Journal of Cellular Physiology

Modulation of Mammary Stromal Cell Lactate Dynamics by Ambient Glucose and Epithelial Factors

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Hyperglycemia is a risk factor for a variety of human cancers. Increased access to glucose and that tumor metabolize glucose by a glycolytic process even in the presence of oxygen (Warburg effect), provide a framework to analyze a particular set of metabolic adaptation mechanisms that may explain this phenomenon. In the present work, using a mammary stromal cell line derived from healthy tissue that was subjected to a long-term culture in low (5 mM) or high (25 mM) glucose, we analyzed kinetic parameters of lactate transport using a FRET biosensor. Our results indicate that the glucose pre-culture and soluble epithelial factors constitute a stimulus for lactate stromal production, factors that also modify the kinetic parameters and the monocarboxylate transporters expression in stromal cells. We also observed a vectorial flux of lactate from stroma to epithelial cells in a co-culture setting and found that the uptake of lactate by epithelial cells correlates with the degree of malignancy. Glucose preconditioning of the stromal cell stimulated epithelial motility. Our findings suggest that lactate generated by stromal cells in the high glucose condition stimulate epithelial migration. Overall, our results support the notion that glucose not only provides a substrate for tumor nutrition but also behaves as a signal promoting malignancy.

J. Cell. Physiol. 232: 136–144, 2017.

Current data strongly supports the hypothesis that cell microenvironment is a major determinant of tumor progression (Cirri and Chiarugi, 2012). Cell components are responsible for a variety of functions that allow tumors to grow. Among these functions, it has been proposed that stromal cells play a significant role in the metabolic reprogramming that characterizes the energetic behavior of tumors (Fiaschi et al., 2012). Since the pioneering studies of Warburg (Warburg et al., 1927), evidence has accumulated that tumor cells display high glucose consumption associated with an up-regulation of glycolysis (Gatenby and Gillies, 2004). On this basis, glucose usage, measured using the radiolabeled glucose analogue tracer [^{18}F]-fluorodeoxyglucose by positron emission tomography (PET), constitutes a useful clinical tool to identify distant metastases (Kelloff et al., 2005). This clinical assay is also supported by the fact that many forms of cancer are associated with higher rates of glycolysis, reduced pyruvate oxidation, and increased lactate production (Altenberg and Greulich, 2004).

The existence of aerobic glycolysis in invasive human tumors, its persistence under normoxic conditions and its correlation with aggressiveness, strongly suggest that the glycolytic phenotype confers a significant proliferative advantage during the evolution of cancer and must constitute a crucial component of the malignant phenotype (Gatenby and Gillies, 2004). Besides increased glucose consumption, a variety of tumor cells also show greater avidity for glutamine (Gln) which, in addition to lactate, are substrates for anabolic growth in cancer cells (Wise and Thompson, 2010). Together, metabolic changes that ensure the availability of lactate and Gln for cell proliferation are fundamental to the task of incorporating nutrients into biomass (e.g., nucleotides, amino acids, and lipids) (Vander Heiden et al., 2009). Tumors have tissue lactate levels ranging from 4 to 40 mM, versus 2 mM in normal tissues (Dhup et al., 2012). Metastatic tumors are more abundant in lactate compared to non-metastatic tumors, establishing a correlation with poor prognosis and shorter patient survival (Schwickert et al., 1995).

Until recently, metabolic studies of tumors had been restricted to the epithelial component. However, recent data provide evidence of a system of metabolic coupling, where tumor stromal fibroblasts behave as a catabolic phenotype, meanwhile carcinoma cells assume an anabolic role (Martinez-Outschoorn et al., 2014). In this model, catabolic (glycolytic) stromal cells are functionally coupled with their epithelial counterpart by: (i) enhanced expression of membrane monocarboxylate transporters (MCT4 in stroma and MCT1 in epithelia); and (ii) production of energy substrates (predominantly lactate and ketone bodies), which incorporated by epithelial cells, promote cell proliferation and growth (Vander Heiden et al., 2009). Metabolite translocation studies have been improved with the advent of genetically

Contract grant sponsor: Fondo Nacional de Investigación Científica y Tecnológica of Chile (FONDECYT);
Contract grant number: 1120187.
Contract grant sponsor: Chilean Government through the Centers of Excellence Basal Financing Program of CONICYT.

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Manuscript Received: 15 December 2015
Manuscript Accepted: 31 March 2016

Accepted manuscript online in Wiley Online Library (wileyonlinelibrary.com): 2 April 2016.
DOI: 10.1002/jcp.25398

encoded nanosensors that allow, via noninvasive methods, the determination of relative levels, concentrations and even metabolic fluxes in individual cells in vivo (San Martin et al., 2013).

Clinical and epidemiological studies have provided evidence that the glycemic load constitutes a risk factor for the development of some forms of human cancer. An association has been suggested between diabetes, especially type 2 diabetes mellitus (T2DM), and cancer incidence and progression (Vigneri et al., 2009). Hyperglycemia can influence cancer cells through increased levels of growth factors (as insulin/IGF-1) and inflammatory cytokines in circulation. Beyond that, there are also reasons to believe that hyperglycemia has a direct impact on cancer cell proliferation, apoptosis and metastasis (Ryu et al., 2014). Despite the fact that high glucose induces a variety of intracellular processes, direct effects of high glucose in cancer cell behavior are relatively unexplored. In the present study, we show that mammary fibroblastic stromal cells exposed to high glucose levels produce more lactate, display an enhanced expression of MCT4, and are capable of transferring lactate to epithelial cells. These results suggest that stromal cells cultured in hyperglycemic conditions are better equipped to sustain a metabolic coupling phenomenon in which stromal cells transfer lactate to epithelial cells. Our analysis is particularly relevant in the study of desmoplastic tumors, which, like in breast lesions, the abundance of stromal cells may account for glucose uptake and lactate production.

Materials and Methods

Cell culture, cell lines, and chemicals

Human cell line RMF-621 corresponds to a hTert-immortalized fibroblasts from a reduction mammoplasty, was a generous gift from Dr. Charlotte Kuperwasser (Tufts University, MA). Cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM) of low or high glucose (Invitrogen, Carlsbad, CA), supplemented with 10% fetal bovine serum (FBS) (Hyclone, Logan, UT) and maintained in a humidified atmosphere of 37°C, 5% CO2 (15). MCF-7 and MDA-MB 231, both human breast cancer cell lines, were purchased in ATCC (Manassas, VA) and were cultured in DMEM/F12 supplemented with 10% FBS and maintained in the same condition as above. MCT1 inhibitor AR-C155858 was acquired from Chem Express (Princeton, NJ).

Cell imaging

To evaluate lactate transport in living cells, we used the FRET nanosensor Laconic (San Martin et al., 2013) incorporated in adenoviral particles (Vector Biolabs, Philadelphia, PA). Laconic is a fusion protein composed by a ligand-binding moiety (LldR) specific to lactate and the fluorescent proteins mTFP and Venus. Imaging was carried out in a Nikon Ti microscope with a 40× objective equipped with a monochromator (Cairn Research, Kent, UK), which allows discrete excitation at 430 ± 10 nm. Two windows of emitted light were simultaneously collected at 490–520 nm (mTFP) and over 535 nm (Venus) by means of an optical splitter (Cairn Research). Images were digitized by a CCD camera (ORCA3, Hamamatsu, Japan) and data expressed as ratio between mTFP and Venus fluorescence. Experiments were conducted 48–72 h after infection at room temperature (24–26°C) in KRH buffer (in mM: 140 NaCl, 4.7 KCl, 20 Hepes, 1.25 MgSO4, 1.25 CaCl2, pH 7.4) supplemented with lactate. In experiments which include glucose and/or lactate as substrates, cells were also treated with the MCT inhibitor AR-C155858 (1 μM).

To evaluate the capacity of stromal RMF-621 cells to release lactate to epithelial cells, we established monocultures of MCF-7 and MDA-MB231 cells in a coverslips that were further infected with Laconic. Forty-eight hours later, cells were washed and incubated with KRH buffer supplemented with 0.3 mM glucose and basal intracellular lactate was registered. After 25 min an inoculum of 2.5×10^5 RMF-621 cells in KRH/0.3 mM, glucose was added. Variations of intracellular lactate were registered as above.

Cell migration assay

MCF-7 cell migration was studied using a 6.5-mm Transwell chamber with a pore size of 8 μm (Corning, Corning, NY). MDA-MB231 cell invasion was studied using the same system but coating the Transwell membrane with 10 mg/ml of Matrigel on the topside. We performed three types of experiments. In the first type (1×10^5), MCF-7 and MDA-MB231 cells were allowed to migrate (or invade) to the stimulus of a previously established monolayer of RMF-621 cells derived from high- and low-glucose culture. In a second type of experiment, a group of epithelial cells were pretreated 1 h before migration/invasion started, with a MCT1 inhibitor AR-C155858 (1 μM) and migrated/invaded media conditioned by RMF-621 cells previously cultured in high and low glucose. In a third type of experiment, both types of epithelial cells were pretreated with 10 mM lactate for 48 h previous to migration/invasion. In this case, cells were allowed to migrate against 10% FCS. MCF-7 cells migrate against different stimuli for a 24 h period and MDA MB-231 invades for 16 h. After this, membrane was fixed in methanol and migratory cells were stained on the lower side of the membrane with 0.2% crystal violet (Tobar et al., 2010). Migration values correspond to the average of three independent experiments by counting 16 fields from four pictures (×20) per chamber (two chambers per experimental condition).

Western blot and antibodies.

The expression of MCT4 and MCT1 protein in RMF-621 and epithelial MCF-7 and MDA-MB 231 cells was evaluated by Western-blot. Briefly, cells were lysed in lysis buffer (50 mM N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid, pH 7.4, 150 mM $NaCl_2$, mM $MgCl_2$, 2 mM ethyleneglycol-bis (aminoethylether)-tetraacetic acid, 1% Triton X-100 and 10% glycerol) supplemented with complete protease inhibitors (Roche, Mannheim, Germany). The protein concentration of cell lysates was determined using Pierce BCA Protein Assay kit (Thermo, Rockford, IL). Protein extracts were heat denatured in sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis loading buffer 4× (240 mM Tris–HCl, pH 6.8, 8% SDS, 40% glycerol, and 20% 2-mercaptoethanol). Equal amounts of protein from different treatments were resolved by SDS–polyacrylamide gel electrophoresis in 10% acrylamide gels and electrotransferred to polyvinylidene difluoride membranes using a buffer containing 24 mM Tris, 194 mM glycine, and 20% methanol. Proteins were further analyzed using the Pierce ECL Western Blotting Substrate (Thermo). The immunoreactions were achieved by incubation of the membranes, previously blocked with a solution containing 5% bovine serum albumin in Tris-buffered saline and 0.05% Tween 20 (Sigma, St. Louis, MO), with anti MCT4 (H-90) rabbit polyclonal antibody (sc-50329), anti MCT1 (H-1) mouse monoclonal antibody (sc-365501) both from Santa Cruz (Santa Cruz, CA), mouse anti-beta actin (A5441) and anti-tubulin (T5168) from Sigma. Densitometric analysis of western blot bands was performed using Molecular Imaging Software, Kodak version 4.0 (Rochester, NY).

Quantitative PCR

Total RNA was isolated with Trizol (Ambion, Carlsbad, CA) from RMF-621 and epithelial MCF-7 and MDA-MB 231 cells according to manufacturer instructions. Reverse transcription to complementary DNA was performed with 1 μg of RNA from each sample using M-MLV reverse transcriptase and oligo-dT (Promega, Madison, WI) as primer, according to manufacturer protocol.

MCT4 messenger RNA (mRNA) expression was assessed by real-time PCR using a Light Cycler instrument (Roche). The reaction was performed using 100 ng of complementary DNA and LightCycler® FastStart DNA Master SYBR Green I kit (Roche) in a final volume of 20 μl. All the reactions were performed in duplicate and negative controls were included. The primers used were: MCT1 (forward), 5′-GTGGAATGCTGTCCTGTCCTC-3′, MCT1 (reverse), 5′-TCGATAATTGATGCCCATGCC-3′, MCT4 (forward), 5′-ATTGGCCTGGTGCTGCTGATG-3′, MCT4 (reverse), 5′-CGAGTCTGCAGGAGGCTTGTG-3′, and 18s (forward) 5′-GGACACGGACAGGATTGACA-3′, 18s (reverse) 5′-GGACATCTAAGGGCATCACAG-3′. The level of MCT4 and MCT1 expression was normalized using 18s gene as loading control.

Lactate assay

Lactate was evaluated in media conditioned by RMF-621 cells (1×10^5 cells in a 12 well plate) derived from high- and low-glucose culture treated or not with 30% media conditioned by mammary epithelial cells for 48 h. Next, media conditioned were removed and 1 ml of culture media (phenol red-free) was added and incubated by 4 h. Afterwards, media were collected and lactate was evaluated by gas chromatography coupled to mass spectrometry (GC-MS) equipped with a capillary column HP5M. Briefly, an internal standard was added to each sample and the organic acid was oximated and extracted twice with Ethyl acetate as described in Kumps et al. (1999). The organic fraction obtained was dried under nitrogen and derivatized with BSTFA and TMS (1%). Afterwards, one microliter of derivatized sample was injected in the GC-MS. Each metabolite is identified based on its own mass spectra by matching with a spectral library of known metabolites from NIST, NIH. Quantification of acids lactic and pyruvic was carried out through prior elaboration of a calibration curve (quadratic equation) with increasing amounts of each metabolites normalized to Tropic Acid (Internal Standard).

Results

Environmental glucose modulates lactate transport in human mammary fibroblasts

To evaluate the influence of extracellular glucose on lactate transport, we used RMF-621 human mammary fibroblasts cultured in low and high glucose, expressing the genetically-encoded lactate-sensitive FRET nanosensor Laconic (San Martin et al., 2013). Regardless of the net direction of lactate flux, MCTs transport in both directions, and therefore their activity may be studied by monitoring influx or efflux, the former being more practical. Measurements of lactate influx upon exposure with 0.1, 1.0, 2.5, 5, 10, and 20 mM lactate of RMF-621 mammary stromal cells (Fig. 1A) resulted in an apparent K_m value of 6.4 ± 2.3 mM for cells cultured in 5 mM glucose and 6.7 ± 0.8 mM for cells cultured in 25 mM glucose. V_{max} values were 0.31 ± 0.04 ΔR/min for cells cultured in low glucose and 0.65 ± 0.03 ΔR/min for cells cultured in high glucose. The higher V_{max} value in cells cultured in high glucose suggested a transporter expression effect. To test this possibility, the expression of MCT1 and MCT4 were evaluated by qPCR and western blot. As Figure 1B–D shows, higher glucose content in culture selectively enhanced the expression of MCT4 mRNA and protein. Therefore, we could explain the enhancement of lactate permeability (V_{max}) in high glucose-derived cells by the improved activity of a specific isoform.

Epithelial soluble factors modulate lactate transport in glucose-pretreated human mammary fibroblast

Next, we analyzed whether epithelial soluble factors were able to modify the expression of MCT4. RMF-621 cells cultured in 5 or 25 mM glucose were exposed for 48 h to media conditioned by MCF-7 (weakly invasive) and MDA-MB231 (strongly invasive) epithelial breast cancer cell lines. As Figure 2 shows: (i) the basal expression level of MCT4 measured by qPCR (A) and western blot (B and C) was higher in cells derived from high glucose cultures; and (ii) the stimulus of media conditioned by epithelial cells on MCT-4 expression was related with cell malignancy, that is, more malignant cells produce a more powerful stimulation of MCT4 protein expression. We analyzed the lactate transport kinetics in RMF-621 cells derived from low and high glucose and treated with media conditioned by MDA-MB 231 cells (CM MDA), which showed the highest stimulus on stromal MCT4 expression. Stimulus of CM MDA on RMF-621 cells derived from low glucose resulted in a small change in lactate transport, with V_{max} values of 0.31 ± 0.04 ΔR/min and 0.46 ± 0.09 ΔR/min, and K_m values of 6.47 ± 2.33 mM and 7.62 ± 3.45 mM). Conversely, stimulus of MC MDA on RMF-621 cells derived from high glucose produce a significant change in V_{max} (from 0.65 ± 0.03 ΔR/min to 1.97 ± 0.18 ΔR/min) and a more expressive enhancement of Km (from 6.7 ± 0.8 mM to 24.8 ± 3.8 mM) (Fig. 2D). These results suggest that stromal RMF-621 cells derived from high glucose culture, in contact with malignant epithelial factors, exhibit a higher responsiveness compared to low glucose to a wider repertoire of lactate concentrations reflected in the expression of a higher average K_m value for the metabolite. These data correlate with the stimulus of CM MDA on MCT4 expression and allow us to suggest that, in these conditions, RMF-621 cells derived from high glucose acquired a predisposition to lactate export.

RMF-621 cells behave as lactate producers. Effect of high glucose and epithelial stimulus

Our results suggested that RMF-621 stromal cells are potential producers of lactate for eventual epithelial consumption. To analyze whether this assumption was correct, we performed two types of experiments. First, to assay whether in stromal cell, lactate was metabolized at a lower rate than is generated, the lactate signal was recorded in RMF-621 cells in the presence of 2 mM glucose and 2 mM lactate for 10 min (Fig. 3A). At this time, lactate was withdrawn from the culture and the signal decayed steeply. From minute 18, coincident with the addition of the MCT inhibitor AR-C155858 (1 μM), the signal increased indicating lactate accumulation. The most plausible explanation for this phenomenon is that lactate produced from glucose was not metabolized and was available for export. Next, we measured lactate levels in culture media generated by cells derived from high and low glucose using gas chromatography coupled to mass spectroscopy (GC-MS). To assess the effect of epithelial factors in lactate production, we pre-incubated (48 h) either low or high glucose-derived cells with (30%) media conditioned by MCF-7 and MDA- MB231 tumor mammary cells. After treatment, in the absence of stimuli, cells accumulated lactate for 4 h. As Figure 3B shows, at basal level, RMF 621 cells that derive from high glucose cultures produced and exported significantly more lactate than those maintained in low glucose. The combination of high glucose pre-culture and incubation with media conditioned by the malignant MDA-MB231 cells resulted in better stimulus for lactate production.

Co-culture of epithelial and stromal cells suggested a lactate unidirectional flux

To assess whether stromal lactate could be transferred to epithelial cells, a co-culture system was designed. For this purpose, adherent epithelial mammary cells expressing Laconic were cultured in 0.3 mM glucose without lactate. Twenty-five

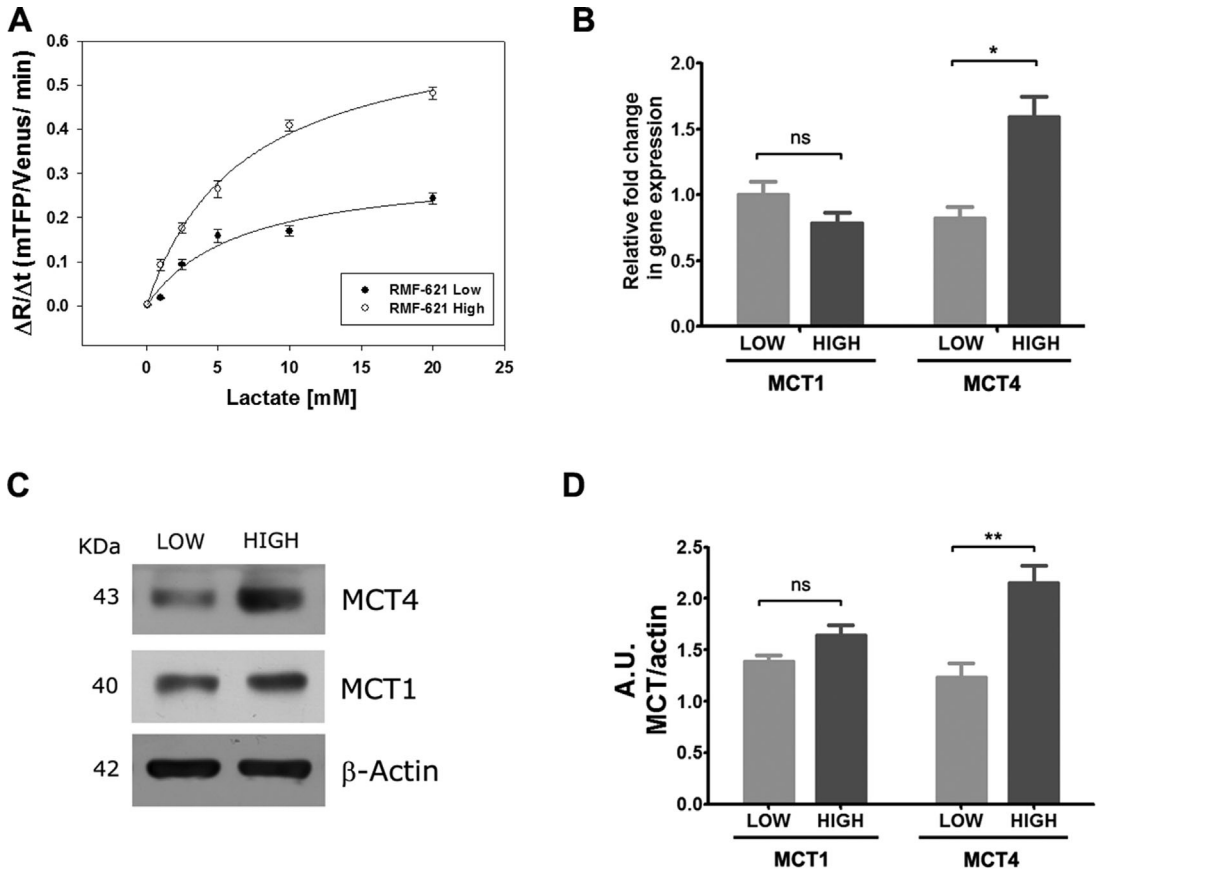

Fig. 1. Glucose preconditioning modulates lactate transport in mammary stromal cells. (A) Dose response of lactate uptake by stromal RMF-621 cells pre-treated with low (full symbols) and high (empty symbols) glucose. ΔR/Δt values were obtained calculating mTFP/Venus ratio per minute obtained measuring relative intracellular lactate concentration and plotted in function of lactate concentrations. Data correspond to average of 10 experiments with at least three cell recordings. (B) mRNA expression measured by qPCR of MCT1 and MCT4 in RMF-621 cells cultured in the presence of low (5.5 mM) or high (25 mM) glucose. Values are referred to those obtained for a MCT1 in low glucose. (C) Protein expression of MCT4 and MCT1 in RMF-621 cells cultured in high and low glucose. Image represents a representative western blot of three independent experiments. (D) Densitometric analysis of protein expression of MCT1 and MCT4 in RMF-621 cells cultured in the presence of low or high glucose. Data correspond to three independent experiments. Columns represent mean ratio of MCT1 and MCT4 signal to Actin signal; bars represent ±SD. * and ** indicates statistically significant differences with $P < 0.05$ or $P < 0.01$, respectively.

minutes later, a suspension of intact stromal RMF 621 cells derived from low- and high-glucose culture was added over the epithelial cell monolayer. In this experimental setting, the only source of external lactate for epithelial cells is the stromal cells and the increase of fluorescence ratio corresponds to epithelial lactate uptake from stromal release. As Figure 4 shows, epithelial cells showed a consistent tendency to incorporate more lactate from RMF-621 cells derived from high glucose and the slope values of lactate accumulation rate were higher in MDA-MB 231 than in MCF-7. At baseline, we found that the intracellular lactate signal was higher in MDA-MB 231 compared with MCF-7 cells.

In epithelial cells, MCT expression is related with lactate uptake and malignancy

The results of the co-culture experiment suggest that malignant epithelial cells have a high capacity to capture lactate. To test this hypothesis, we performed two types of experiments: (i) first, qPCR and western blot were used to evaluate the expression of monocarboxylate transporters in two types of epithelial mammary epithelial cells: MCF-7 (weakly invasive) and MDA-MB 231 (strongly invasive). As Figure 5A and B show, the MDA-MB 231 cell line (the more malignant line) expressed a higher protein level of MCT4 transporter compared to MCF-7, the less malignant line. On the contrary, the expression of MCT1 transporter was higher in MCF-7 than in MDA-MB231; and (ii) additionally, by performing saturation curves on lactate, we evaluated the kinetic values for lactate transport in both epithelial cell lines. As Figure 5C shows, both types of cells displayed very different saturation curves. Kinetic parameters of MCF-7 cells showed a Km value for lactate of 4.3 ± 0.11 mM and Vmax of 0.05 ± 0.006 ΔR/min whereas MDA-MB 231 cell line expressed a Km for lactate of 35 ± 8.9 mM and Vmax of 2 ± 0.35 ΔR/min.

Mammary stromal-produced lactate stimulated epithelial migration

To test whether stromal lactate was able to modulate phenotypic properties associated with epithelial malignancy, we performed migration/invasion experiments using MCF-7 (weakly migratory) and MDA-MB231 (highly migratory) cells. Three types of experiments were carried out: (i) we evaluated whether glucose pre-treatment modulated stromal capacity to stimulate epithelial migration. The results illustrated in

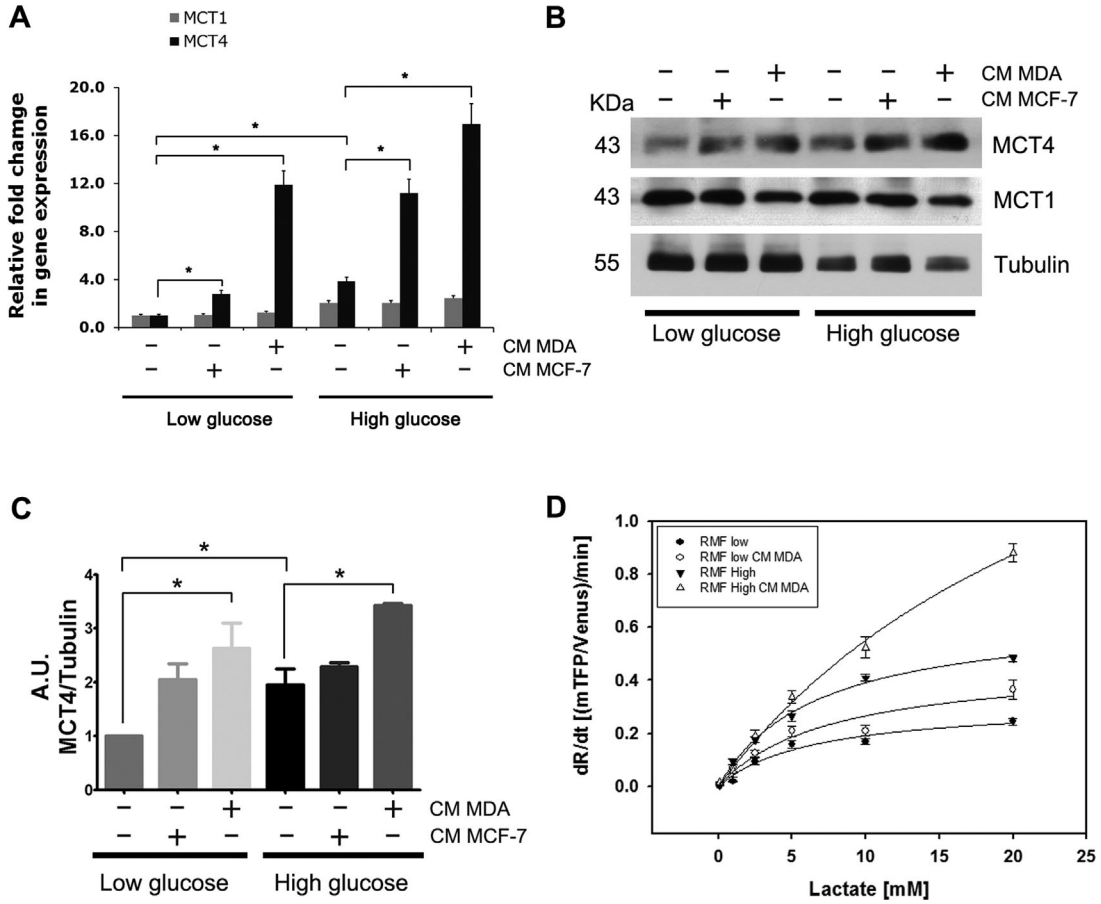

Fig. 2. Soluble epithelial factors stimulate MCT4 expression in RMF-621 cells. (A) mRNA expression of MCT 1 and 4 in RMF-621 cells cultured in low and high glucose and exposed to 30% media conditioned by MCF-7 and MDA-MB231 cells. (B) Protein expression of MCT4 and MCT1 in RMF-621 cells cultured in the same conditions as A. (C) Densitometric analysis of protein expression of MCT4 from three independent experiments performed in the same experimental conditions as in A. Columns represent mean ratio of MCT4 signal to Tubulin signal; bars represent ±SD. (D) Saturation curve for lactate of RMF-621 cells derived from low (circles) and high (triangles) glucose culture treated or not with 30% media conditioned by MDA-MB231 cells. *Indicates statistically significant differences with $P < 0.05$.

Figure 6A, showed that RMF-621 cells pre-cultured in high glucose and plated in the lower chamber of Transwell, constituted a more potent stimulus to epithelial migration (MCF-7 cells) and invasion (MDA MB-231 cells) than their counterpart cultured in low glucose; (ii) using a different experimental setting, we replaced RMF-621 cells as a stimulus for epithelial motility with media conditioned derived from high- and low-glucose culture. A group of epithelial cells (MCF-7 and MDA-MB231) were pre-treated with the MCT inhibitor AR-C155858 (1 μM) and allowed to migrate (or invade) in the presence or absence of the inhibitor. As Figure 6B shows, conditioned media stimulated epithelial motility in both type of cells and AR-C155858 was able to block this stimulus suggesting the participation of a monocarboxylate in this phenomenon; and (iii) finally, MCF-7 and MDA-MB231 cells were pre-treated for 48 h with 10 mM lactate and allowed to migrate against 10% FCS. Figure 6C shows that pre-treatment with lactate significantly stimulated both, cell migration and invasion.

Discussion

Enhanced glucose metabolism is a hallmark of cancer. Within tumors, glucose is largely metabolized to lactate even in a normoxic environment (Warburg, 1956). Most of the knowledge on tumoral metabolism has been gleaned from studies on homotypic populations of epithelial cancer cells, with less attention paid to the influence of the non-epithelial tumor cellular partners, such as tumor infiltrating fibroblasts (Martinez-Outschoorn et al., 2014). By association with cancer cells, fibroblasts acquire new secretory and contractile properties becoming cancer-associated fibroblasts (CAFs), the most prominent cell type of tumor stroma (Pietras and Ostman, 2010). Metabolic studies suggest that CAFs behave in a catabolic manner, contributing to the provision of energetic substrates like lactate to carcinoma cells (Martinez-Outschoorn et al., 2014). However, other studies have shown that stromal fibroblasts are lactate consumers (Rattigan et al., 2012).

Our results support the notion that fibroblasts are lactate producers and that this property is modulated by ambient glucose. We also found that stromal lactate constitutes an efficient stimulus for epithelial migration. These results are in line with the purported role of hyperglycemia as a risk factor for cancer (Sieri et al., 2013; Ryu et al., 2014), a role which remains contentious (Johnson et al., 2012). In molecular terms, it has been suggested that hyperglycemia modulates key cancer cell pathways, such as cell proliferation, apoptosis, migration and invasion (Ryu et al., 2014). The present findings suggest

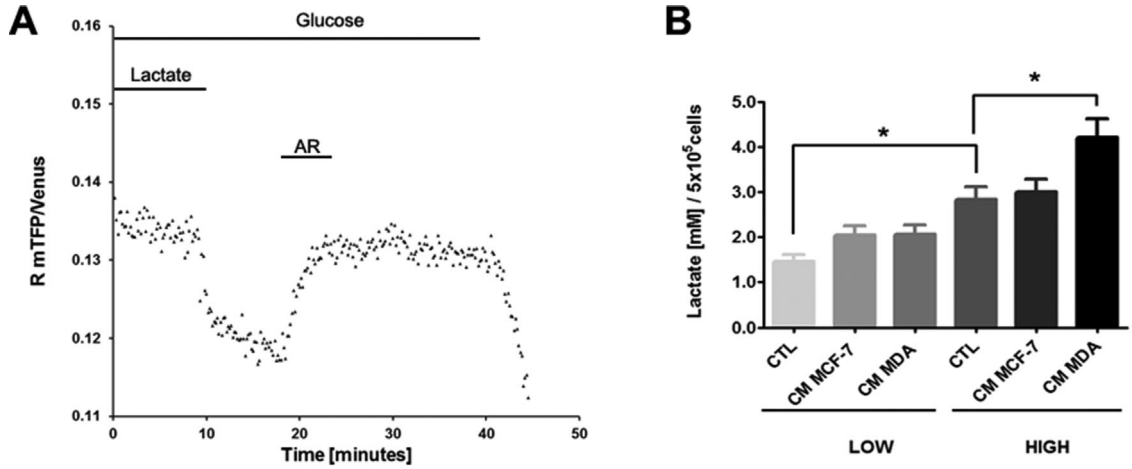

Fig. 3. RMF-621 cells behave as a lactate producer cells. Effect of epithelial soluble factors on lactate production. (A) Full trace of a time course of the fluorescence ratio of Laconic biosensor in RMF-621 cells derived from high glucose culture. Horizontal bars indicate the time in which different substrate were incubated with cells. AR is the MCT inhibitor AR-C155858. (B) Lactate abundancy (determined by GC-MS) in culture media of RMF-621 cells cultured in low and high glucose and treated or not with 30% media conditioned by MCF-7 and MDA-MB231 cells. *Indicates statistically significant differences with $P < 0.05$.

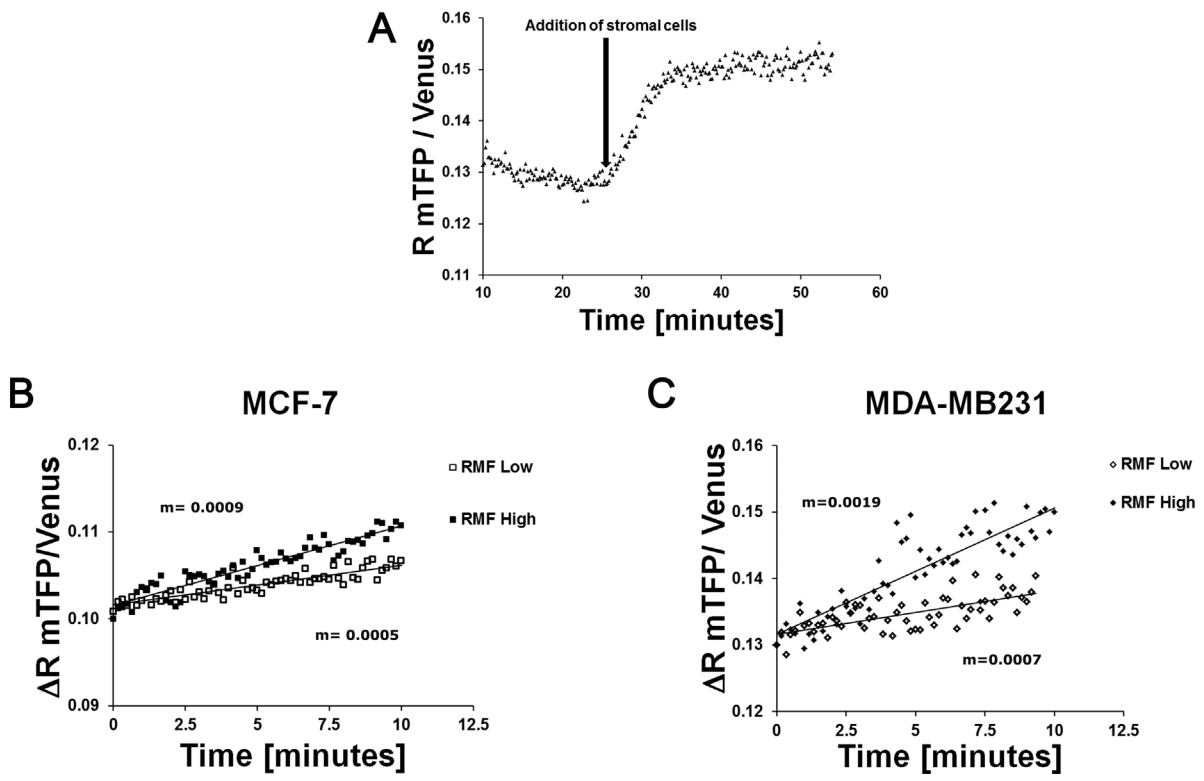

Fig. 4. Co-culture experiments suggest that RMF-621 cells behave as a lactate donor and epithelial cells as lactate receiver. (A) Time course of fluorescence ratio from a representative protocol indicating changes in intracellular lactate in MDA-MB231 cells infected with Laconic. Arrow indicates the time in which stromal RMF-621 cells (2.5×10^5) were added. (B) Time course of intracellular lactate variation in MCF-7 cell starting from the time in which intact RMF-621 (derived from low- and high-glucose culture) were added. (C) Time course of intracellular lactate variation in the MDA-MB231 cell starting from the time in which intact RMF-621 derived from low- (open symbols) and high-glucose culture (closed symbols) were added.

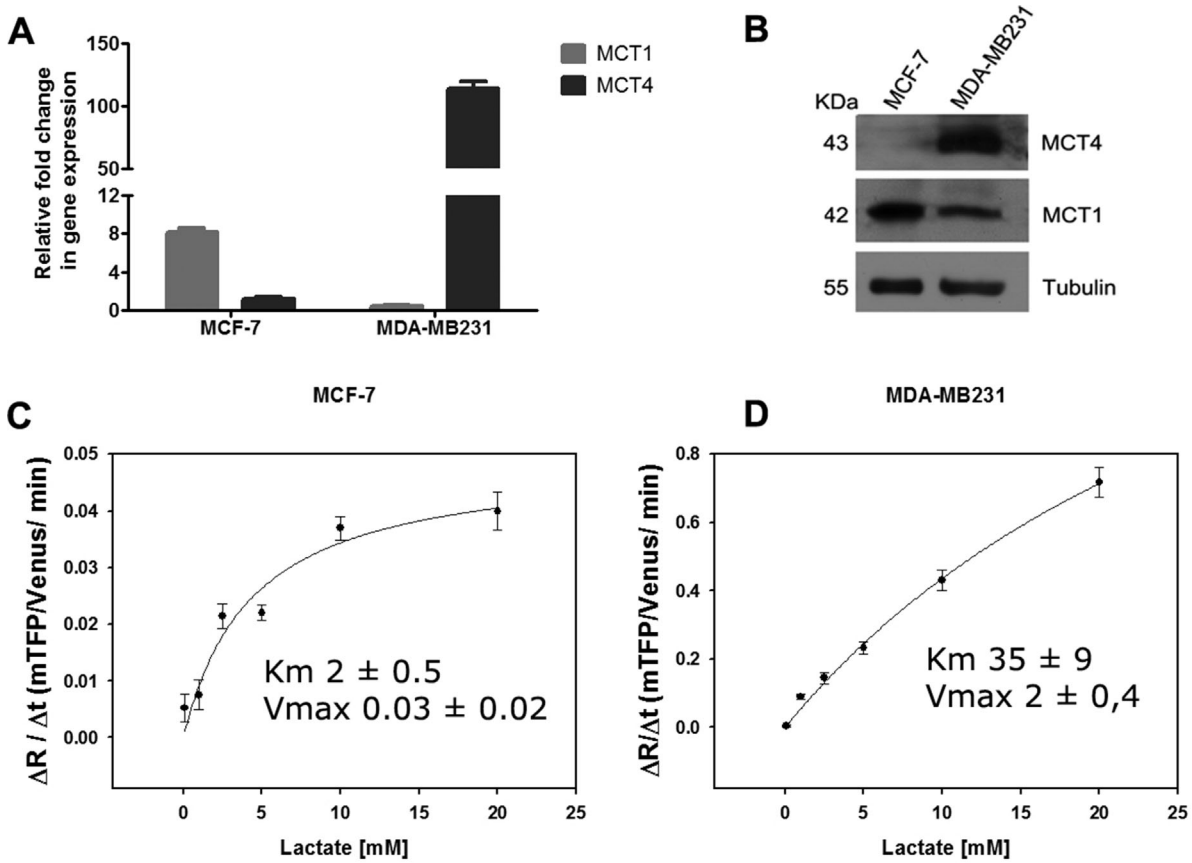

Fig. 5. MCT expression and kinetic values for lactate transport in mammary epithelial cells. (A) mRNA expression for MCT1 and MCT4 in intact MCF-7 and MDA-MB231 epithelial cells. (B) Western blot of MCT1 and MCT4 in intact MCF-7 and MDA-MB231 epithelial cells. (C) Saturation curve for lactate transport to MCF-7 cells measured with Laconic. (D) Saturation curve for lactate transport to MDA-MB231 cells measured with Laconic.

that, under the stimulus of glucose and epithelial factors, the expression of monocarboxylate transporters (MCTs) and the resulting impact on the transport and metabolism of lactate, become key elements in tumor metabolism.

MCTs play a role in the adaptation to cancer by regulating both energy supply and intracellular pH. RMF-621 cells primarily express MCT1 and MCT4 transporters, which differ mainly in their affinity for lactate, MCT1 being the more avid (Km for lactate = 3.5–6 mM) versus MCT4 (Km lactate = 22–35 mM). Results in Figures 1 and 3 show that RMF-621 cells cultured in high glucose have an enhanced expression of MCT4 and increased export of lactate. These findings are in line with previous data establishing that in white muscle glycolytic cells (one of the most glycolytic tissues), MCT4 appears to be the main mechanism adapted to the export of lactate (Dimmer et al., 2000). This stimulus on lactate transport and secretion of high glucose-cultured cells, occur with low Km values of lactate, suggesting the participation of MCT1. Nevertheless, when these cells were pre-treated with CM MDA, a condition that is more representative of a malignant microenvironment, the Km value for lactate increased significantly. This finding is consistent with the participation of MCT4 in lactate transport. Next, we attempted to verify the existence of a directional lactate flux from stroma to epithelia in a co-culture experiment. Two findings appear of particular significance. First, the fact that RMF-621 cells cultured in high glucose released more lactate than those cultured in low glucose and, second, that lactate uptake of MDA-MB231 (more malignant) cells was more effective than those of MCF-7 cell line, a weakly invasive cell line. Interestingly, kinetic analysis for lactate transport showed that malignant cells not only displayed a higher Vmax value, but also a Km value corresponding to MCT4. Consistently, MDA-MB231 cells were found to express predominantly MCT4. Our data shows that epithelial MCT4 expression correlated with malignancy degree in breast cancer cell lines. MDA-MB231 cells, the most malignant cell line in this study, expressed abundant copies of this transporter and showed a higher Km. MDA-MB231 cells also expressed some mesenchymal traits resulting from the epithelial to mesenchymal transition phenomenon, and corresponds to a triple-negative (TN) isotype, characterized by its aggressiveness (Chavez et al., 2010). Interestingly, this type of breast cancer is more frequent in women who are obese or have type II diabetes and that display a unique sensitivity to Metformin, one of the most used anti-diabetes drugs which has also been shown to have a relevant anti-tumoral effect (Liu et al., 2009). TN cells are characterized by high levels of MCT4 caused by stabilization of HIF-1, a well-known player in the acquisition of malignant properties (Pouyssegur et al., 2006).

We have previously shown that mammary stromal cells secrete soluble factors that stimulate epithelial migration (Tobar et al., 2010). The same experimental setting was used here to verify that RMF-621 cells pre-cultured with high glucose express a higher stimulus on epithelial migration and invasion compared to low glucose. By blocking the access of

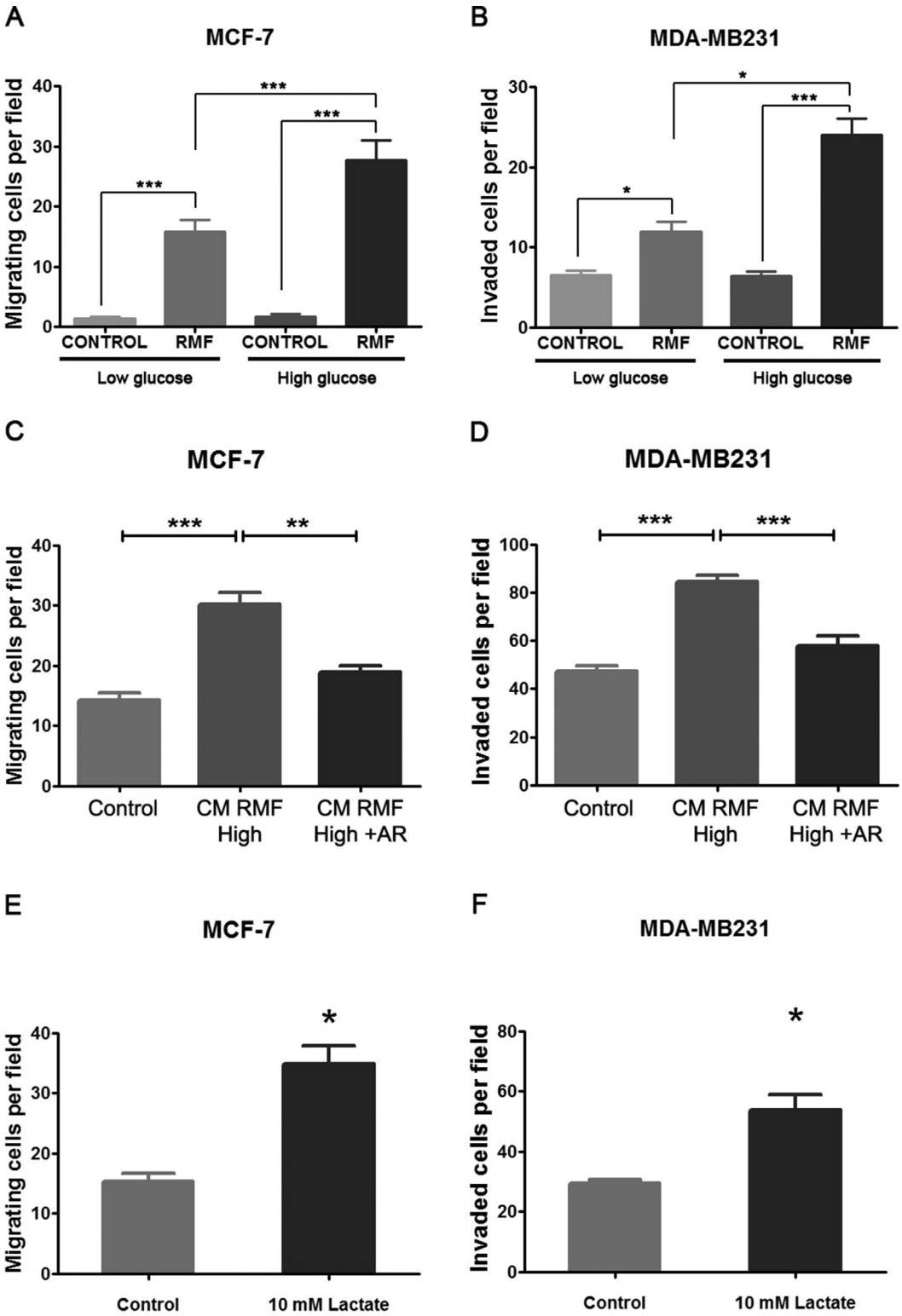

Fig. 6. Estromal lactate stimulates epithelial motility. (A) MCF-7 cells were stimulated to migrate for 24 h and (B) MDA-MB231 stimulated to invade for 16 h in a Transwell system. In this case, RMF-621 cells derived from low- and high-glucose culture were plated in the lower compartment of the Transwell. In control conditions, both cell lines migrated/invaded against culture media. (C and D) MCF-7 and MDA-MB231 cells pre-treated or not with AR-C155858 by 1 h and were allowed to migrate (C) or invade (D) against media conditioned by RMF-621 cells derived from low and high glucose culture. In control conditions both cell lines migrated/invaded against culture media. (E and F) MCF-7 and MDA-MB231 cells were pretreated with 10 mM lactate for 48 h previous to migration (E) or invasion experiment (F). Pre-treated and control cells migrated/invaded against 10% FCS. *, **, and * indicates statistically significant differences with $P < 0.05$, $P < 0.01$, and $P < 0.001$, respectively.**

lactate to epithelial migrating cells using a MCT blocking agent (AR-C155858), we postulate that lactate in media conditioned by RMF-621 cells derived from high- and low-glucose culture, was most likely, an important element in the stromal stimulus of epithelial migration. A further experiment in which epithelial migration/invasion was stimulated by lactate treatment confirmed this suggestion.

The present findings support the inductive effects of hyperglycemia on tumor development. Indeed, we propose that stromal cells, the more abundant phenotype in mammary tumors, subjected to a glycemic stimulus are able to establish a metabolic response characterized by the lactate production which not only provides a fuel for epithelial growth, but also constitutes a stimulus for cell migration and invasion, significant properties of the malignant phenotype.

Acknowledgments

The present work was supported by the grant 1120187 from Fondo Nacional de Investigación Científica y Tecnológica of Chile (FONDECYT; PI: JM). Authors thank Dr. Alejandro San Martin (CECs) for the training support on the use of imaging system. The Centro de Estudios Científicos (CECs) is funded by the Chilean Government through the Centers of Excellence Basal Financing Program of CONICYT.

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