Research Article

High levels of the type III inorganic phosphate transporter PiT1 (SLC20A1) can confer faster cell adhesion

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ABSTRACT

The inorganic phosphate transporter PiT1 (SLC20A1) is ubiquitously expressed in mammalian cells. We recently showed that overexpression of human PiT1 was sufficient to increase proliferation of two strict density-inhibited cell lines, murine fibroblastic NIH3T3 and pre-osteoblastic MC3T3-E1 cells, and allowed the cultures to grow to higher cell densities. In addition, upon transformation NIH3T3 cells showed increased ability to form colonies in soft agar. The cellular regulation of PiT1 expression supports that cells utilize the PiT1 levels to control proliferation, with non-proliferating cells showing the lowest PiT1 mRNA levels. The mechanism behind the role of PiT1 in increased cell proliferation is not known. We, however, found that compared to control cells, cultures of NIH3T3 cells overexpressing PiT1 upon seeding showed increased cell number after 24 h and had shifted more cells from G0/G1 to S+G2/M within 12 h, suggesting that an early event may play a role. We here show that expression of human PiT1 in NIH3T3 cells led to faster cell adhesion; this effect was not cell type specific as it was also observed when expressing human PiT1 in MC3T3-E1 cells. We also show for NIH3T3 that PiT1 overexpression led to faster cell spreading. The final total numbers of attached cells did, however, not differ between cultures of PiT1 overexpressing cells and control cells of neither cell type. We suggest that the PiT1-mediated fast adhesion potentials allow the cells to go faster out of G0/G1 and thereby contribute to their proliferative advantage within the first 24 h after seeding.

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Introduction

The mammalian type III sodium-dependent inorganic phosphate (P₃) symporters PiT1 (SLC20A1) and PiT2 (SLC20A2) are part of the Pi transport (PiT) family (TC#2.A.20) [1,2], which is represented in all kingdoms of life [3]. Originally, human PiT1 (formerly GLVR1) and human and rat PiT2 (formerly GLVR2 and Ram-1, respectively) were discovered as receptors for different gammaretroviruses [4–6] and were later found to transport Pi into cells [7–13]. The two PiT paralogs are highly related, they show about 60% amino acid identity and chimeras between PiT1 and PiT2 can change their retroviral specificities [14–18]. They have similar Pi transport functions [11,19,20], and are broadly expressed in mammalian tissues [21]. Moreover, knockdown of PiT1 expression can lead to upregulation of the PiT2 mRNA level [22–24]. These observations suggest that PiT1 and PiT2 possess overlapping functions in maintaining cellular phosphate homeostasis.

PiT1 and PiT2, however, also have non-overlapping functions [24–29], and PiT-transport-independent functions of PiT1 have been discovered [27,29,30]. Knockout of PiT1 in mice retards growth of embryos, slows proliferation of liver cells, and impairs erythroid and early B-cell development [24–27]. The effects of knockout of PiT1 in mice on erythroid and early B-cell development have been associated with defects in cell cycle progression [26,27]. Knockdown of PiT1 impairs proliferation of the transformed cell lines HeLa and HepG2 and tumorigenesis of HeLa cells in nude mice [29], and proliferation of the murine density-inhibited cell line MC3T3-E1 [23]. Thus, a certain level of PiT1 is important for cell proliferation. Moreover, overexpression of PiT1 is sufficient to increase proliferation of the murine density-inhibited cell lines NIH3T3 and MC3T3-E1 [23]. In agreement with that PiT1 overexpression leads to a general proliferative advantage, cultures of these cells grew to higher cell densities, but they remained density-inhibited [23]. However, when transformed, NIH3T3 cells overexpressing PiT1 formed more colonies in soft agar than control cells [23]. The cellular regulation of the endogenous PiT1 expression in NIH3T3 and MC3T3-E1 cells supports that cells utilize their PiT1 levels to control proliferation, with non-proliferating cells showing the lowest PiT1 mRNA levels [23].

There is direct and indirect evidence that the role of PiT1 in cell proliferation is not dependent on its Pi transport function [23,24,29]. Thus although mouse embryonic fibroblasts from PiT1 knockout mice [24] and MC3T3-E1 cells with knocked down PiT1 expression [23] exhibited increased PiT1 expression, they still showed impaired proliferation. In addition, overexpression of PiT1 leading to increased Pi uptake, did not rescue impaired proliferation of HeLa cells caused by reduced PiT1 expression, while expression of a PiT1 transport knockout mutant did [29]. Moreover, overexpression of PiT1 in NIH3T3 and MC3T3-E1 cells in general does not lead to regulation of PiT2, and while it does lead to increased proliferation of both cell types, only NIH3T3 cells show increased ability to import Pi [23]. Together these results suggest that PiT1’s role in regulation of cell proliferation is independent on its Pi uptake ability.

PiT1 overexpression was found to confer increased proliferation upon NIH3T3 and MC3T3-E1 cells under standard cultivation conditions, i.e., in standard growth media containing 10% bovine serum and tissue-culture treated plastic ware, all negatively charged (when wetted) tissue-culture treated polystyrene (TCP5) [23]. When cells are seeded in serum-containing cell medium onto TCP5, the proteins of the serum will very quickly adsorb to the negative charges of the polystyrene. Depending on the quality of the TCP5 (i.e., charge density and topology of the polystyrene) adsorption of more or less adhesion-promoting proteins will occur. The seeded cells adhere to the adhesion-promoting proteins, which predominantly are vitronectin (spreading factor) and plasma fibronectin, or via adhesion-promoting protein already attached to the cells. Specific contact with the adhesion-promoting proteins through integrins allows the cells to spread out [31–36] (reviewed in Elbert et al. [37] and Wilson et al. [38]). It is presently not known why cultures of cells with increased expression of PiT1 show enhanced proliferation. We have, however, previously observed that after just one day in culture, fibroblastic NIH3T3 cells overexpressing PiT1 had proliferated faster, and already 12 h after seeding, a lower percentage of the cells were in G0/G1 in the PiT1 overexpressing cultures compared to cultures of control cells [23]. We have here addressed how a high expression of PiT1 allows the cells to increase proliferation within the first day in culture. Using the same conditions as in the proliferation experiments, i.e., standard cultivation conditions, we found that overexpression of PiT1 in NIH3T3 cells leads to faster adhesion and spreading compared to control cells. The adhesion advantage of PiT1 overexpressing NIH3T3 cells compared to control cells was, moreover, found to be independent of serum concentrations during cultivation prior to seeding and during seeding, and of the quality of the TCP5, which the cells had been cultivated on prior to seeding. In addition, adhesion of MC3T3-E1 cells was also investigated under standard cultivation conditions, and overexpression of PiT1 was also found to confer faster adhesion on this pre-osteoblastic cell line.

Materials and methods

 Constructs

The vector pLXSN [39] was modified. The original HindIII site in the plasmid was removed by site-directed mutagenesis and HindIII and NotI sites introduced in the multiple cloning region; the modified pLXSN vector is referred to as pLXSN-ΔHindIII+HindIII+NotI and the retroviral vector as LXSN+HindIII+NotI. The PiT1 encoding sequence was cloned as a HindIII-XhoI fragment from pOJ75 [16] into the HindIII – XhoI sites of pLXSN-ΔHindIII+HindIII+NotI resulting in the plasmid pLPIT1SN-ΔHindIII+HindIII+NotI; the retroviral vector is referred to as LPIT1SN+HindIII+NotI.

Malachite green based assay for Pi₃ determination

To determine the contribution of Pi₃ from 10% newborn calf serum (NCS) (Gibco BRL) and 10% fetal bovine serum (FBS) (Gibco BRL) supplemented to Dulbecco’s Modified Eagles Medium (DMEM) and Minimum Essential Medium alpha (α-MEM) (Gibco BRL), respectively, the total Pi₃ contents of Pi₃-free DMEM containing 10% NCS or 10% FBS were determined using a malachite green based method basically as described by Baykov et al. [40] with the exception that 50 μL samples were added to 100 μL assay solutions in a 96-well plate and the developed malachite green-phosphomolybdate complex was measured at 650 nm.

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2 Inorganic phosphate, Pᵢ₃

3 Tissue-culture treated polystyrene, TCP5.
Cell culture

The murine fibroblastic cell line NIH3T3 (ATCC CRL-1658) and derivatives of this were cultivated in DMEM (0.916 mM Pi) supplemented with 10% NCS and 1% penicillin and streptomycin (PS) (Gibco BRL) (DMEM-NCS-PS). The Pi content of DMEM-NCS-PS was 0.92 mM, which is the Pi level NIH3T3 cells have been adapted to. NIH3T3 cells were grown according to the 3T3 scheme and even though NIH3T3 cells overexpressing PiT1 grow denser than control NIH3T3 cells, they maintain strict culture-density inhibited proliferation when cultivated in NCS. The murine pre-osteoblastic MC3T3-E1 cells [41] (a kind gift from Dr. H. Kodama) and derivatives of these were cultured in α-MEM (1.014 mM Pi) supplemented with 10% FBS and 1% PS (α-MEM-FBS-PS). The Pi content of α-MEM-FBS-PS was 0.94 mM, which is the Pi concentration undifferentiated MC3T3-E1 cells were adapted to. MC3T3-E1 cells and derivatives below the 5th passage were used for experiments.

Establishment of NIH3T3 and MC3T3-E1 cells transduced with the retroviral vector LXSN (NIH3T3-LXSN and MC3T3-E1-LXSN, respectively) or with the human PiT1 expressing retroviral vector LPiT1SN (NIH3T3-LPiT1SN and MC3T3-E1-LPiT1SN, respectively), have been described previously [23]. NIH3T3 cells were transduced with the retroviral vectors LXSN+ HindIII+NotI or LPiT1SN+ HindIII+NotI and the cell populations NIH3T3-LXSN+ HindIII+NotI (referred to here as NIH3T3-control) and NIH3T3-LPiT1SN+ HindIII+NotI (referred to here as NIH3T3-PiT1) were established by selecting with G418 (Gibco BRL) as described previously for NIH3T3-LXSN and NIH3T3-LPiT1SN [23].

Unless otherwise indicated, the cells were cultivated in their standard growth medium. Before seeding, an aliquot of the cells were established by selecting with G418 (Gibco BRL) (DMEM-NCS-PS). The Pi content of the wells were determined on known numbers of pelleted cells; the cells were counted as described above. NIH3T3-LPiT1SN and NIH3T3-LXSN contained the same average amount of protein per cell, and the protein contents of the wells were used as a relative measure of the number of adhered cells.

The effects of serum concentration and TCPS quality on adhesion were analyzed as follows. NIH3T3-PiT1 and NIH3T3-control cells were cultivated at equal densities using cell culture flasks of low quality TCPS (Sarstedt Red Cap tissue culture flasks) or of high quality TCPS (NUNC, Nunclon TM surface) in DMEM-PS supplemented with varying serum concentrations (10%, 5%, or 0.5% of NCS) for 48 h before seeding. The cells were seeded at 5,000 cells/cm² in 4-well plates (NUNC, Nunclon TM surface) in DMEM-PS containing serum concentrations corresponding to their treatment before seeding. Three hours after seeding, the protein contents of the wells were determined as described above. The average amount of protein per cell was determined as described above. NIH3T3-PiT1 and NIH3T3-control contained the same average amount of protein per cell, and the protein contents of the wells were used as a relative measure of the number of adhered cells.

Quantitative reverse transcription PCR (qRT-PCR) analysis

Cells seeded in 4-well plates (NUNC, Nunclon TM surface) were lysed on the plates and RNA was purified using Ambion® RNAqueous® -4PCR kit (Applied Biosystems) as described by the manufacturer. RNA was reversely transcribed to cDNA immediately after purification using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). The qRT-PCR were performed using the following TaqMan® Gene Expression Assays (Applied Biosystems) as described by the manufacturer: human β2-microglobulin (Hs00965596_m1), and as an endogenous control, mouse β-2-microglobulin (Mm00437762_m1). For each sample, three technical replicates were made. The individual qRT-PCR reactions contained: 10 μL TaqMan® Universal Fast PCR Master Mix (Applied Biosystems), 1 μL Hs00965596_m1, 1 μL Mm00437762_m1, and 8 μL cDNA (approximately 10 ng). The PCR cycles employed were: 95 °C for 10 min, 40 cycles of 95 °C for 1 s, and 60 °C for 20 s. The efficiencies of each set of primers were determined on dilution series of cDNA and were used in calculations of relative gene expression as described [42].

Evaluation of cell adhesion

Cells were cultivated using NUNC (Nunclon TM surface) plastic wares (TCPS) and seeded in 6-well plates (NUNC, Nunclon TM surface) in their standard growth medium. At different time points after seeding, the un-attached cells were removed by washing the cells in phosphate-buffered saline (PBS). The adhered cells were fixed in glutaraldehyde, and stained in 0.1% crystal violet (MERCK). The cells were photographed and 12 randomly picked fields per data-point were counted. Alternatively, NIH3T3- LPiT1SN and NIH3T3-LXSN cells were seeded in 4-well plates (NUNC, Nunclon TM surface), un-attached cells removed by washing the cells in PBS, and adhered cells lysed in 0.5% Triton X-100 in ddH2O. As a measure of the number of adhered cells, the protein content of the wells were determined using BCA Protein Assay Reagent kit (Pierce) as described by the manufacturer. The average amount of protein per cell was determined on known numbers of pelleted cells; the cells were counted as described above. NIH3T3-LPiT1SN and NIH3T3-LXSN contained the same average amount of protein per cell, and the protein contents of the wells were used as a relative measure of the number of adhered cells.

Cell cycle analysis

Cell cycle analysis was performed as previously described [23]. Cells 3 were seeded at 20,000 cells/cm² in T25-flasks in triplicates. They were detached using trypsin, pelleted by centrifugation, and re-suspended in PBS. The cells were then cooled on ice, mixed 1:1 with ice-cold 99.9% ethanol, incubated on ice, and stored at 4 °C until staining. In the staining procedure, the cells were pelleted by centrifugation, re-suspended in 1 ml PBS containing 20 μg/ml RNase A, and stained by addition of 100 μl propidium iodide (1 mg/ml). The cells were analyzed by flow cytometry (FL2 channel) on a FACS Calibur flow cytometer (Becton Dickinson). CellQuest software was used for acquisition, followed by doublet subtraction, and data analysis employing the Watson Pragmatic model using FlowJo software.

32P transport assay

Cells were seeded in 4-well plates (NUNC, Nunclon TM surface), and 32P transport assay was performed as previously described [23] using 100 μM total [Pi] in the uptake experiments. The protein contents in the cell lysates were determined using BCA Protein Assay Reagent kit (Pierce) as described by the manufacturer.
F-actin staining

Cells were seeded at 5,000 cells/cm² in 8-well chamber slides (TCPS, Sarstedt). At different time points after seeding, the adhered cells were fixed in 4% paraformaldehyde, washed, permeabilized using 0.1% Triton-X100, and incubated in the dark with phalloidin conjugated with Alexa Fluor 594 (Life Technologies) (1U/well). The cells were mounted with fluorescence mounting medium (Dako), studied under a Zeiss Axiovert 200M microscope, and photographed at 20X and 40X magnification (Photometrics CoolSNAP HQ camera).

Statistical analysis

All data are presented as means±standard deviation (SD). The hypothesis that two values were identical was tested using a two-tailed Student’s t test. In Fig. 4B, factorial ANOVA was employed. A value of \( p \leq 0.05 \) was considered statistically significant.

Results and discussion

High PiT1 expression levels increase the adhesion rates of fibroblastic NIH3T3 and pre-osteoblastic MC3T3-E1 cells

We have previously shown that NIH3T3 cells expressing human PiT1 (NIH3T3-LPiT1SN) proliferate faster than NIH3T3 cells harboring the empty transfer vector (NIH3T3-LXSN) under standard cultivation conditions \[23\]. Specifically, analyses of the progression through the cell cycle showed a statistically higher percentage of cells in S+G2/M in PiT1 overexpressing cultures than in control cell cultures after just 12 h in culture \[23\], and we therefore studied an early event in cell culture: adhesion. To determine whether the level of PiT1 expression affects cell adhesion, we studied the adhesion rate of NIH3T3-LPiT1SN and control cells under standard cultivation conditions. The cells were seeded at 5,000 cells/cm² in 6-well plates, fixed, and counted after
After 1 h in culture, 78% more NIH3T3-LPiT1SN cells than control cells had adhered, and after 2 h in culture, 73% more NIH3T3-LPiT1SN cells than control cells had adhered (Fig. 1A and B). Thus, an increased level of PiT1 expression was sufficient for the NIH3T3 cells to adhere faster than control cells; however, the PiT1 level did not affect the total number of adhered cells.

In the experiment shown in Fig. 1A and B, we seeded the cells at 5,000 cells/cm². In the previous studies analyzing cell cycle progression and cell proliferation, the cells were seeded at a density of 20,000 cells/cm² [23]. To address whether the difference in seeding density influenced the adherence characteristics, we seeded the cells at 20,000 cells/cm² in the experiment shown in Fig. 1C. We determined the protein content of the cells prior to seeding and of each well 2 h after seeding. The two seeded cell populations had the same average amount of protein per cell (data not shown) and the protein contents of the wells were used as a relative measure of the number of adhered cells. Almost 92% more NIH3T3-LPiT1SN than control cells adhered during these 2 h (Fig. 1C).

We also addressed whether the observed faster cell adhesion mediated by increased PiT1 expression in fibroblastic NIH3T3 cells is cell type specific. Overexpression of human PiT1 in pre-osteoblastic MC3T3-E1 cells also results in increased proliferative potential under standard cultivation conditions [23]. We therefore investigated adhesion of MC3T3-E1 cells transduced with the human PiT1 expressing vector LPiT1SN (MC3T3-E1-LPiT1SN) or the transfer vector LXSN (MC3T3-E1-LXSN) under standard cultivation conditions. The cells were seeded at 5,000 cells/cm² in 6-well plates and fixed and counted after 0.5, 1, 2, 6, and 24 h in culture (Fig. 2). Already 0.5 h after seeding, statistically significantly more PiT1 overexpressing cells than control cells had adhered (250% more) (Fig. 2). After 1 h in culture, 240% more MC3T3-E1-LPiT1SN cells than control cells had adhered (Fig. 2). At 2 h after seeding, the total number of adhering cells did not differ between the two cultures. Thus, as we found for NIH3T3 cells (Fig. 1A and B), the total number of cells adhering did not differ between PiT1 overexpressing MC3T3-E1 and control cells. However, at 6 and 24 h, statistically significantly more MC3T3-E1-LPiT1SN cells than control cells were present in agreement with increased cell division in PiT1 overexpressing cultures. Thus, PiT1 overexpression in the pre-osteoblastic MC3T3-E1 cells also led to faster adhesion.

Thus overexpression of PiT1 leads not only to increased proliferation of the NIH3T3-LPiT1SN and MC3T3-E1-LPiT1SN cells compared to their respective control cells [23] but also to faster adhesion under the same standard cultivation conditions (Figs. 1 and 2).

Fig. 2 - Effect of PiT1 overexpression on adhesion of MC3T3-E1 cells. MC3T3-E1-LPiT1SN (human PiT1 expressing) and MC3T3-E1-LXSN (control) cells were seeded in 6-well plates at 5,000 cells/cm². After 0.5, 1, 2, 6, and 24 h, the cells were washed, fixed, and stained with 0.1% crystal violet. A) Representative pictures of the wells after staining with crystal violet. B) Average numbers of cells in each microscope field in a 10x magnification. Results are means of 12 random fields ± SD. Size bars: 200 μm. * indicates statistically significantly different from control cells at the same day, p < 0.05.
Characterization of a second population of NIH3T3 cells overexpressing PiT1

To further validate that overexpression of PiT1 was sufficient to give NIH3T3 cells an adhesion advantage, we established other populations of human PiT1 expressing and control NIH3T3 cells. NIH3T3-PiT1 refer to NIH3T3 cells stably transduced with a slightly modified LXSN vector encoding human PiT1, and NIH3T3-control refer to NIH3T3 cells transduced with the empty transfer vector. The mRNA expression level of human PiT1 in NIH3T3-PiT1 cells is shown in Fig. 3A. Human PiT1 supports Pi uptake in NIH3T3 cells, and the NIH3T3-PiT1 cells show increased Pi uptake function compared to the NIH3T3-control cells (Fig. 3B), and at a similar level as NIH3T3-LPiT1SN cells (Fig. 3B) [23]. These results show that there is an increased expression of functional PiT1 protein at the cell surface of NIH3T3-PiT1 cells and at a level comparable to that of the NIH3T3-LPiT1SN cells. The NIH3T3-PiT1 cells also showed increased proliferation compared to NIH3T3-control cells (not shown). We performed cell cycle analysis to address whether the NIH3T3-PiT1 cells, as the NIH3T3-LPiT1SN cultures [23], showed different cell cycle profiles after just 12 h in culture. In agreement with previous results, overexpression of PiT1 led to statistically significantly lower percentage of cells in the G0/G1 phases and increased percentage of cells in S+G2/M phases at 12 and 24 h after seeding (Fig. 3C). We also included a cell cycle analysis 6 h after seeding in the present study. Albeit the differences are small, already 6 h after seeding, the PiT1 overexpressing cultures and the control cultures showed statistically significantly different in their cell cycle profiles, with a higher percentage of PiT1 overexpressing cells in G0/G1 and a lower percentage in S+G2/M phases (Fig. 3C). We next investigated whether the

Fig. 3 – Characterization of NIH3T3-PiT1 and NIH3T3-control cells. A) Analysis of the human PiT1 mRNA level in a second NIH3T3 cell population transduced with human PiT1 expressing vector. NIH3T3-PiT1 (human PiT1 (hPiT1) expressing) and NIH3T3-control (transduced with the transfer vector) cells were seeded at 20,000 cells/cm² in 4-well plates. After one day in culture, the human PiT1 mRNA level was determined using qRT-PCR. Results from cell lysates from three wells and triplicate qRT-PCR analyses of each cell lysates are shown. B) Pi-transport assay of NIH3T3-PiT1 and NIH3T3-control cells. The cells were seeded at 20,000 cells/cm² in 4-well plates. The next day, 32Pi import in Pi-free medium supplemented with 5 µM 32P and 95 µM P was analyzed over 5 min. Each column represents 32P import per mg protein per hour of four wells. Data are means ± SD. * indicates statistically significantly different from control cells, p < 0.05. C) Cell cycle analysis of NIH3T3-PiT1 and NIH3T3-control cells. The cells were seeded at 20,000 cells/cm² in T25-flasks in triplicates. After 6, 12, and 24 h, the cells were fixed in ethanol. The cells were stained with propidium iodide and analyzed by flow cytometry. The percentages of cells in the respective phases of the cell cycle 6, 12, and 24 h after seeding obtained using the Watson Pragmatic model are shown. In each sample, 20,000 cells have been counted. Each column represents the mean of triplicate set ups ± SD. * indicates statistically significantly different from control cells in the same phases of the cell cycle, p < 0.05.
NIH3T3-PiT1 and NIH3T3-control cells exhibited different adhesion potentials.

**Fast adhesion mediated by high PiT1 expression levels is independent of serum concentrations and growth on different TCPS qualities**

Both cell division and adhesion can be affected by the concentration of different serum factors. When seeding cells in varying serum concentrations, the amount of vitronectin compared to fibronectin adsorbed onto the TCPS changes. However, even at as low as 0.1% serum, adhesion-promoting proteins from the serum are adsorbed to the TCPS prior to cell adhesion. Using human serum, the variation in adsorbed adhesion-promoting proteins from the serum has been seen to affect the speed of adhesion and spreading of baby hamster kidney (BHK) cells, with cells seeded in low serum concentrations (0.1–1%) adhering and spreading faster than cells seeded in higher serum concentrations (e.g., 10%) [32].

We therefore also addressed a possible influence of serum factors in the experiment addressing whether the NIH3T3-PiT1 and NIH3T3-control cells showed different adhesion potentials. The cells were grown in the presence of 5% or 0.5% NCS for 48 h before seeding in the same serum concentrations. The protein contents per well were determined 3 h after seeding, and independent on the serum concentration, statistically significantly more protein per well was present in cultures of PiT1 overexpressing cells compared to control cells (Fig. 4A). We also found that the serum concentration did not have an effect on the adhesion rates of control (compare two left most columns in Fig. 4A) or PiT1 overexpressing cells (compare two right most columns in Fig. 4A). Thus, the ability of PiT1 overexpressing cells to adhere faster than control cells was not affected by the nutritional state of the cells or by the concentration of external serum factors during seeding.

Differences in the modifications of the TCPS affect which and how serum proteins are adsorbed [37,38]. This could influence the cell-adhesion rate in that cells can adjust to growth on the TCPS surface they are cultivated on. We therefore tested whether the fast adhesion of NIH3T3-PiT1 cells was affected by the quality of the TCPS they had been cultivated on prior to seeding. The cells were, for 48 h before seeding, cultivated on two types of TCPS, Sarstedt Red Cap (low quality TCPS) and NUNC, Nunclon™Δ surface (high quality TCPS), respectively. Both polystyrene surfaces are negatively charged when wetted, but flasks with the Nunclon™Δ surface have an optimized and guaranteed uniform charge density allowing adsorption of more adhesion-promoting proteins from the serum than standard treated TCPS as Red Cap TCPS flasks from Sarstedt, which are designed for adhesion of robust and easily adhering cells. As seen in Fig. 4B, both control and PiT1 overexpressing cells adhered faster after growth on high quality TCPS when re-seeded on TCPS of high quality, than after growth on low quality TCPS followed by seeding on high quality TCPS. Thus, the abilities of the cells to adhere were influenced by the TCPS quality they had previously been cultivated on. PiT1 overexpression, however, still resulted in increased adhesion rate independent of the TCPS quality of the cultivation flasks.

Thus, the increased adhesion rate of PiT1 overexpressing cells was neither affected by the TCPS surface that the cells had been cultivated on nor by serum concentrations.
NIH3T3 cells overexpressing Pit1 spread out faster after seeding compared to control cells

In order to initiate proliferation upon adhesion, anchorage-dependent cells are dependent on spreading by interaction with extracellular adhesion-promoting proteins [43–46]. During cell adhesion and spreading, F-actin is rearranged, thus staining of the actin filaments can be used to follow the adhesion and spreading processes. To address whether the increased adhesion rate of Pit1 overexpressing cells compared to control cells leads to faster spreading of NIH3T3-Pit1 cells, we did an F-actin staining after 0.5, 1, 2, and 6 h in culture (Fig. 5). As seen in Fig. 5A, more NIH3T3-Pit1 cells had adhered after both 0.5, 1, and 2 h compared to NIH3T3-control cells in agreement with the results shown in Figs. 1 and 4. Furthermore, NIH3T3-Pit1 cells appear to spread out at earlier time points compared to NIH3T3-control cells (1 h and 2 h) (Fig. 5A). When studying the cells at 40X magnification (Fig. 5B), it was seen that NIH3T3-Pit1 cells had started spreading already after 30 min whereas NIH3T3-control cells only showed initial spreading at 1 h and only after 6 h had both NIH3T3-Pit1 and NIH3T3-control cells spread out. Thus overexpression of Pit1 not only led to faster adhesion but also to faster spreading of the anchorage-dependent NIH3T3 cells.

Our previous results showed that when cultivated under standard cultivation conditions, overexpression of Pit1 led to increased proliferation rates of MC3T3-E1 and NIH3T3 cells [23]. We have here shown that overexpression of Pit1, under standard cultivation conditions, also leads to faster adhesion of MC3T3-E1 and NIH3T3 cells and, at least for NIH3T3 cells, to faster spreading after seeding. Using NIH3T3 cells, we have moreover shown that the increased adhesion rate induced by overexpression of Pit1 was unaffected by changes in serum-factor concentrations and TCPS quality during cultivation.

In order to proliferate, anchorage-dependent cells are dependent on signals from growth-factors in G1 and signals from adhesion in G1 and during cytokinesis [44,47]. The observation that growth factors are only required until mid G1 phase, while adhesion also is required later in the G1-phase as well as during cytokinesis underscores adhesion as an important regulator of cell proliferation in anchorage-dependent cells [44–47]. The signals from adhesion necessary in G1 and during cytokinesis are dependent on interactions between integrins and extracellular...
adhesion-promoting proteins and, e.g., allow the cells to spread out [46,48,49]. Specifically, spreading in G1 is critical for progression to the S phase [43,46]. Faster adhesion/spreading could therefore explain at least initial increased proliferation of anchorage-dependent cells after seeding. We hypothesize that the observed faster adhesion, and for NIH3T3 also faster spreading, after seeding of cells overexpressing PiT1 compared to control cells are contributing to an initial faster cell cycle progression of the PiT1 overexpressing cells after seeding and thereby also to at least the initial increased proliferation after seeding.

As mentioned above and shown in Byskov et al. [23], the PiT1 overexpressing cells do not just show an initial faster cell cycle progression after seeding, they proliferate faster, and cultures of these grow to higher cell densities than cultures of control cells [23]. Cell adhesion, cell shape, and cell density have long been recognized as tightly coupled during cell division [50]. Studies in which the cell shape was controlled suggest that cell shape is involved in controlling density-inhibited proliferation, i.e., decreased cell flattening in dense cultures relates to decreased proliferation [43,51]. If the level of PiT1, which indeed is regulated by the cells themselves [23], in general, and not only after seeding, is involved in determining the shape of the cells, this could also explain the observed increase in culture density of PiT1 overexpressing cells.

Beck et al. reported that knockdown of PiT1 in HeLa cells resulted in delayed progression through mitosis due to an extended metaphase stage. They also reported an impaired ability to proceed through anaphase and telophase/cytokinesis resulting in many large multinucleated cells [29]. Although the transformed status of the HeLa cells does make a direct comparison difficult, it is however interesting that knockdown of PiT1 expression impairs the ability of the cells to proceed through specific stages of the cell cycle, which, for attached cells, are dependent on changes in the ability of the cells to interact with extracellular adhesion-promoting proteins, e.g., rounding up and migration [47-50]. Thus interestingly, the effects of overexpression of PiT1 on NIH3T3 and MC3T3-E1 cells and the effects of knockdown of PiT1 expression in HeLa cells are all in agreement with a role of PiT1 in regulation of processes involving interaction with an extracellular matrix.

We have previously shown that the pre-osteoblastic MC3T3-E1 cells, unlike NIH3T3 cells, regulate the overexpressed PiT1 in a manner, so it does not support P1 transport into the cell [23], while increased PiT1 expression does lead to faster adhesion and proliferation of both cell types. Since PiT1 overexpression does lead to increased adhesion rate and proliferation of MC3T3-E1 cells, this could suggest that the increased adhesion and proliferation rates of PiT1 overexpressing cells are independent of the PiT transport ability of PiT1. The reduced proliferation of HeLa cells observed with knocked down PiT1 expression could be rescued by expression of a P1-transport-defect PiT1 protein [29]. These results strongly suggest that P1 transport per se is not involved in the effects that changes in the PiT1 expression level have on cell proliferation. Although a P1-sensor function of mammalian PiT1 has so far not been directly shown, it has been reported for the yeast P1 transporter Pho84 [52]. As the P1-transport-defect PiT1 used by Beck and co-workers [29] potentially could still convey a P1 signal or be captured in a signaling conformation, it is thus possible that the role of PiT1 in increased adhesion/spreading and proliferation rates relates to a P1-sensor function of PiT1.

Conclusion

The present study provides insight into how PiT1 overexpressing cells can proliferate faster than control cells. Thus we have here shown that elevated expression level of PiT1 can confer faster adhesion on MC3T3-E1 and NIH3T3 cells, and for NIH3T3 cells also faster spreading, under conditions which support increased cell proliferation and increased cell-density. Using NIH3T3 cells, we showed that the increased adhesion rate induced by overexpression of PiT1 was not affected by changes in serum concentrations or by the TCP5 quality during cultivation. We suggest that the here described faster adhesion and spreading are contributing to, at least the initial increase in proliferation after seeding of cells overexpressing PiT1 compared to control cells. It is notable that a role of PiT1 in processes regulating cell adhesion and spreading is in agreement with the observed later proliferative advantage of PiT1 overexpressing cells and increased cell density of the cultures as well as with the by others reported delayed progression through mitosis and impaired cytokinesis induced by knocking down PiT1 in HeLa cells growing attached.

Sources of Funding

This work was supported by the Novo Nordisk Foundation, the Danish Medical Research Foundation (Grant number 09-066064 (271-08-1005), the Danish Heart Foundation, the Danish Diabetes Association, and the Intramural Budget at the Department of Clinical Medicine at Aarhus University. IBK was supported by a scholarship from the Danish Cancer Society and in part by The Graduate School of Science and Technology at Aarhus University. KB was supported by a scholarship from the Danish Cancer Society and a fellowship from the Department of Clinical Medicine at Aarhus University.

Conflict of interest

None.

Acknowledgments

We thank Merete Scheving for excellent technical assistance. We thank Bryan O’Hara for P0J75.

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