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# 6-phosphofructo-2-kinase/fructose 2,6-bisphosphatase-3 is required for transforming growth factor β1-enhanced invasion of Panc1 cells *in vitro*

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ABSTRACT

Transforming growth factor β1 (TGFβ1) is a well-established inducer of the epithelial-mesenchymal transition (EMT) that is essential for the acquisition of malignant properties, such as invasion, in tumor cells. Although recent studies suggest that the EMT in tumor cells is associated with reprogramming of energy metabolism and TGFβ1 has been shown to stimulate glycolysis in multiple primary cell lines, little is known about TGFβ1's effect on glycolysis and glycolytic regulators in transformed cells. Given the known regulatory role of 6-phosphofructo-2-kinase/fructose 2,6-bisphosphatase-3 (PFKFB3) in glycolysis and association of glycolytic activity with malignant features such as invasion, we sought to investigate whether TGFβ1 regulates PFKFB3 expression and if PFKFB3 is involved in the TGFβ1-mediated increase in the invasive ability of the Panc1 cell cline—a well-established model of TGFβ1-initiated EMT. Herein we demonstrate that TGFβ1 induces PFKFB3 expression and stimulates glycolysis in Panc1 cells. We also show that siRNA silencing of PFKFB3 prevents the stimulation of glycolysis and in vitro invasive ability of Panc1 cells by TGFβ1. Furthermore, PFKFB3 silencing suppresses the TGFβ1-mediated induction of the Snail protein, suggesting that PFKFB3 is required for the regulation of Snail expression by TGFβ1. Taken together, our study identifies PFKFB3 as a key TGFβ1 effector protein that mediates TGFβ1's effect on Snail expression, invasion, and glycolysis.



## 1. Introduction

The transforming growth factor beta (TGFβ) family members initiate and regulate a wide range of cellular functions, such as differentiation, migration, invasion, and apoptosis, by binding to cell surface type I and type II serine/threonine kinase receptors (TGFβRI and TGFβRII, respectively) [1]. The TGFβ subfamily consists of three members—TGFβ1, TGFβ2, and TGFβ3—which, upon binding to TGFβRII, cause a heterodimeric formation with TGFβRI, leading to the activation of the SMAD family proteins—major mediators of the canonical TGFβ signaling [2]. TGFβ has a dual role in neoplastic transformation and tumor progression—acting as a tumor suppressor during neoplastic transformation and tumor initiation and as a tumor promoter during tumor progression and metastasis. Consistent with its tumor promoting function and association of TGFβ signaling with malignant properties, TGFβ proteins have been shown to be highly expressed during the late states of tumor progression [1–3]. Malignant properties such as heightened invasive ability and metastasis promoted by TGFβ signaling have been attributed to a cell biological process known as the epithelial-mesenchymal transition (EMT), during which tumor cells

*Abbreviations:* TGFβ, transforming growth factor-β; EMT, epithelial-mesenchymal transition; PFKFB, 6-phosphofructo-2-kinase/fructose 2,6-bisphosphatase; F2,6BP, fructose 2,6-bisphosphate.

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of epithelial origin lose polarity and intercellular adhesions and acquire fibroblast-like mesenchymal characteristics such as enhanced motility and invasiveness [2]. These observations suggest that targeting TGFβ signaling may be a valid approach for the treatment of cancers that produce or respond to TGFβ proteins. The EMT induced by TGFβ is orchestrated and mediated by several transcription factors, including the zinc finger transcription factors Snail (encoded by *SNAI1*) and the ZEB family of transcription factors. Given the regulatory roles of upstream TGFβ components in normal cell development and non-enzymatic nature of the known downstream of TGFβ effectors, identification of enzymes that mediate TGFβ-associated malignant features may lead to the development of better approaches aimed at specifically targeting TGFβ-associated phenotype in cancer.

Altered energy metabolism is now recognized as a hallmark of neoplastic transformation and recent studies [4,5] suggest that activation of the EMT by TGFβ signaling is associated with a metabolic transition that favors increased energy production [4]. However, the metabolic mediator(s) of TGFβ signaling are poorly understood. An essential regulatory step in glycolysis involves the phosphorylation of fructose 6-phosphate by 6-phosphofructo-1-kinase (PFK-1), which is allosterically activated by fructose 2,6-bisphosphate (F2,6BP)—the product of bifunctional 6-phosphofructo-2-kinase/fructose 2,6-bisphosphatases (PFKFB). Although the third and fourth isoforms of PFKFBs—PFKFB3 and PFKFB4—have been shown to stimulate glycolysis in tumor cells due to their higher kinase activities relative to bisphosphatase activities [6,7], their co-expression in many tumor types [6,8] suggests non-overlapping functions.

In the current study, we show, for the first time, that TGFβ1 increases glycolysis in Panc1 cells by inducing PFKFB3 expression and that PFKFB3 is required for TGFβ1-mediated invasion of Panc1 cells *in vitro*. Furthermore, we demonstrate that the effect of TGFβ1 on Snail induction is largely abrogated by PFKFB3 silencing. These data suggest that PFKFB3 is involved in the regulation of Snail and acquisition of an invasive and glycolytic phenotype by TGFβ1 in Panc1 cells.

## 2. Materials and methods

### 2.1. Cell culture and TGFβ1 treatment

Panc1 cells were purchased from ATCC (Cat. #CRL-1469) and were cultured in Dulbecco's Eagle's medium supplemented with 10% fetal bovine serum, 50 units/ml penicillin, 50 μg/ml streptomycin, and 250 ng/ml amphotericin B. Cells were grown at 37 °C in 5% $CO_2$. TGFβ1 protein (Sigma Cat. #11412272001) was added to the culture media at 10 ng/ml concentration.

### 2.2. Fructose 2,6-bisphosphate assay

Cells were lysed with a buffer containing 100 mM NaOH and 50 mM Tris-acetate (pH = 7) and F2,6BP levels were analyzed following a coupled enzyme method described by Van Schaftingen et al. [9].

### 2.3. Glucose and lactate assays

Glucose and lactate levels in the media were measured colorimetrically using kits employing enzyme-based assays following the manufacturer's protocol (Biovision Cat. #K606-100 and K607-100).

### 2.4. siRNA transfections

Two separate mammalian non-targeting control (Thermo Fischer Sci. Cat.# 4390843 and 4390846) and two PFKFB3-specific siRNA species (Thermo Fischer Sci. Cat.#s10358 and s10359) were used in all transfections. Transfections were achieved using Lipofectamine RNAiMAX following the manufacturer's recommendations.

### 2.5. Real-time quantitative PCR

Total RNA was reverse-transcribed using an mRNA to cDNA synthesis kit (Thermo Fischer Sci. Cat.# 4387406) following the manufacturer's protocol. The quantitative (q) PCR was performed in StepOne Plus (ThermeFischer Sci, NY, USA) using TaqMan probes (ThermeFischer Sci Cat #s: PFKFB3, Hs00998698_m1; Snail, Hs00195591_m1; E-cadherin, Hs01023895_m1; β-actin, Hs99999903_m1). β-actin was used as the internal control for normalization of cDNA.

### 2.6. SDS-PAGE and Western blotting

SDS-PAGE and Western blotting were performed following standard protocols. PVDF membranes were probed with primary antibodies specific to PFKFB3 (Proteintech Cat. #13763-1-AP), Snail (Cell Signaling Cat.#3879), E-cadherin (Cell Signaling Cat.#3195), β-actin (Cell Signaling Cat.#3700), and α-tubulin (Cell Signaling Cat.#2125) proteins. HRP-conjugated goat anti-rabbit (Cell Signaling Cat.#7074) or anti-mouse (Cell Signaling Cat.#7076) secondary antibodies were used. Bound antibodies were detected using ECL prime (Amersham) and bands were visualized in ChemiDoc MP (BioRad).

### 2.7. Immunfluorescence analysis

Cells grown in chamber slides were fixated with %4 formalin. An anti-E cadherin primary (Cell Signaling Cat.#14472) and an Alexa Fluor (488)-conjugated goat anti-mouse secondary (Cell Signaling Cat.#4408) antibodies were used to visualize the E-cadherin protein.

### 2.8. In vitro Matrigel invasion assay

Invasion assays were performed in 24-well modified Boyden chambers (Corning Cat.#354578). Twenty four hours after transfection of cells in 6-well plates as described above, TGFβ1 protein was added (10 ng/ml) and cells were further incubated for 24 h. Cells were trypsinized and resuspended in DMEM containing %1 FBS. Cells ($3 \times 10^4$ cells/well) were seeded onto trans-membranes that were precoated with 1 μg/ml Matrigel (Corning Cat.#354234) for 2 h. Full growth medium containing %10 FBS as chemoattractant was included in the lower chamber. After 18 h, cells that invaded Matrigel were fixed with methanol and stained with 0.2% crystal violet. Stained cells were visualized under 10× magnification and were counted in 5 randomly captured fields. Cells treated with TGFβ1 after transfection were separately grown in adherent/immobile conditions to normalize the effect of cell number on invasion.

### 2.9. Statistical analysis

All data are expressed as the mean ± s.d. of three experiments. Statistical significance was assessed by the two-sample *t*-test (independent variable).

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