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Breast cancer metabolic cross-talk: Fibroblasts are hubs and breast cancer cells are gatherers of lipids

Filipa Lopes-Coelho [a, b, 1], Saudade André [a, c, 1], Ana Félix [a, c], Jacinta Serpa [a, b, *]

[a] Centro de Estudos de Doenças Crónicas (CEDOC), NOVA Medical School/Faculdade de Ciências Médicas, Universidade Nova de Lisboa, Campo Mártires da Pátria 130, 1169-056, Lisbon, Portugal
[b] Unidade de Investigação Em Patobiologia Molecular Do Instituto Português de Oncologia de Lisboa Francisco Gentil (IPOLFG), Rua Prof Lima Basto, 1099-023, Lisbon, Portugal
[c] Pathology Department, IPOLFG, Rua Prof Lima Basto, 1099-023, Lisbon, Portugal

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ABSTRACT

The cellular components of microenvironment are partners of cancer cells, sharing soluble factors and organic molecules to accomplish tumor energy and biomass demands. We tested the role of fibroblasts in fatty acids metabolism in breast cancer, addressing fatty acid synthase (FASN) expression and activity, the expression of lipids chaperons (FABPs) and transporters (FATPs) and lipids cellular content.

We showed that the amount of lipids increased in cancer cells exposed to fibroblasts conditioned media, showing that lipids transfer is crucial in this metabolic cross-talk. Accordingly, it was seen in those cancer cells a concomitant decrease in the expression of FABP2 and FABP3 and an increase in FATP1 expression, whose function is independent of FABPs. The *in vivo* experiment corroborates the role of CAFs in tumor growth.

Our study is one more step toward the understanding of metabolic dynamics between cancer cells and CAFs, disclosing FATP1 as a putative target to disturb the transfer of lipids between CAFs and breast cancer cells.

1. Introduction

Breast cancer is the most frequent cancer in women worldwide, with an estimated 1.7 million cases and 521,900 deaths in 2012, accounting for 25% of all cancers and 15% of female cancer deaths (Torre et al., 2015). In cancer, a tissue phenomenon called desmoplasia occurs frequently (DeClerck, 2012), consisting in a deposition of fibrotic stroma surrounding cancer cells driven by an increased proliferation of fibroblasts. Hence, besides cancer cells, cancer associated fibroblasts (CAFs) are the predominant cellular component in tumor microenvironment, being thought to influence cancer initiation, progression and therapeutic response (Corsa et al., 2016; Luo et al., 2015). Obviously, their modulation will also be performed by the regulation of the availability of relevant metabolic compounds in the microenvironment (Martinez-Outschoorn et al., 2014; Micke and Arne, 2004; Cirri and Chiarugi, 2012; Koukourakis et al., 2006; Ou et al., 2015; Liu et al., 2016). Cancer cell metabolism must be fitted to answer the energetic and biomass demands within the organ/tissue microenvironment (Hanahan and Weinberg, 2011; Serpa and Dias, 2011). Several compounds are important but fatty acids have a central role, since they can be used as fuel and as construction blocks, sustaining cell renewal and mitogenesis (Serpa et al., 2010; Röhrig and Schulze, 2016; Nakamura et al., 2014).

In the present study, we aimed to explore the role of CAFs as fatty acids suppliers in breast cancer, in order to find the main intervenients in this metabolic cross-talk between fibroblasts and cancer cells, with and without hormone stimuli. From the complex fatty acid metabolic network, we will address the contribution for breast cancer metabolic dialog of: fatty acid binding proteins (FABP) (Furuhashi and Hotamisligil, 2008; Chmurzyńska, 2006; Storch and Thumser, 2000), working as chaperons in intracellular fatty acids transport; fatty acid synthase (FASN) (Menendez and Lupu, 2007;

Abbreviations: CAFs, cancer associated fibroblasts; FASN, fatty acid synthase; FABP, fatty acids binding proteins; FATP, fatty acids transporter proteins; GAFs, gynecomastie associated fibroblasts; SFs, skin fibroblasts.

* Corresponding author. Centro de Estudos de Doenças Crónicas (CEDOC), NOVA Medical School/Faculdade de Ciências Médicas, Universidade Nova de Lisboa, Campo Mártires da Pátria 130, 1169-056, Lisbon, Portugal.
E-mail address: jacinta.serpa@fcm.unl.pt (J. Serpa).

[1] Equal contribution.

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Mashima et al., 2009), and fatty acid transporter proteins, including FATP family and CD36 (Glatz et al., 2010; Heather et al., 2006; Hegarty et al., 2002; Dourlen et al., 2015), which are mediators of intercellular transport of fatty acids.

2. Experimental procedures

2.1. Cell lines and cell culture

Cell line derived from a patient with breast adenocarcinoma (MDA-MB-231: ATCC® HTB-26™) was obtained from American Type Culture Collection (ATCC, Manassas, VA, USA); skin fibroblasts (SFs) were gently assigned by Dr. Jose Casado and Dr. Juan Bueren - Hematopoiesis and Gene Therapy Division, CIEMAT, Madrid, Spain; gynecomastia associated fibroblasts (GAFs) and female breast cancer associated fibroblasts (CAFs) were established in our lab. For primary cell lines establishment the tissue derived from patients were dissociated mechanically followed by enzymatic digestion using collagenase type II (1 mg/mL) (17,101–015, Invitrogen, Carlsbad, CA, USA) for 3 h at 37 °C with agitation. Fibroblasts were characterized by flow cytometry with vimentin (IC2105A, R&D systems, Minneapolis, MN, USA) and cytokeratin (ab52460, Abcam, Cambridge, UK) staining.

Cells were maintained at 37 °C in a humidified 5% CO_2 atmosphere in Dulbecco's modified Eagle's medium (DMEM) 1X (41,965–039, Invitrogen) containing 4.5 g/L of D-glucose and 0.58 g/L of L-glutamine, 10% Fetal bovine serum (FBS) (S 0615, Invitrogen), 1% Antibiotic-Antimycotic (15,240,062, Invitrogen). Before any *in vitro* experiment, cells were synchronized under starvation (culture medium without FBS), for 8 h at 37 °C and 5% CO_2. For experimental conditions cells were cultured in 1% FBS with and without estradiol (1 nM; E8875, Sigma Aldrich, St Louis, MO, USA), estrone (0.1 μM; E9750, Sigma Aldrich) and testosterone (35 nM; T1500, Sigma Aldrich), for 16 h. MDA-MB-231 cells were also cultured in the presence of fibroblasts conditioned media, both with and without hormonal stimuli. Fatty acids exposure was performed by culturing cells in the presence of C16- palmitic acid (4.1 mM; P0500, Sigma Aldrich) and C18- oleic acid (3.2 mM; O1008, Sigma Aldrich), fatty acids concentrations were determined based on human serum levels described by Abdelmagid et al. (2015) (Abdelmagid et al., 2015). Proliferation curve was determined by using trypan blue (T8154, Sigma Aldrich) exclusion counting method.

2.2. Quantitative real-time PCR

RNA was extracted using RNeasy Mini Extraction kit (74,104, Qiagen, Hilden, Germany) and cDNA synthesized from 1 μg RNA and reversely-transcribed by SuperScript II Reverse Transcriptase (18080–44, Invitrogen), both according to the manufacturer's protocol. Quantitative Real-Time PCR was performed using Power SYBR Green PCR Master Mix (4367659, AB), according to manufacturer's protocol. Primers used were in Table 1. Real-time PCR was carried out in an ABI Prism® 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA, USA). Hypoxanthine-guanine phosphoribosyltransferase (HPRT) was used as housekeeping gene. Experiments were performed in biological triplicates.

2.3. Nile red staining

Nile red staining was performed based on Greenspan et al. (1985) (Greenspan et al., 1985) and Yao et al. (2011) (Yao et al., 2011). For flow cytometry analysis, cells were detached with trypsin, washed PBS 1x and stained with Nile Red (1 μg/mL) for 15 min at room temperature. After incubation, cells were centrifuged, PBS 1X was added and samples were analyzed on FACSCalibur flow cytometer (Becton-Dickinson). Data were analyzed using FlowJo software (http://www.flowjo.com/). Experiments were performed in biological triplicates.

2.4. Fatty acid synthase (FASN) enzymatic activity

Cell extracts were obtained after cell lysis (phosphate potassium buffer (pH 6.5), 1% Triton X100 and proteases inhibitor). FASN activity was calculated as follow based in Moustaid et al. (1995). and Bazin and Ferré (2009). Buffer A (acetyl-CoA (25 μM; A2056, Sigma Aldrich), NADPH (100 μM; 2646-71-1, Sigma Aldrich), phosphate potassium buffer, pH 6.5) was added to cell lysates and incubated at 37 °C for 5 min. After temperature equilibrium, buffer B was added (malonyl-CoA (60 μM; M 4263, Sigma Aldrich), phosphate potassium buffer, pH 6.5) and the reaction carried into automated spectrophotometer (Biotek ELx808) with temperature control (37 °C) at λ 340 nm for 2 h.

The fatty acid enzymatic activity was calculated by the determination of the amount of NADPH oxidized, using the formula:

$$\Delta C = \Delta A / E(340nm)$$

Where, ΔC corresponds to changes in NADPH concentration, ΔA

Table 1
Primers used to quantify FASN, FABPs and FATPs by quantitative real-time PCR.

Primer	Forward (5′-3′)	Reverse (5′-3′)
FABP1	CTTCATGAAGGCAATCGGTCTG	CCACCGTGAATTCGTTTTGGATC
FABP2	GGGTGTTAATATAGTGAAAAGGAAGC	GTTCAGTTCCGTCTGCTAGATTG
FABP3	CTCGGTGTGGGTTTTGCTAC	CTTCCTGTCATCTGCTGTTGTC
FABP4	GAAGTAGGAGTGGGCTTTGC	GCCCAGTATGAAGGAAATCTCAG
FABP5	CAGACTGTCTGCAACTTTACAG	GTGACATTGTTCATGACACACTC
FABP6	CACATAAAGGAAAGCCTCCCAG	CAAGGAGCTTCATGAACTCATC
FABP7	CTGTAAGTCTGTTGTTAGCCTGG	CTTACCATAACCATTTTGCCATCC
FASN	GCACCAATACAGATGGCTTCAAGGAGC	GCTCGGGTGATGCCATTCAGCTCC
FATP1	CAACATGGACGGCAAGGTC	CAGCAGCTCCATTGTGTCCTC
FATP2	GGATGTATTGTGGCTGGTGC	CCGAAGCAGTTCACCGATATAC
FATP3	CGTGCTTCCTGGCTTTACAAG	CCTGGCTCACCTGGAGATG
FATP4	CTACCTTACTGGTGATGTGCTG	GCGGCTGAGTGTGCCTTCCAC
FATP5	CTGCTTAGATCTCGGAGCC	CGCCCACATACAGGATCACTG
FATP6	GGATGTGTTGAGTTGGGTGCC	GATTGTTTGCAAAGGTAGCGAC
FATpm	GTGAGAACAGCGAAGTCTTGAAG	GCAGAAAACTGGCTCCGATC
CD36	GGTACAGATGCAGCCTCATTTC	GCAAAGGCCTTGGATGGAAG
HPRT	TGACACTGGCAAAACAATGCA	GGTCCTTTTCACCAGCAAGCT

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