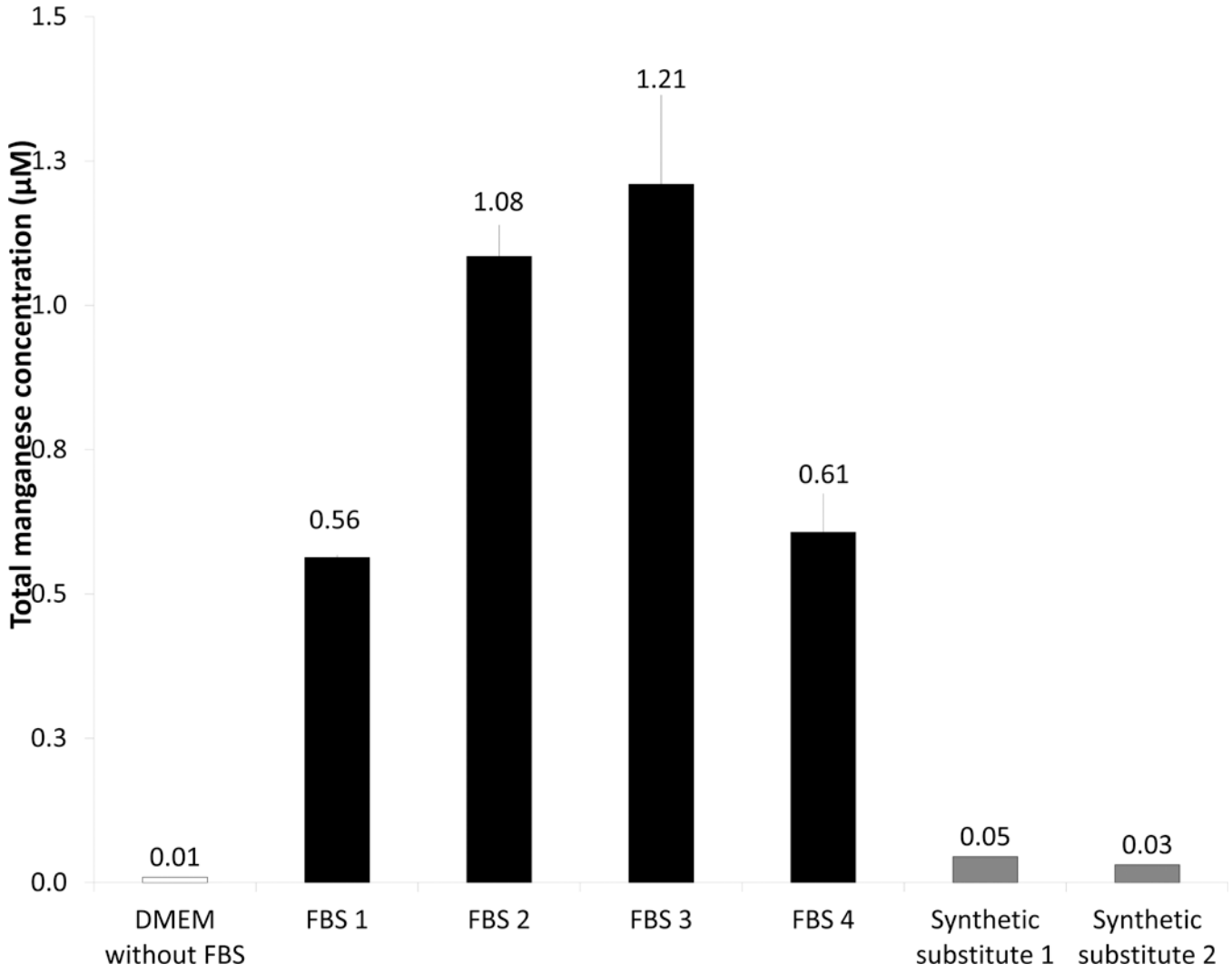


Figure 7: ICP-MS Mn^{2+} levels differ according to different FBS sources.

500 µL of DMEM and 500 µL of each FBS were prepared as described in Material and Methods section for ICP-MS analysis and total manganese concentration was measured.

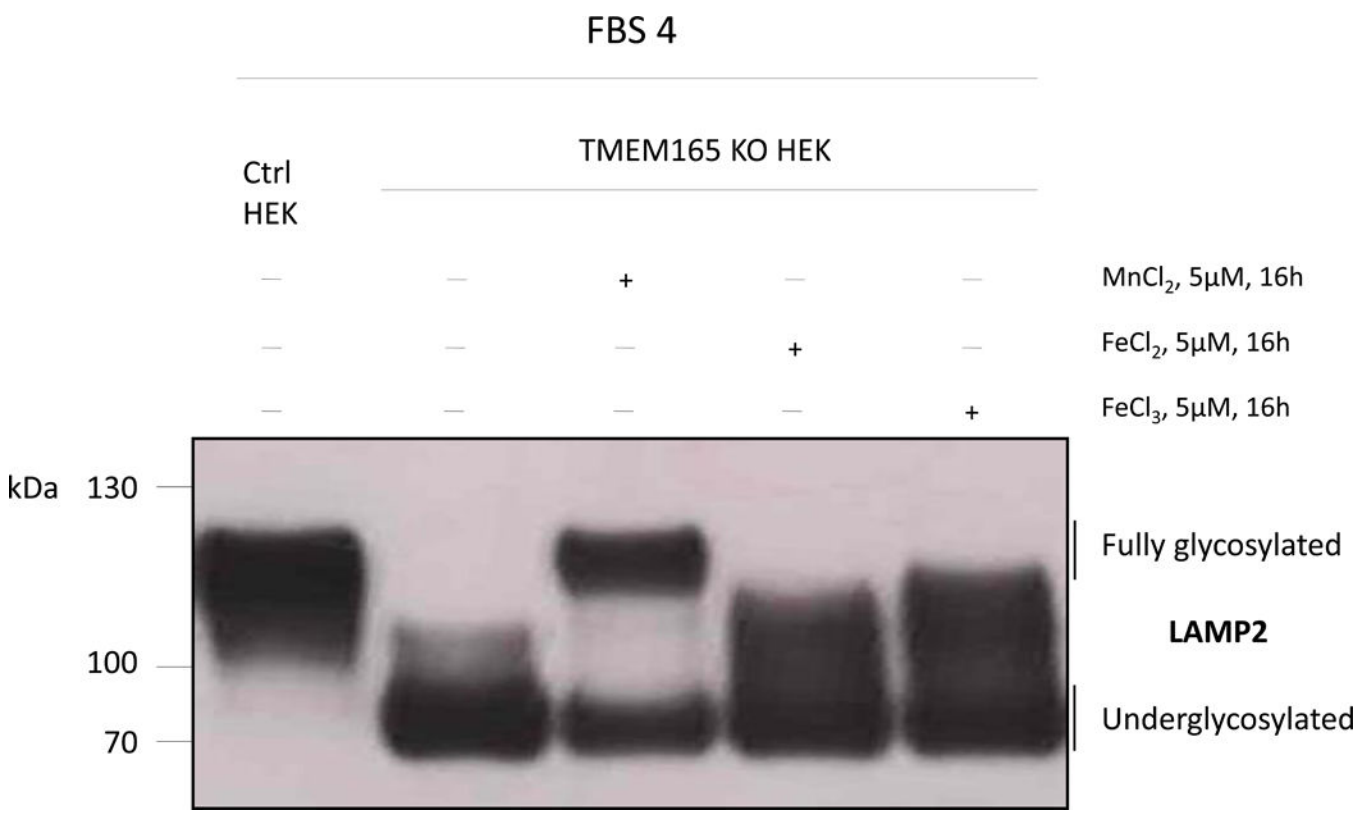


Figure 8: Comparative efficacy of Mn^{2+}, Fe^{2+} and Fe^{2+} addition on the suppression of the LAMP2 glycosylation defects.

Control and TMEM165 KO HEK cells were cultured in DMEM supplemented with 10% FBS 4. Cells were incubated with or without 5 µM $MnCl_2$, 5 µM $FeCl_2$ and 5 µM $FeCl_3$ for 16h. Total cell lysates were prepared, subjected to SDS-PAGE and western blot with the indicated antibody.

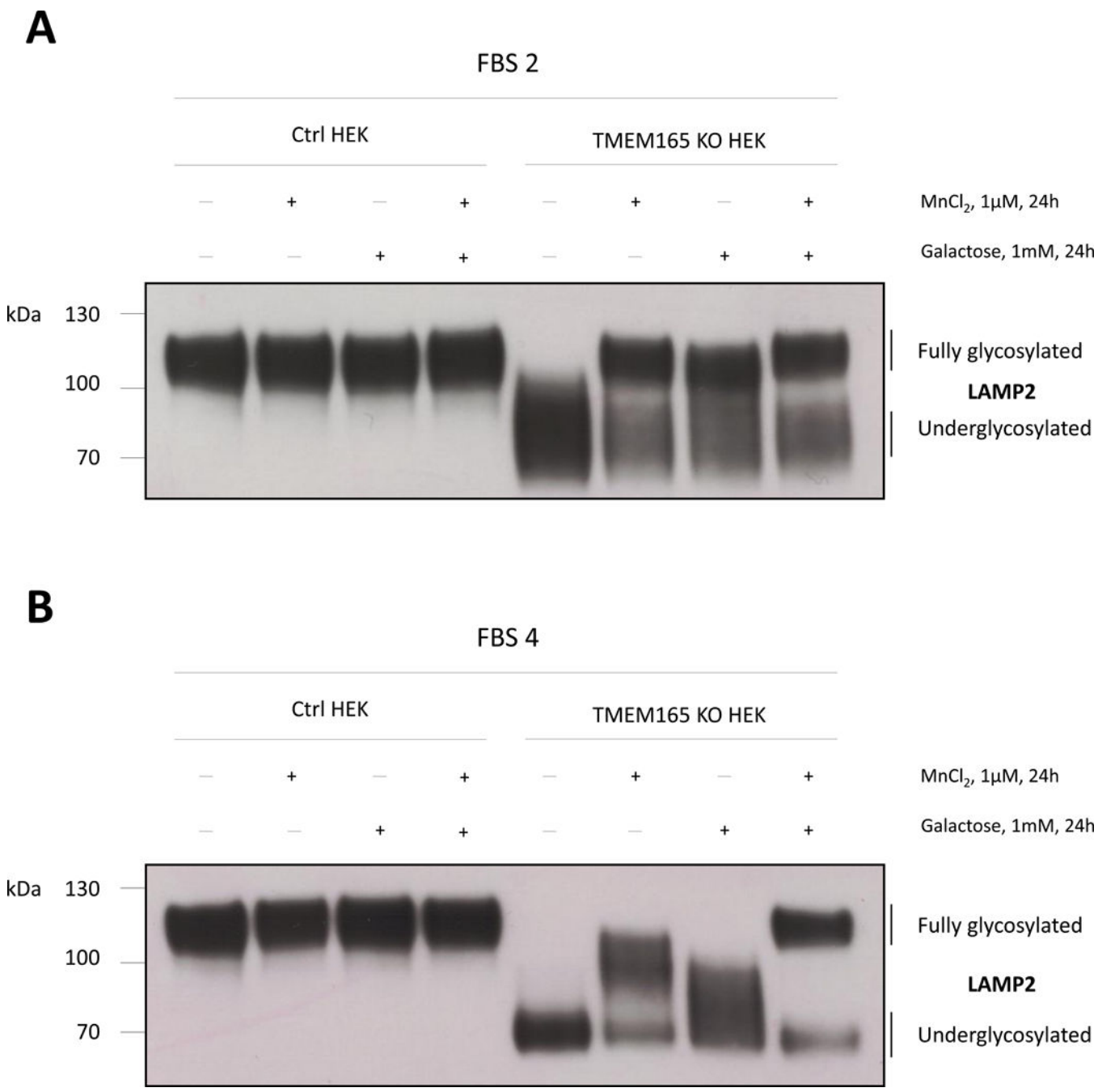


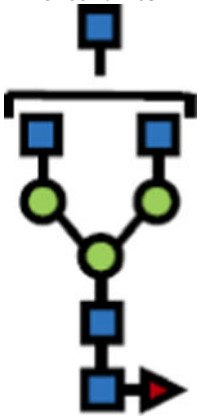
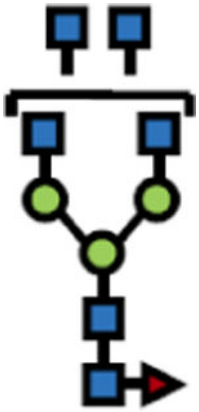
Figure 9: The suppression of the LAMP2 glycosylation defects by galactose supplementation depends on the FBS used for cell culture.

Control and TMEM165 KO HEK cells were cultured in DMEM supplemented with 10% FBS 2 (panel **A**) or 4 (panel **B**). Cells were incubated with or without 1 µM $MnCl_2$ and 1mM galactose for 24h. Total cell lysates were prepared, subjected to SDS-PAGE and western blot with the indicated antibody.

Table 1:

Comparison of relative intensity of specific ions (m/z) observed in control and TMEM165 kO HEK cells cultured with different FBS.

Symbols represent sugar residues as follow: blue square, N-acetylglucosamine; green circle, mannose; red triangle, fucose. Linkages between sugar residues have been removed for simplicity

	Ion at m/z 1591	Ion at m/z 1836	Ion at m/z 2081	Ion at m/z 2326
				
A (Ctrl FBS 1)	27 %	29 %	25 %	13 %
B (Ctrl FBS 2)	19 %	26 %	27 %	13%
C (Ctrl FBS 3)	29 %	30 %	28 %	13%
D (Ctrl FBS 4)	22 %	24 %	28 %	10 %
E (KO FBS 1)	31 %	50 %	56 %	34 %
F (KO FBS 2)	24 %	39 %	42 %	23 %
G (KO FBS 3)	24 %	38 %	43 %	18 %
H (KO FBS 4)	**36 %**	**56 %**	**77 %**	**58 %**