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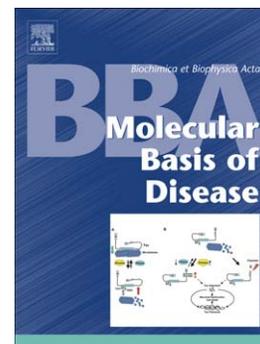
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***GALNT3*, a gene associated with Hyperphosphatemic Familial Tumoral Calcinosis, is transcriptionally regulated by extracellular phosphate and modulates matrix metalloproteinase activity**

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SUMMARY

GALNT3 encodes UDP-N-acetyl-alpha-D-galactosamine: polypeptide N-acetylgalactosaminyl-transferase 3 (ppGalNacT3), a glycosyltransferase which has been suggested to prevent proteolysis of FGF23, a potent phosphaturic protein. Accordingly, loss-of-function mutations in *GALNT3* cause hyperphosphatemic familial tumoral calcinosis (HFTC), a rare autosomal recessive disorder manifesting with increased kidney reabsorption of phosphate, resulting in severe hyperphosphatemia and widespread ectopic calcifications. Although these findings definitely attribute a role to ppGalNacT3 in the regulation of phosphate homeostasis, little is currently known about the factors regulating *GALNT3* expression. In addition, the effect of decreased *GALNT3* expression in peripheral tissues has not been explored so far. In the present study, we demonstrate that

GALNT3 expression is under the regulation of a number of factors known to be associated with phosphate homeostasis, including inorganic phosphate itself, calcium and 1,25-dihydroxyvitamin D₃. In addition, we show that decreased *GALNT3* expression in human skin fibroblasts leads to increased expression of FGF7 and of matrix metalloproteinases, which have been previously implicated in the pathogenesis of ectopic calcification. Thus, the present data suggest that ppGalNacT3 may play a role in peripheral tissues of potential relevance to the pathogenesis of disorders of phosphate metabolism.

INTRODUCTION

Inorganic phosphate (Pi) is an element of essential importance for skeletal mineralization, mineral homeostasis, and a wide range of cellular processes involving signaling and synthesis of energy-generating compounds. Pi dietary intake, renal and intestinal absorption and exchange with intracellular and bone storage pools, are tightly regulated to maintain its serum level within a narrow range [1]. Elevated Pi levels have been linked to increased mortality in patients with end-stage or even early stage chronic kidney disease [2, 3]. Moreover, serum Pi levels in the upper normal range were found to be associated with a significant increase in mortality among individuals with normal renal function [4].

Although hyperphosphatemia is associated with many common acquired conditions[5], only a few inherited (monogenic) disorders manifesting with increased serum Pi levels are known [6]. Hyperphosphatemic familial tumoral calcinosis (HFTC; MIM211900) is a rare autosomal recessive condition characterized by the progressive deposition of basic calcium phosphate crystals in peri-articular spaces and soft tissues. HFTC is associated with marked hyperphosphatemia in the face of inappropriately normal parathyroid (PTH) and normal or slightly elevated levels of 1,25-dihydroxyvitamin D ($1,25(\text{OH})_3\text{D}$) [7]. HFTC may result from mutations in three different genes, encoding proteins which are part of a common metabolic pathway. The first mutations associated with HFTC were initially reported in *GALNT3* [8-15], which encodes UDP-N-acetyl-

alpha-D-galactosamine: polypeptide N-acetylgalactosaminyl-transferase 3 (ppGalNacT3), a glycosyltransferase that initiates mucin-type O-glycosylation[16]. Loss of function mutations in the fibroblast growth factor 23 (*FGF23*) gene were subsequently found to also cause HFTC [17-20]. *FGF23* codes for a circulating molecule that promotes renal phosphate excretion by decreasing phosphate reabsorption in the proximal renal tubule. In addition, FGF23 further contributes to lowering circulating Pi levels by down-regulating the biosynthesis and increasing the catabolism of 1,25(OH)₃D [21, 22], resulting in decreased 1,25(OH)₃D levels. ppGalNacT3 was shown *in vitro* to be essential for FGF23 processing and secretion; moreover, decreased ppGalNacT3 activity was found to result in decreased levels of active FGF23 in the circulation [23-25]. Recently, a mutation in a third gene, *KL*, was found to cause a phenotype very much resembling HFTC [26, 27]. *KL* encodes Klotho, which functions as a co-receptor for FGF23 [28]. Although it is clear that ppGalNacT3, FGF23 or Klotho deficiency affects Pi homeostasis systemically through dysregulation of phosphate reabsorption in the kidney (and possibly intestinal mucosa [29]), little is currently known about non-systemic, kidney-independent, features of ppGalNacT3 regulation and metabolic effects. Interestingly, ectopic calcification in HFTC is prominently (although not exclusively) observed in cutaneous and subcutaneous tissues [7]. To investigate these aspects of ppGalNacT3 physiological roles, we used *in vitro* systems and identified Pi as a major regulator of *GALNT3* gene expression. In addition, we found out that as a consequence of decreased ppGalNacT3 expression, fibroblasts

release increased amounts of matrix metalloproteinases (MMPs), which have been previously implicated in the pathogenesis of ectopic calcification [30-33]. These data highlight the possible role of ppGalNacT3 in peripheral tissues

MATERIALS AND METHODS

Materials

Parathyroid Hormone Fragment 1-34 (PTH), $1\alpha,25$ Dihydroxyvitamin D₃, calcium chloride, and sodium phosphate dibasic dehydrate were obtained from Sigma (St Louis, USA). Phosphonoformic acid (PFA) was purchased from MBL International (Woburn, MA, USA). FGF7 was purchased from Pepro Tech (Rocky Hill, NJ, USA).

Cell cultures

Primary fibroblast cell cultures were derived from punch biopsies obtained from patients or healthy controls after having received their written and informed consent according to a protocol reviewed and approved by our Institutional Review Board. Primary human fibroblasts immortalized with a hTERT-expressing retroviral vector (hTERT-fibroblasts) [34] were kindly provided by Dr. Sarah Selig. All cells were maintained in DMEM-low glucose medium (Biological Industries, Beit Haemek, Israel) supplemented with 10% fetal calf serum, 1% L-Glutamine and 1% penicillin/streptomycin. Human embryonic kidney cells (HEK 293) were maintained in DMEM/F12 medium with the same supplements.

Quantitative RT-PCR (qRT-PCR)

For quantitative real-time PCR, cDNA was synthesized from 1 μ g of total RNA extracted from cultured fibroblasts using the Reverse-iT™ first strand synthesis kit (ABgene, Epsom, UK) and random hexamers. cDNA PCR amplification was carried out using the Absolute™ QPCR SYBR® Green Mix (ABgene) on a Rotor-Gene 3000 multi-filter system (Corbett Research, Sydney, Australia) with primer pairs specific for *GALNT3*, *GALNT1*, *GALNT4*, *GALNT6*, *FGF7*, *MMP9*, *MMP8* and *ACTB* (Table 1). To ensure the specificity of the reaction conditions, at the end of the individual runs, the melting temperature (T_m) of the amplified products was measured to confirm their homogeneity. Cycling conditions were as follows: 95°C for 10 min; 95°C for 10 sec, 62°C for 25 sec and 72°C for 15 sec for a total of 40 cycles. Each sample was analyzed in triplicate. For quantification, standard curves were obtained using serially diluted cDNA amplified in the same real-time PCR run. Results were normalized to *ACTB* mRNA levels, and for confirmation to *GAPDH* mRNA levels. After the quantification procedure, the products were resolved by 2.5 % agarose gel electrophoresis to confirm that the reaction had amplified the DNA fragments of expected size.

Small interfering RNA (siRNA) transfection

Fibroblasts cells were grown to log phase, trypsinized and maintained in 2 ml of growth medium without antibiotics into 6-well tissue culture plates at 50% confluence for 24 hours. Six μ l of lipofectamine (Invitrogen, Carlsbad, CA) were mixed in a total volume of 500 μ l of Opti-MEM medium with GALNT3-specific siRNA or scrambled siRNA (20nM) (Invitrogen) and added to the cells for 6 hours, at which time, the transfection medium was replaced with complete growth medium. The GALNT3-specific siRNA duplex used consisted of 5'-rGrGrCrArUrCrArAUrArCrArGrCUUrCrATT-3' and 5'-UrGrArArGrCUrGUrAUUrGrAUUrGrCrCTT3'.

Western blotting

Cells were homogenized in lysis buffer (25 mM HEPES, 300 mM NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA and protease inhibitors mix including 1 mM PMSF, 1 mg/ml aprotinin and leupeptin; (Sigma, St Louis, MO, USA). Following centrifugation at 10,000g for 10 min at 4°C, proteins were electrophoresed through a 10% SDS-PAGE and transferred onto a nitrocellulose membrane (Trans-Blot Bio-Rad, Hercules, CA, USA). After 1 hour blocking with 1xTBS (20 mM Tris, 150 mM NaCl) with 5% skim milk and 0.01% Tween 20, blots were incubated with a rabbit polyclonal anti-ppGalNacT3 antibody [35]. The specificity of this antibody has been demonstrated in previous publications [36]. The blots were washed three times with

TBS-Tween (20 mM Tris HCl, 4 mM Tris base, 140 mM NaCl, 1 mM EDTA, 0.1% Tween-20). After incubation with secondary HRP-conjugated anti-rabbit antibody (Sigma-Aldrich, St. Louis, MO, USA) and subsequent washings, proteins were detected using the EZ-ECL chemiluminescence detection kit (Biological Industries, Beit Haemek, Israel).

In order to compare the amount of protein in the different samples, the blots were reprobbed with mouse monoclonal antibody to β -actin (ABcam, Cambridge, UK) and secondary HRP-conjugated anti-mouse antibody (Sigma-Aldrich, St. Louis, MO, USA). For quantitative determination of signal intensity, membranes were scanned with ImageMaster VDS-CI (Amersham Pharmacia Biotech) and analyzed by digital densitometry (Tina 2.10g software).

FGF7 ELISA

Human FGF7 Eliza Quantikine kit (R&D, Minneapolis, MN, USA) was used to detect FGF7 in the media of cultured primary fibroblasts, according to the manufacturer's instructions.

Gelatin zymography

Conditioned medium (CM) was collected from cultured fibroblasts maintained under serum-free conditions for 24hr and concentrated 1:20 using Vivaspin 6 concentrators (Sartorius, Goettingen, Germany). The samples were then mixed with non-reducing SDS gel sample buffer and applied without boiling to a 10%

polyacrylamide gel containing 0.1% SDS and 1mg/ml gelatin. After electrophoresis, the gels were washed in 50 mmol/l Tris-HCl (pH 7.5) containing 0.15 mol/l NaCl, 5 mmol/l CaCl₂, 0.02% NaN₃, 0.25% Triton X-100 (three changes) at room temperature, and then incubated in the same buffer without Triton X-100 at 37°C for 20 h. Proteins were stained by Coomassie Brilliant Blue R-250 solution (Bio-Rad, Hercules, CA, USA).

MMP Profiling

MMP activity in the CMs was determined using Enzolyte MMP fluorometric assay kit (AnaSpec, Inc., San Jose, CA, USA). The CM samples were incubated with MMP-specific peptide substrates following the manufacturer's instructions. Cleavage of substrates by MMPs removed the quenching effect of QXL520 on 5-carboxyfluorescein, resulting in increased fluorescence with excitation at 490 nm and emission at 535 nm[37]. Quantification of signal emission was performed using FLUOstar galaxy microplate reader (BMG Labtech GmbH, Jena, Germany).

RESULTS

Identification of major regulators of ppGalNacT3/*GALNT3* expression in human cells

Although abnormal expression of ppGalNacT3 is clearly associated with marked derangements of Pi metabolism[1, 6, 7, 21, 22], it is not known whether Pi itself is

involved in the regulation of ppGalNacT3/*GALNT3* expression. We therefore assessed the effect of increasing Pi concentrations on ppGalNacT3 expression in human hTERT-fibroblasts in culture. ppGalNacT3 expression was strongly induced in response to Pi (Fig. 1a) up to extracellular concentration of 5 mM, which is known to trigger apoptosis [38]. To determine the molecular basis of Pi-induced ppGalNacT3 up-regulation, we assessed *GALNT3* expression by qRT-PCR. Consistent with the induction observed at the protein level, treatment with Pi for 24 hr at 3.5 mM up-regulated *GALNT3* expression 2.4-fold, whereas lower Pi concentrations had little effect (Fig. 1b). Cells were then cultured in medium with 3.5mM Pi for different time periods. We observed an initial increase in *GALNT3* RNA level 12 hours after raising Pi concentrations, reaching maximal induction after 22 hours (Fig. 1c).

To determine whether the effect of Pi on *GALNT3* expression is cell lineage-specific, we assessed *GALNT3* expression in HEK293 cells (a cell line derived from human embryonic kidney tissue) exposed to varying concentrations of Pi. Here again, a Pi dose-dependent increase in ppGalNacT3 protein (Fig. 2a) and *GALNT3* RNA (Fig. 2b) levels was observed. *GALNT3* gene induction was most likely dependent upon Pi influx into the cells as the effect of Pi on *GALNT3* expression could be blocked by exposing the cells to phosphonoformic acid (PFA), a known inhibitor of Pi transport[39] (Fig. 2c).

We also determine the effects of other molecules previously implicated in the regulation of Pi circulating levels on *GALNT3* expression. Although PTH lacked

any significant effect (data not shown), both calcium and $1,25(\text{OH})_3\text{D}$ had a suppressive dose-dependent effect on *GALNT3* gene expression (Fig. 2d).

***GALNT3* down-regulation induces *FGF7* expression**

As mentioned above, the reason for the preferential accumulation of calcium deposits in the skin and subcutaneous tissues in HFTC [7] is still unclear. Recently, *FGF7* (also known as keratinocyte growth factor; KGF) was identified as capable of promoting Pi excretion through the kidney [40]. The major source of *FGF7* under physiological conditions are dermal fibroblasts [41]. We therefore postulated that as we found that Pi induces the expression of both *GALNT3* (Fig. 1) and *FGF7* (Fig. 2S), *GALNT3* gene expression may possibly influence the expression of *FGF7*. To assess this possibility, we used siRNA transfection to down-regulate the expression of *GALNT3* in hTERT-fibroblasts (Fig. 1S). Significantly increased *FGF7* RNA levels were found in cells transfected with *GALNT3*-specific siRNA as compared with control siRNA-transfected cells (Fig. 3a). To assess the relevance of these findings to the pathogenesis of HFTC, we also quantified *FGF7* gene expression levels in primary fibroblast cell cultures derived from two HFTC patients. These individuals were previously shown to carry a splice site mutation [15], which was found to result in complete absence of the ppGalNAcT3 protein in the skin[42]. *GALNT3* gene transcription was decreased by 70% in primary fibroblasts derived from HFTC patients as compared with healthy controls (data not shown). In contrast, *FGF7* RNA expression was 3.5 fold higher in patient cells than

in control primary fibroblasts (Fig. 3b). Accordingly, HFTC patients' primary fibroblasts were found to secrete much higher concentrations of FGF7 (80 + 10 vs 27 + 5 pg/ml), as compared with control fibroblasts (Fig.3c).

***GALNT3* down-regulation is associated with augmented MMP activity**

Among the various potential downstream paracrine effects of FGF7, whose secretion seems to be triggered by *GALNT3* down-regulation, we noted that FGF7 has been shown to induce the activity of several MMPs in different tissues [43-46], while MMPs have in turn been previously implicated in the pathogenesis of ectopic calcification [30-33]. Thus we first assessed the capacity of FGF7 to trigger MMPs secretion in dermal hTERT-fibroblasts. Gelatin-zymography analysis of CM collected 24 hr after exposure to FGF7 revealed an increase in gelatinase activity in response to FGF7 (Fig. 4a).

Since *GALNT3* down-regulation induces FGF7 secretion (Fig. 3), and FGF7 was found both in this (Fig. 4a) and previous studies [43-46] to up-regulate MMP expression, we hypothesized that *GALNT3* down-regulation may also affect MMP activity in dermal hTERT-fibroblasts. Indeed, significant up-regulation of *MMP9* (1.7-fold) and *MMP8* (7.9-fold) mRNA levels was found upon down-regulation of *GALNT3* expression by siRNA in human fibroblasts (Fig. 4b) as well as in HFTC primary fibroblasts as compared with control cells (not shown). Of note, increased Pi concentration did not affect the extent of gene induction associated with

GALNT3 down-regulation (Fig. 3S) and no change in Pi extracellular concentration was observed 3 and 5 days after siRNA transfection (not shown).

We then assessed human hTERT-fibroblasts transfected with *GALNT3*-specific siRNA for MMP activity using a panel of MMP-specific fluorescent substrates. We observed a significant elevation in the specific activities of MMP-8 (collagenase), MMP-9/2 (gelatinases) and MMP-3 (but not MMP-1) in cells down-regulated for *GALNT3* expression as compared with control fibroblasts (Fig. 4c). Gel zymography analysis demonstrated increased gelatinase activity in CM obtained from *GALNT3*-down-regulated hTERT-fibroblasts as compared with control cells (Fig. 4d).

DISCUSSION

Pi homeostasis has been shown to play an important role in human physiology, determining to a large extent survival in selected groups [5, 47, 48]. The study of rare inherited disorders often leads to insights into physiological processes of general relevance [49]. In this regard, the initial discovery of the cause of HFTC unveiled ppGalNacT3 as a new element critical for maintaining Pi homeostasis [15]. Specifically, ppGalNacT3 catalyzes the initial steps of O-linked oligosaccharide biosynthesis, which consists in the transfer of an N-acetyl-D-galactosamine residue to a serine or threonine.

ppGalNacT3 deficiency has been shown to cause two distinct diseases, HFTC [8-15, 42] and hyperostosis hyperphosphatemia syndrome [23, 50, 51], a recessive bone disorder. Thus, despite the fact that O-linked oligosaccharide biosynthesis occurs ubiquitously, ppGalNacT3 deficiency results in a very restricted spectrum of clinical manifestations. Functional redundancy among glycosyltransferases [52] has been invoked to explain this discrepancy [15]. However this is unlikely to account alone for the preferential accumulation of calcium deposits in the skin, suggesting a specific effect of ppGalNacT3 deficiency in this tissue. We therefore sought to assess the effects of *GALNT3* down-regulation on dermal fibroblasts in an *in vitro* setting to address this issue in isolation from the systemic effects of ppGalNacT3. We show here that Pi induces ppGalNacT3 expression in dermal hTERT-fibroblasts and kidney epithelial cells. The fact that Pi modulates ppGalNacT3 expression in peripheral tissues may be interpreted to suggest that ppGalNacT3 plays a physiological role in peripheral tissues of relevance to the pathogenesis of HFTC. Accordingly, we used cultures of HFTC patients' primary fibroblasts as well as human hTERT-fibroblasts transfected with *GALNT3*-specific siRNA to mimic the consequences of decreased ppGalNacT3 in an *in vitro* system. We discovered that decreased *GALNT3* expression results in increased secretion of both FGF7 and MMPs, the expression of which is induced by FGF7. MMPs have been linked to ectopic calcification in vascular tissues. In particular, MMP-9 was found to be up-regulated during vascular calcification, and inhibition of MMPs activity was found to prevent calcification in the same model [30, 31]. Thus, the

present data together with previous observations suggest a causative role for MMPs in the pathogenesis of ectopic calcification in skin tissues. A number of studies indicate that Pi is able to induce MMPs expression in fibroblasts and other tissues [53-56], suggesting that both ppGalNacT3 deficiency and induced MMP expression may have an additive detrimental (tissue-specific?) effect. This hypothesis is further supported by the finding that tetracyclines, which are known inhibitors of MMPs, have beneficial therapeutic effects in patients with cutaneous calcinosis [30, 31, 57, 58]. This therapeutic response to tetracyclines is of particular importance as a large panoply of other treatments aimed at reducing circulating Pi levels shows very limited therapeutic benefit to HFTC patients [7, 59-61].

Collectively, the present observations imply that in addition to the well-known systemic effects of ppGalNacT3 deficiency on renal Pi re-absorption, the pathogenesis of HFTC may also involve local/peripheral processes. A number of natural modulators of ectopic calcification, such as fetuin-A, matrix Gla protein and osteopontin, may also play a local role in the pathogenesis of ectopic calcification in various tissues including the skin [62-64]. The existence of such a wide array of biological molecules and pathways contributing to the formation of ectopic calcifications suggests that variant alleles encoding these proteins may account for the extensive clinical heterogeneity observed in HFTC and related disorders.

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TABLE

Table 1. qRT-PCR oligonucleotide sequences

Sequence	Size (bp)	Gene
8F-CTCTATGTCTGGATGTTGG 9R-TCATGTTGAGCAGAGTATTC	109	<i>GALNT3</i>
1F-CTTGATTTGGGTA CTCTTGG 2R-GAGGCTTTTGTACTGGCTCTAG	122	<i>GALNT1</i>
1F-GTGGAGCTCTTGGTCTCT 1R-TCGTTGAGCTGGAGTTTG	187	<i>GALNT4</i>
1F-ACGAGATGAATGAGGAGC 3R-CTGCGTCAGCTCTGAGT	206	<i>GALNT6</i>
12F-CTTTGGACACGCACGAC 13R-GGATGTCATAGGTCACGTAGC	131	<i>MMP9</i>
1F-GACGCTTCCATTTCTGCT 2R-CGATCACATTAGTGCCATTC	164	<i>MMP8</i>
1F-CAATGACATGACTCCAGAGC 2R-CAACTGCCACTGTCCTG	274	<i>FGF7</i>
3F-CGATCACATTAGTGCCATTC 4R-AGGTGGACAGCGAGGCCAGGA	127	<i>ACTB</i>

FIGURE LEGENDS

Figure 1. Effect of Pi on pPGalNacT3/GALNT3 expression in human fibroblasts. (a) hTERT fibroblasts were cultured for 24 hr in the presence of increasing concentrations of Pi, and pPGalNacT3 expression was ascertained by Western Blotting (upper panel). Results were quantified by digital densitometry and the expression of pPGalNacT3 was normalized to that of β -actin (Tina 2.10g software; lower panel); hTERT fibroblasts were cultured for 24 hr in the presence of (b) increasing concentrations of Pi or (c) for increasing time periods in the presence of 3.5 mM Pi, and *GALNT3* gene expression was quantified by qRT-PCR. Results are expressed as % of expression relative to control cells + standard deviation (SD).

Figure 2. Regulation of ppGalNacT3/*GALNT3* in HEK293 cells. (a) HEK293 cells were cultured in the presence of 1 mM or 3.5 mM Pi, and ppGalNacT3 expression was assessed by (a) Western blotting and (b) qRT-PCR 24 hr later; (c) To assess the effect of Pi transport on *GALNT3* induction, we maintained the cells in 3.5 mM Pi in the presence (+) or absence (-) of PFA, a known inhibitor of Pi transport (100% refers to the level of expression of *GALNT3* in the presence of 1mM phosphate); (d) We also measured *GALNT3* expression in HEK293 cells cultured with increasing concentrations of calcium and 1,25(OH)₃D. Results in (b) and (d) are expressed as % of expression relative to control cells + SD.

Figure 3. FGF7 expression in ppGalNacT3-deficient human fibroblasts. (a) hTERT fibroblasts were transfected with *GALNT3*-specific siRNA (siRNA) or a non-relevant siRNA (control), and FGF7 expression was assessed by qRT-PCR after 3 days. Results are presented as % of expression relative to control cells + SD; (b) Primary human fibroblasts derived from HFTC patients (HFTC) and control individuals (control) were assessed for FGF7 expression by qRT-PCR. Results are given as % of expression relative to control cells + SD; (c) FGF7 secretion in the medium obtained from patient (HFTC) and control (control) primary fibroblasts cultures was determined using a quantitative ELISA assay. Results are expressed in pg/ml + SD.

Figure 4. GALNT3 down-regulation increases MMPs expression and activity.

(a) Gelatinase activity in the culture medium was assessed by gel zymography, as described in Materials and Methods; (b) Human fibroblasts were transfected with *GALNT3*-specific siRNA (siRNA) or a non-relevant siRNA (control), and *MMP8* and *MMP9* gene expression was assessed by qRT-PCR. Results are given as % of expression relative to control cells + SD; (c) The same cells were comparatively assessed for MMP activity using a fluorometric assay as described in Materials and Methods; (d) Gelatinase activity in the culture medium of both types of cells was assessed by gel zymography.

Figure 1S. Effect of GALNT3-specific siRNA on GALNT3 expression.

(a) hTERT-fibroblasts were transfected with *GALNT3*-specific siRNA (siRNA) or a non-relevant siRNA (control), and mRNA and protein were extracted 3 and 5 days later respectively. Expression levels of *GALNT3* and ppGalNacT3 were assessed by (a) qRT-PCR and (b) immunoblotting. (c) We confirmed the specificity of *GALNT3*-down-regulation by demonstrating the lack of effect of the siRNA chosen on the expression of 2 other glycosyltransferase isoforms (*GALNT1* and *GALNT4*) while *GALNT6* was not expressed at all in hTERT-fibroblasts.

Figure 2S. Effect of phosphate on FGF7 expression. hTERT-fibroblasts were cultured for 24 hr in the presence of increasing concentrations of Pi and *GALNT3*

gene expression was quantified by qRT-PCR. Results are expressed as % of expression relative to control cells + SD.

Figure 3S. Effect of phosphate on gene expression change associated with *GALNT3* down-regulation. The change in gene expression levels for *FGF7* and *MMP8* as assessed by qRT-PCR were measured 3 days after siRNA transfection in the presence of 1 or 3 mM of phosphate. Results are expressed as change-fold in gene expression in cells transfected with *GALNT3*-specific siRNA as compared with cells transfected with a non-relevant siRNA + SD.

