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Adipocytes promote pancreatic cancer cell proliferation *via* glutamine transfer

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ABSTRACT

Adipocytes promote progression of multiple cancers, but their role in pancreatic intraepithelial neoplasia (PanIN) and ductal adenocarcinoma (PDAC) is poorly defined. Nutrient transfer is a mechanism underlying stromal cell-cancer crosstalk. We studied the role of adipocytes in regulating *in vitro* PanIN and PDAC cell proliferation with a focus on glutamine metabolism. Murine 3T3L1 adipocytes were used to model adipocytes. Cell lines derived from PKCY mice were used to model PanIN and PDAC. Co-culture was used to study the effect of adipocytes on PanIN and PDAC cell proliferation in response to manipulation of glutamine metabolism. Glutamine secretion was measured with a bioanalyzer. Western blotting was used to study the effect of PanIN and PDAC cells on expression of glutamine-related enzymes in adipocytes. Adipocytes promote proliferation of PanIN and PDAC cells, an effect that was amplified in nutrient-poor conditions. Adipocytes secrete glutamine and rescue PanIN and PDAC cell proliferation in the absence of glutamine, an effect that was glutamine synthetase-dependent and involved PDAC cell-induced down-regulation of glutaminase expression in adipocytes. These findings suggest glutamine transfer as a potential mechanism underlying adipocyte-induced PanIN and PDAC cell proliferation.

1. Introduction

Stromal cell-tumor crosstalk contributes to carcinogenesis. Study of tumor microenvironment stromal cells has focused on fibroblasts, but a growing literature implicates adipocytes. Peritumor adipocytes predict poor prognosis in multiple cancers [1,2], and adipocytes promote proliferation and invasion of multiple types of cancer cells in *in vitro* and *in vivo* models [3–10]. Similar data support a role for adipocytes in PDAC: pancreatic steatosis in humans predisposes to PanIN, PDAC, and to more advanced disease [11,12], while human adipose tissue stem cells promote pancreatic cell proliferation and invasion *in vitro* [13]. Finally, pancreatic adipocytes are associated with PDAC progression in murine models [14–16].

The mechanisms by which adipocytes promote cancer are unknown. An important candidate mechanism is reciprocal metabolic programming that promotes energy transfer from stromal cells to cancer cells. Such metabolic crosstalk has been demonstrated in fibroblast-tumor lactate shuttling in breast and prostate cancers [17,18], while reciprocal changes in lipolysis and fatty acid oxidation promote fatty acid delivery from adipocytes to ovarian cancer [8].

Abbreviations: FCS, fetal calf serum; GPNA, L-glutamic acid-γ-p-nitroanilide-hydrochloride; Gln, glutamine; GSI, glutamine synthetase inhibitor; GLS, glutaminase; GDH, glutamate dehydrogenase; GS, glutamine synthetase; PanIN, pancreatic intraepithelial neoplasia; PDAC, pancreatic ductal adenocarcinoma; QRTPCR, quantitative real-time polymerase chain reaction

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Many cancers are glutamine-dependent, including PDAC, which undergoes metabolic reprogramming towards a non-canonical glutamine catabolic pathway to produce NADPH for reducing capacity [19]. Transfer of glutamine from cancer-associated fibroblasts to breast cancer cells drives breast cancer cell proliferation, with reciprocal metabolic reprogramming that promotes glutamine synthesis in fibroblasts and glutamine catabolism in breast cancer cells [20]. A single report implicates adipocytes as a source of glutamine for cancer cells in the context of leukemia, with reciprocal induction of glutamine synthetase (GS) in adipocytes that promotes glutamine transfer to leukemia cells [21]. The specific effects of adipocytes on PDAC cells *in vitro* are poorly defined, and whether adipocytes provide glutamine to PDAC is unknown. The goal of this study was to define the effects of adipocytes on PanIN and PDAC cell proliferation and determine if adipocytes influence PanIN and PDAC cell proliferation *via* glutamine-dependent metabolic crosstalk, utilizing murine PanIN and PDAC cell lines and the murine 3T3L1 adipocyte line.

2. Methods

2.1. Cell culture

All cell culture was performed in standard tissue culture conditions of 37 °C, 5% CO_2. Mature 3T3L1 adipocytes were generated by culturing 3T3L1 preadipocytes (American Type Culture Collection, Manassas, VA, USA) in DMEM, 10% FCS, 500 μM IBMX, 1 μg/ml insulin, 0.25 μM dexamethasone, 2 μM rosiglitazone for 3 days, followed by culture in DMEM, 10%FCS, 1 μg/ml insulin for 3 days, followed by culture in DMEM, 10% FCS for 7 days. Trypan Blue staining was used to determine adipocyte viability.

Murine PI34 and PD7591 cell lines are derived from a PanIN lesion (PI34) and a PDAC tumor (PD7591) from PKCY mice. The PKCY strain contains a codon-12 K-ras gene mutation, present in 95% of human PDAC tumors and a floxed p53 allele, expression of which is targeted to pancreatic epithelial cells *via* a Pdx1 promoter-driven Cre-recombinase gene, thus generating a pancreas-specific heterozygous p53 knockout on a mutant K-ras background. PKCY mice develop autochthonous PDAC by 18–20 weeks of age with histopathologic progression similar to human disease [22,23] Monoculture and co-culture experiments with mature adipocytes, preadipocytes, and cancer cells were performed in DMEM, 0.5% FCS with indicated glucose and glutamine concentrations. Conditioned media was generated by culturing mature adipocytes or PI34/PD7591 cells for 72 h in substrate conditions matching the corresponding experiment, after which media was harvested and used in a 50:50 mixture with fresh media containing the same substrates as the conditioned media. The irreversible glutamine synthetase inhibitor L-methionine sulfoximine (Sigma-Aldrich Inc., St. Louis MO, USA) was used at 10 mM to pre-treat adipocytes for 24 h followed by thorough washing of adipocytes 3 × with PBS prior to their use in co-culture. The glutamine exporter inhibitor L-glutamic acid γ-p-nitroanilide-hydrochloride (GPNA) (Sigma-Aldrich Inc.) was used at 1 mM and added directly to co-culture media, given that its effect is not irreversible, thus precluding pre-treatment of adipocytes prior to co-culture.

2.2. Oil Red-O staining

Differentiated adipocytes were washed with 500 μL 1X PBS, then 200 μL 4% formalin was added and cells fixed for 15 min, then formalin was removed, and cells washed twice with 1X PBS, then 200 μL of 60% isopropanol added and cells incubated 5 min, then isopropanol removed and 200 μL Oil Red-O solution (American Master Tech Scientific Inc., Lodi, CA, USA) added, then cells incubated 15 min, then Oil Red-O solution removed and cells washed with 500 μL 1X PBS 3 times and imaged with light microscopy.

2.3. QRTPCR

Equal amounts of input RNA prepared from cells using RNeasy kit (Qiagen, Inc., Germantown, MD, USA) were used for quantitative real-time polymerase chain reaction (QRTPCR). RNA was reverse-transcribed using a high capacity cDNA kit (Applied Biosystems, Inc., Foster City, CA, USA), and QRTPCR performed using transcript-specific Taqman primer-probes using actin as an endogenous control on an AB StepOnePlus Thermocycler (Applied Biosystems, Inc., Foster City, CA, USA). The 2^{-ddCT} quantification method was used to calculate fold-difference in transcript levels. The following murine-specific primer-probes were used (ThermoFisher Scientific Inc., Rockford, IL, USA): peroxisome proliferator-activated receptor gamma (PPARg:Mm01184322_m1), fatty acid synthase (FASN:Mm00662319_m1), adipose triglyceride lipase (AGTL:Mm00503040_m1), sterol regulatory element-binding transcription factor 1c (SREBP1c, Mm00550338_m1), CCAAT/Enhancer Binding Protein-alpha (CEBPa, Mm00514283_s1).

2.4. Western blotting

Adipocyte protein lysates in RIPA buffer (25–50 μg) underwent 7.5% SDS-PAGE gel electrophoresis, were transferred to PVDF membrane, blocked in TBST + 5%BSA for one hour at 25 °C, washed 3 times in TBST, incubated in TBST + 1%BSA overnight at 4 °C with primary antibodies specific for glutamine synthetase (rabbit polyclonal, 1:5000, ThermoFisher Scientific Inc., Rockford, IL, USA, Cat#PA1-46165), glutaminase (rabbit polyclonal, 1:1000, Abcam Inc., Cambridge, MA, USA, Cat#ab93434), or glutamate dehydrogenase (rabbit monoclonal, 1:1000, Abcam Inc., Cambridge, MA, USA, Cat#-ab166618), then washed in TBST and incubated with IRDye800-conjugated goat anti-rabbit IgG (1:10,000, Rockland Immunochemicals Inc., Gilbertsville, PA, USA, Cat#611-145-122) in TBST/1%BSA for one hour at 25 °C. Parallel blots loaded with identical amounts of the same lysates were probed with actin-specific primary antibody (mouse monoclonal, 1:1000, ThermoFisher Scientific Inc., Rockford, IL, USA, Cat#MA5-15739) and Alexa Fluor 700-conjugated secondary antibody (goat anti-mouse IgG, 1:1000, ThermoFisher Scientific Inc., Rockford, IL, USA, Cat#A-20136). Densitometry was performed using an Odyssey Infrared Imaging System and software (LI-COR Biosciences Inc., Lincoln, NE, USA); densitometry signals for glutamine synthetase, glutaminase, and glutamate dehydrogenase were normalized to actin densitometry levels.

2.5. Proliferation assay

Semipermeable transwell membrane inserts (0.4 μm, Corning Costar Inc., Tewksbury, MA, USA) containing 15,000 3T3L1 preadipocytes or mature adipocytes were placed above 2000 PanIN/PDAC cells in 24-well culture plates in 500 μl DMEM, 0.5%FCS with glucose/glutamine concentrations as indicated, cultured 72 h, transwells removed, and 50 μl XTT reagent (Biotium Inc., Hayward, CA, USA) added, cells incubated overnight, then 100 μl of media was removed and added to a 96-well assay plate (Corning Costar Inc., Tewksbury MA, USA, Cat#3795) and absorbance read at 450 nm with background subtraction reading at 650 nm on an Biotek Epoch plate reader (BioTek Inc., Winooski, VT, USA).

2.6. Glutamine measurements

Glutamine levels were measured in cell culture media using an immobilized enzyme electrode-based YSI 2950 Biochemistry