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Forkhead box transcription factors Foxa1 and Foxa2 are important regulators of Muc2 mucin expression in intestinal epithelial cells

Maria van der Sluis^{a,1}, Audrey Vincent^{c,1}, Janneke Bouma^a, Anita Korteland-Van Male^{a,b}, Johannes B. van Goudoever^a, Ingrid B. Renes^a, Isabelle Van Seuning^{c,*}

^a Department of Paediatrics, Division of Neonatology, Erasmus MC-Sophia Children's Hospital, Rotterdam, The Netherlands

^b Department of Paediatrics, Division of Gastroenterology, Erasmus MC-Sophia Children's Hospital, Rotterdam, The Netherlands

^c Department of Paediatrics, Inserm, U837, Centre de Recherche Jean-Pierre Aubert, Place de Verdum, 59045 Lille, France

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ABSTRACT

The mucin Muc2 is the main component of the intestinal mucus layer and thus plays important roles in intestinal protection. Therefore, it is important to understand its regulation during goblet cell differentiation. Foxa1 and Foxa2 forkhead box transcription factors (TFs) participate in transcriptional programs governing intestinal cell differentiation. Using immunohistochemistry, we showed a spatio-temporal pattern of expression of both TFs in developing and adult mouse intestine and their expression in Muc2-expressing intestinal cells. Down-regulation of Foxa1 and Foxa2 by RNA interference in cultured intestinal cells decreased *Muc2* mRNA level by half, and abolished Muc2 protein expression. Chromatin immunoprecipitation and gel shift assays showed that these two TFs directly bind to the *Muc2* promoter. Co-transfection experiments indicated that both TFs activate the *Muc2* promoter and that mutations of three Foxa *cis*-elements inhibit *Muc2* transactivation. In conclusion, this work identifies Foxa1 and Foxa2 as important regulators of *Muc2*-expression in the intestine.

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The mucin Muc2 is the main protein component of intestinal mucus, which provides a physical barrier between epithelium and luminal content [1,2]. The protective role of Muc2 is definitively proven by the development of spontaneous colitis and intestinal tumours in Muc2 knockout mice [3]. Since regulatory systems, which are active during development, are often reactivated in cancer, it is hypothesized that they could be responsible for aberrant MUC2-expression in intestinal cancers and other intestinal disorders [2].

In humans, *MUC2* mRNA is expressed during embryonic development of the intestine, in a specific spatio-temporal pattern of expression [4]. Moreover, Muc2-expression is associated with differentiation of goblet cells and has been identified as a marker of these cells [3]. Altogether, this suggests that *Muc2* gene transcription is under the influence of transcription factors (TFs) that regulate the differentiation of endoderm-derived cell types during intestinal development and more particularly goblet cell differentiation.

Such TFs include families of the caudal-related homeobox (CDX), GATA, and hepatocyte nuclear factors (HNF-3), also known as forkhead box A (FOXA), TFs [2,5–7]. Their participation in maintaining a functional epithelium is the result of a tight spatio-temporal regulation of cell- and/or tissue-specific genes, among which we recently identified both human *MUC2* and murine *Muc2* as targets of CDX1, CDX2 and Gata-4, respectively [8,9]. However, no information regarding *Muc2* regulation by Foxa TFs is available. Foxa1 and Foxa2 play important roles in definitive endoderm formation during gastrulation and differentiation of gut endoderm tissues [7,10]. *Foxa1* and *Foxa2* mRNAs have been found expressed in embryonic endoderm and adult intestine [6,7,10–12]. These TFs belong to the winged helix/forkhead class of DNA binding proteins, possessing highly conserved DNA binding domains [12]. As transcription activators, Foxa TFs bind the DNA consensus sequence 5'-GATTATTGACTT-3' as monomers [13,14]. In a previous study, in which we identified Gata-4 as an important regulator of the murine *Muc2* gene, we also found a Foxa1/Foxa2 *cis*-element in the close vicinity of the Gata-4 site [9]. Knowing that these two families of TFs may synergize to regulate intestine-specific target genes [15–18], we further analyzed the *Muc2* promoter sequence, and found three more putative Foxa binding sites within the promoter.

* Corresponding author. Fax: +33 320 53 85 62.

E-mail address: isabelvs@lille.inserm.fr (I. Van Seuning).

¹ Both authors contributed equally to this work.

Altogether these data suggested that Foxa TFs may be part of the complex transcriptional machinery driving the spatio-temporal expression of *Muc2* in the intestine. In order to test this hypothesis, we undertook to (i) investigate Foxa1, Foxa2, and *Muc2*-expression during differentiation of goblet cells in the developing and adult mouse intestine, (ii) study *Muc2* regulation by Foxa1 and Foxa2, and (iii) evaluate a possible synergy with Gata-4.

Materials and methods

Animals. Pregnant Balb/c mice (Charles River, Maastricht, The Netherlands) were housed as in [16]. Pregnant females were sacrificed by cervical dislocation, embryos were isolated and the intestine was excised at embryonic (E)15.5, E17.5, E18.5, and postnatal (P)1.5 days and adults. The small intestine was fixed in 4% paraformaldehyde in PBS and prepared for light microscopy. All experiments were performed with the approval of the Animal Studies Ethics Committee of the Erasmus Medical Centre (Rotterdam, The Netherlands).

Immunohistochemistry. Five micrometers thick paraffin sections were cut and processed for immunohistochemical stainings [9]. Anti-*Muc2* (sc-15334), anti-Foxa1 (sc-6553X), and anti-Foxa2 (sc-9187X) antibodies (1:1000; Santa Cruz Biotechnology, Tebu-Bio, Le Perray en Yvelines, France) were incubated overnight at 4 °C. To visualize goblet cells, sections were stained with Alcian Blue 8GX (BDH, Brunschwig Chemie, Amsterdam, The Netherlands).

Cell culture, total cellular and nuclear extract preparations. The *Muc2*-expressing murine rectal cancer cell line CMT-93 was cultured as in [9], nuclear extracts were prepared as in [19] and total cellular extracts as in [20]. Protein content (2 µl) was measured using the bicinchoninic acid method (Pierce, Bezons, France). *Muc2* (1:100) Western-blotting was carried out as in [20].

Small interfering RNA (siRNA) assays. CMT-93 cells were seeded 24 h before transfection in 24-well tissue culture plates at a density of 23×10^3 cells/well in antibiotic free medium. Cells were transfected as in [20] with 100 nM of either mouse Gata-4, Foxa1, or Foxa2 SMARTpool® siRNAs, or combinations using 1 µl of DharmaFECT™ two transfection reagent (Dharmacon, Brebières, France). Controls included mock transfected cells, cells transfected with either siCONTROL™ Gapdh or Non-Targeting siRNAs. Each siRNA was assayed in triplicate in at least three experiments. Total RNA was isolated using the Nucleospin RNA II kit (Macherey-Nagel, Hoerd, France) and used (1 µg) to prepare cDNA using oligod(T) (1 µl) and recombinant reverse transcriptase M-MLV (1 µl) (Promega, Charbonnières, France). PCR was performed on cDNA (5 µl) using specific primers (MWG-Biotech, Ebersberg, Germany) for mouse *Muc2* mucin gene and mouse *Gapdh* [8]. Densitometric analysis of DNA bands was carried out using the GelAnalyst-GelSmart software (Clara Vision, Orsay, France).

Cell transfection and luciferase assays. Cell transfections and co-transfections were performed as in [9]. All plasmids used for transfection were prepared using the Endofree plasmid Maxi kit (Qiagen, Courtaboeuf, France).

Site-directed mutagenesis. QuickChange site-directed mutagenesis kit (Stratagene, Amsterdam, The Netherlands) was used to generate site specific mutations in the four Foxa binding sites present in the -221/+29 *Muc2* promoter construct. All mutations were confirmed by DNA sequencing using RV3 and GL2 primers (Promega).

Chromatin Immunoprecipitation Assay (ChIP). ChIP assays with CMT-93 cells (1.0×10^8) and PCR conditions were as in [20]. An aliquot of the total supernatant was removed as a control (input) and the rest was divided into fractions to be precipitated with either 4 µg of specific antibodies (anti-Foxa1, anti-Foxa2, anti-Gata-4 (sc-1237X), SantaCruz Biotechnology) or with normal rabbit IgGs (Upstate, Euro-medex, Mundolsheim, France). Samples (50 ng) were then subjected to PCR analysis with primers covering the -269/-31 region of *Muc2* promoter (forward primer: 5'-AAAGGAATGGAGTTAGTTCAC-3' and reverse primer: 5'-CTTAGCAGGCATCCG AGG-3'). Fifteen microliters of the PCR products (239 bp) were analyzed on a 2% agarose gel.

Results and discussion

Foxa1 and Foxa2 are expressed in Muc2-expressing goblet cells of the mouse small intestine

Immunohistochemical studies performed on developing and adult mouse small intestine, showed that *Muc2* was not detectable at E15.5 (Fig. 1A). As from E17.5, goblet cells expressed the mucin *Muc2* (Fig. 1C, E, and H).

At E15.5, Foxa1 protein was uniformly expressed by all cells in the developing gut (not shown), whereas Foxa2 expression was restricted to the pseudostratified epithelium (Fig. 1B). Foxa1 was expressed by all epithelial and goblet cells in developing

crypts and villi, with a slight decrease in staining intensity towards the tip of the villi (Fig. 1F and I showing E18.5 and adult, respectively). This is in agreement with previous work showing the same pattern of Foxa1 expression along the crypt-villus axis [21]. Based on the expression pattern of Foxa1 within the intestine, and its function as regulator of p27, cyclinD1, and E-cadherin expression [22,23], Foxa1 may be involved in the regulation of the balance between intestinal epithelial proliferation and differentiation. Moreover, binding of Foxa1 facilitates opening of the chromatin, and therefore enhances binding of cofactors and/or repressors [23].

From E17.5 until adulthood the expression of Foxa2 showed a spatio-temporal pattern. More specifically, Foxa2 was observed in the nuclei of the intervillus region (from E17.5 till P1.5, Fig. 1D, G, and Supplemental data), in the crypts (adult mice, Fig. 1J) and in goblet cells (Alcian blue staining of glycoproteins in secretory granules) along the villi (Fig. 1D, G, and J). Foxa2 expression in the region where intestinal stem cells and proliferating cells reside, namely in the intervillus/crypt region, suggests involvement of Foxa2 in maintaining epithelial cells in an undifferentiated state probably to stimulate epithelial proliferation. This hypothesis is supported by results showing that isoforms of the winged helix transcription factors, more specifically, forkhead box homolog 11A and -11B, induced proliferative signals [13]. Localization of Foxa2 in the crypts of the small intestine also corresponds to results previously described [21]. Our results indicate that Foxa1 and Foxa2 are expressed in intestinal cells along the villi with distinct patterns. Very interestingly, whereas Foxa1 was found expressed in nuclei of all cell types, Foxa2 expression was detected almost exclusively in differentiated goblet cells. This strongly suggests that Foxa2 is also an important actor of goblet cell differentiation.

Foxa1 and Foxa2 regulate endogenous Muc2-expression

Since we found that Foxa1 and Foxa2 were expressed in intestinal goblet cells, a cell type for which the mucin *Muc2* is a phenotypic marker, we undertook to study *Muc2* regulation by these two TFs. *Muc2* transcriptional regulation by Foxa1 and Foxa2 was studied in the murine rectal cancer cell line CMT-93, which expresses *Muc2* mRNA (Supplemental data) as well as Foxa1 and Foxa2 proteins (Supplemental data). Down-regulation of Foxa1 and Foxa2, by siRNA, respectively, resulted in a 60% and 40% decrease of the amount of *Muc2* mRNA (Fig. 2A) and abolished *Muc2* protein expression (Fig. 2B). Altogether, this shows that Foxa1 and Foxa2 regulate endogenous *Muc2*-expression.

To identify Foxa responsive regions within *Muc2* promoter, co-transfection experiments were carried out in the presence of the *Muc2*-pGL3 promoter construct -221/+29 (containing three putative Foxa binding sites and the Foxa1/Foxa2 cis-element previously identified [9], Fig. 2C) and Foxa1 (black bars) or Foxa2 (gray bars) expression vectors. The luciferase diagram indicates that overexpression of Foxa1 and Foxa2 resulted in a 6- and 4-fold increase of *Muc2* promoter activity, respectively, demonstrating that both Foxa1 and Foxa2 induce *Muc2* transcription.

Foxa1 and Foxa2 interact with chromatin encompassing the Muc2 promoter by binding to two cis-elements

ChIP assays using specific PCR primers covering the -269/-31 region of the *Muc2* promoter indicate that Foxa1 and Foxa2 bind to the *Muc2* promoter *in vivo* (Fig. 2D). By EMSA we then showed that the two TFs bind to the two cis-elements at -180/-175 (T262, Foxa dist.) and -150/-144 (T275, Foxa ext.) (Supplemental data).

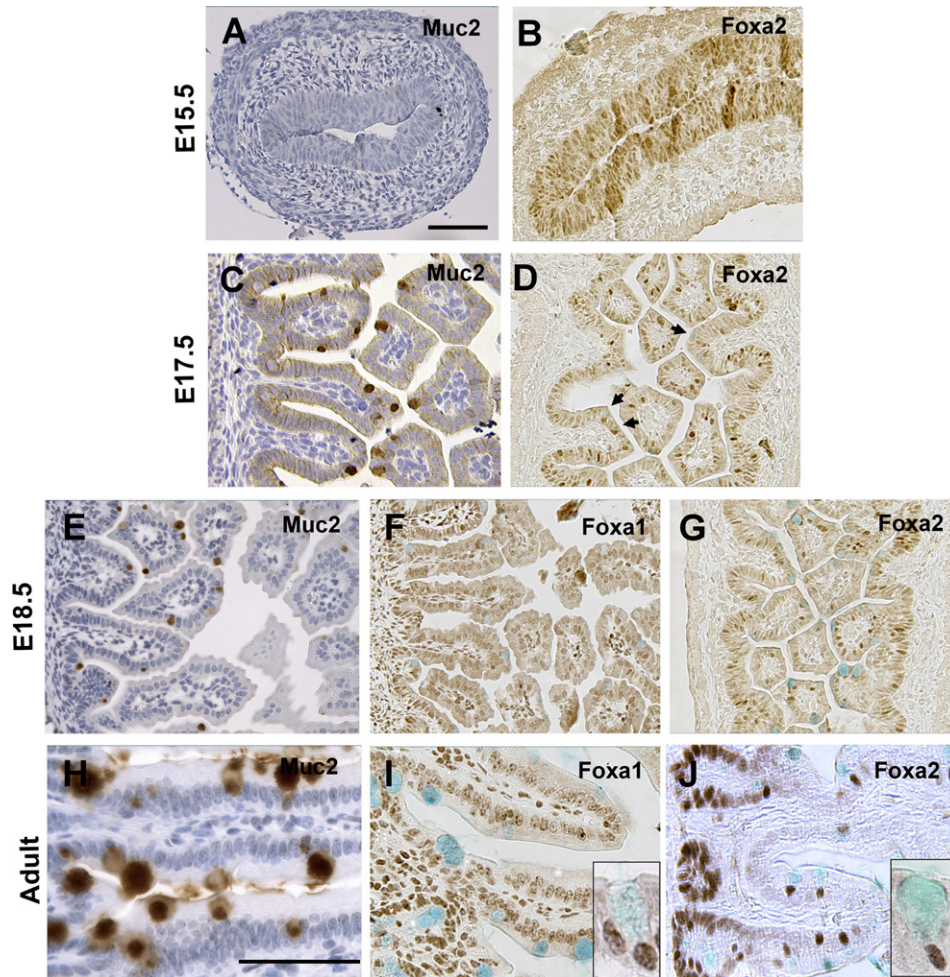


Fig. 1. Spatio-temporal expression of Muc2, Foxa1 and Foxa2 in the developing and adult mouse small intestine. Muc2 (A and C) and Foxa2 (B and D) at E15.5 and E17.5, respectively. Muc2, Foxa1, and Foxa2 stainings of E18.5 (E–G), and adult (H–J) small intestine. Inserts show a close-up of a goblet cell stained with Foxa1 (I) and Foxa2 (J). Magnifications in A–G and H–J are equal, respectively, bar representing 75 μ m.

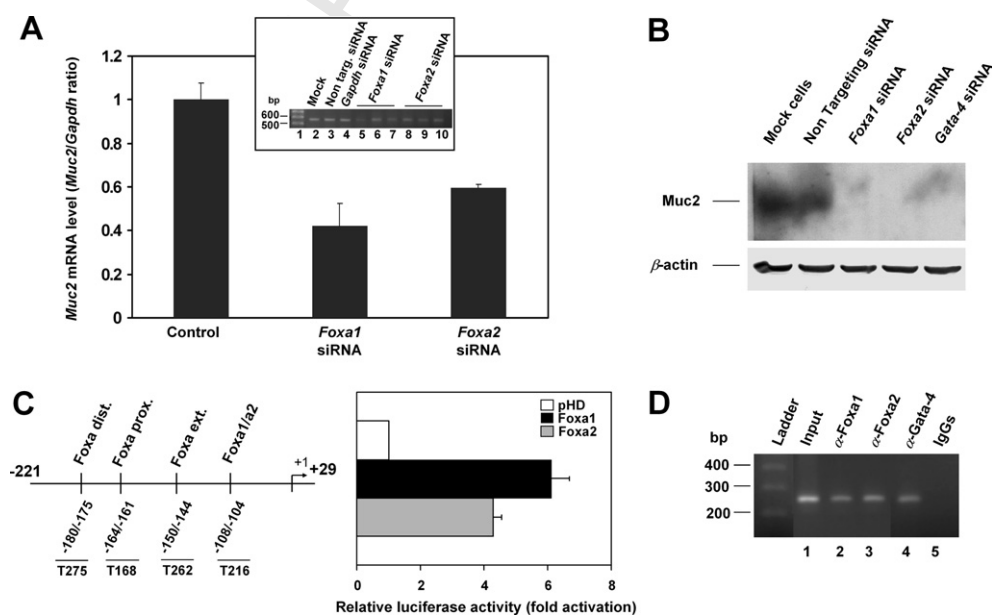


Fig. 2. Foxa1 and Foxa2 regulate endogenous *Muc2*-expression in CMT-93 cells. (A) *Muc2* mRNA level and (B) *Muc2* protein expression in CMT-93 cells transfected with *Foxa1* and *Foxa2* siRNAs. A representative example of the RT-PCR results is shown in A. (C) Luciferase diagram showing the effects of overexpression of Foxa1 (black bar) or Foxa2 (gray bar) on *Muc2* promoter activity. All results represent means \pm SD obtained in triplicate in at least three separate experiments. (D) ChIP analysis of Foxa1 (lane 2), Foxa2 (lane 3), Gata-4 (lane 4), and rabbit IgGs (lane 5) binding to the *Muc2* promoter. Input (lane 1).

213 *Gata-4 and Foxa transcription factors do not synergize to activate the*
214 *Muc2 promoter*

215 In order to establish which Foxa binding site(s), among those
216 identified, were playing a critical role in regulating *Muc2* transcrip-
217 tion, we made single Foxa mutants (Fig. 3A). Mutations of single
218 Foxa sites within the promoter indicated that mutations at sites
219 –164/–161, –150/–144, and –108/–104 led to complete inhibi-
220 tion of transactivation of the *Muc2* promoter by both Foxa1 and
221 Foxa2 (Fig. 3A). Mutation of the most distal Foxa site at –180/
222 –175 led to 60% (Foxa1) and 55% (Foxa2) inhibition of *Muc2* pro-
223 moter activity, respectively. This indicates that each of the Foxa
224 sites is essential for efficient transactivation of the *Muc2* promoter
225 both by Foxa1 and Foxa2.

226 Having previously shown that Gata-4 was an important regula-
227 tor of *Muc2* [9], that down-regulation of Gata-4 by siRNA inhibited
228 *Muc2* protein expression (this report, Fig. 2B) and that Foxa and

229 Gata-4 TFs bind to *cis*-elements close to each other within the
230 *Muc2* promoter, we hypothesized that they could synergize. In
231 order to show this, we made double Gata-4/Foxa mutants. First, sin-
232 gle mutations of Gata sites within the promoter indicated that
233 mutations at sites –168/–165 and –158/–155 (T168) did not lead
234 to full inhibition of *Muc2* promoter transactivation by Gata-4 (55%
235 remaining, Fig. 3B, black bar). This suggested that the most proximal
236 Gata-4 site (–99/–96) was also active. Indeed, when mutating
237 this site, the transactivating effect of Gata-4 on the –221/+29 pro-
238 moter construct was completely lost.

239 Since mutation of the T168 Gata-4 binding site did not lead to
240 full inhibition of *Muc2* promoter activity, double mutants of the
241 T168 Gata site with each of the Foxa elements, were made to assess
242 whether double mutations could lead to total inhibition of the pro-
243 moter activity (Fig. 3C). Similar profiles of *Muc2* promoter transac-
244 tivation were observed with double mutants #1, #2, and #4.
245 Mutations of these Gata-4/Foxa binding sites led to total abroga-

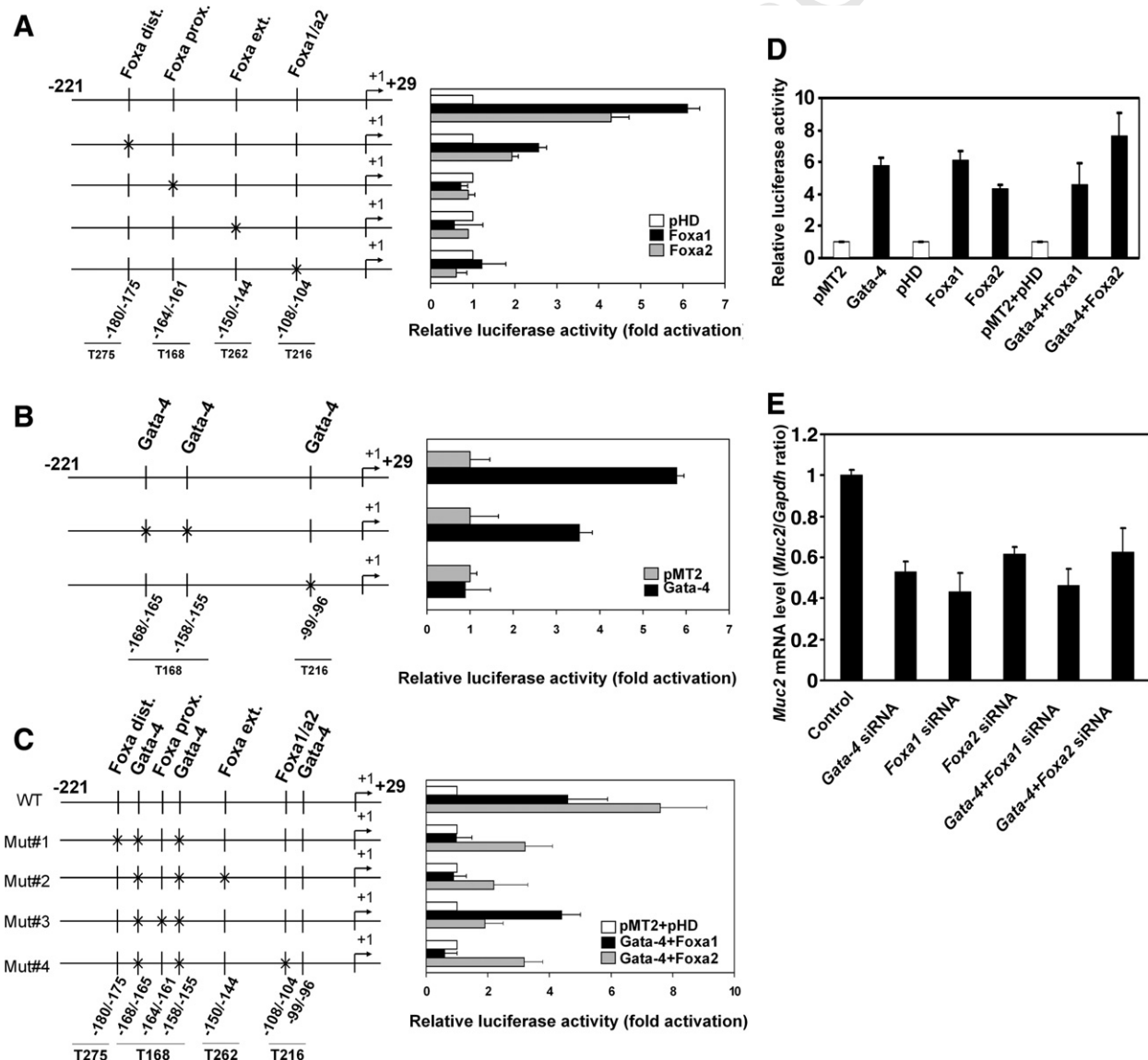


Fig. 3. Gata-4 and Foxa transcription factors do not synergize to activate *Muc2*-expression. (A) Effect of overexpression of Foxa1 (black bars) or Foxa2 (gray bars) or Gata-4 (B) on wt *Muc2* promoter activity and on the *Muc2* promoter bearing either single Foxa mutations (A) or Gata-4 mutations (B) as indicated. (C) Effect of overexpression of Gata-4/Foxa1 (black bars) or Gata-4/Foxa2 (gray bars) combinations on wt *Muc2* promoter activity and on the *Muc2* promoter bearing double Gata/Foxa mutations as indicated. (D) Effect of overexpression of Gata-4, Foxa1 or Foxa2 alone or in combinations on wt *Muc2* promoter activity. Results are expressed as fold activation of luciferase activity in samples co-transfected with the transcription factor of interest compared with the control co-transfected with the corresponding empty vector(s) (white bars). (E) Expression of *Muc2* mRNA in CMT-93 cells transfected with siRNAs specific for *Gata-4*, *Foxa1* or *Foxa2* alone or in combination. All results represent means \pm SD obtained in triplicate in at least three separate experiments.

tion of the transactivation in the presence of Gata-4/Foxa1 (black bars) and partial inhibition (25% remaining) in the presence of Gata-4/Foxa2. This indicates that in these mutants, Gata-4 mutation was preponderant in the presence of Foxa2 when compared to single mutations, whereas it was Foxa mutation that was responsible for the loss of the promoter activity in the presence of Foxa1. Mutant #3 gave a different profile of *Muc2* promoter activity. In that case, promoter activity was similar in the presence of Gata-4/Foxa1 (black bar), whereas 25% remained in the presence of Gata-4/Foxa2 (gray bar) when compared to wild-type (WT) constructs. Compared to single mutants this suggests that some promoter activity was restored in the presence of Foxa1 (black bar), whereas no additive effect was visualized for Gata-4/Foxa2 combination (gray bar).

Cooperation between Gata-4 and Foxa1 or Foxa2 was then studied by co-transfection experiments (Fig. 3D) and by siRNA approach (Fig. 3E). Overexpression of Gata-4, Foxa1 or Foxa2 TFs resulted in an increased luciferase activity of the -221/+29 *Muc2* promoter construct (4- to 6-fold activation, Fig. 3D). No synergistic effect could be visualized when co-transfecting Gata-4 in combination with either Foxa1 or Foxa2. The same absence of synergy was observed in siRNA experiments since double silencing of *Gata-4* with *Foxa1* or *Foxa2* did not lead to increased repression of *Muc2* mRNA expression (40–60% decrease) when compared with single siRNAs (Fig. 3E). Altogether, these studies demonstrate that Gata-4 and Foxa1/Foxa2 do not synergize to induce either *Muc2* promoter activity or *Muc2* mRNA expression.

Our study shows that Foxa1 and Foxa2 each have the individual capacity of activating *Muc2*-expression. Foxa1 has been described as a poor activator of gene transcription compared to Foxa2, and competition has been suggested as they can bind with the same DNA binding sequence [24]. In our studies both Foxa1 and Foxa2 were shown to equally bind the *Muc2* promoter, but functional studies at the protein, RNA and promoter levels indicated that Foxa1 was slightly more efficient as a *Muc2* activator. Gata, HNF, Foxa, and Cdx TFs are known to be able to act in synergy to regulate intestinal cell-specific genes [15–18]. Recruitment of specific combinations of these TFs at the promoter level will then occur and evolve as intestinal cells migrate to the villi and differentiate. In this report we could not show synergistic effect between Gata-4 and Foxa TFs. Foxa binding has been proposed to decompact chromatin and facilitate binding of other TFs, and thereby promote gene activation [12]. However, this does not seem to be the case for *Muc2* in regard to Gata-4. The absence of synergy despite the close localization of the Gata-4 and Foxa1/Foxa2 *cis*-elements within the *Muc2* promoter therefore indicates that these TFs will most likely act as competitors to regulate *Muc2* transcription. Consequently, this also suggests that the amount of each transcription factor in the cell will be critical and will determine which will be controlling *Muc2* transcription.

In summary, we identified Foxa1 and Foxa2 as important regulators of *Muc2* gene expression, and showed that Foxa1 and Foxa2 (i) are both expressed in *Muc2*-expressing goblet cells in the mouse small intestine, (ii) are both potent activators of *Muc2* promoter activity, (iii) mediate *Muc2* regulation by directly interacting with their cognate elements within the *Muc2* proximal promoter, and (iv) are both required for endogenous expression of *Muc2*. Our results identify *Muc2*, a goblet cell marker, as a new target gene of Foxa1 and Foxa2 and point out an important role for these transcription factors in *Muc2* mucin expression in the intestine during goblet cell differentiation.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2008.02.158.

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