

1 **EGFR activity upregulates lactate dehydrogenase A (LDHA) expression, LDH activity, and**
2 **lactate secretion in cultured IB3-1 cystic fibrosis lung epithelial cells**

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15 Running title: EGFR and extracellular pH in IB3-1 CF cells

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26 **ABSTRACT**

27 Cystic fibrosis (CF) is caused by mutations in the CFTR gene. It has been postulated that a reduced
28 HCO_3^- transport in CF through CFTR may lead to a decreased airway surface liquid (ASL) pH. In
29 contrast, others have reported no changes in the extracellular pH (pHe). We have recently reported
30 that in carcinoma Caco-2/pRS26 cells (shRNA for CFTR) or CF lung epithelial IB3-1 cells, the
31 CFTR failure decreased mitochondrial Complex I activity due to an autocrine IL1B loop, and
32 increased lactic acid production. The secreted lactate accounted for the reduced pHe since oxamate
33 fully restored the pHe. These effects were attributed to the IL-1 β autocrine loop and the downstream
34 signaling kinases c-Src and JNK. Here we show that the pHe of IB3-1 cells can be restored to normal
35 pHe values (~7.4) by incubation with the epidermal growth factor receptor (EGFR, HER1, ErbB1)
36 inhibitors AG1478 or PD168393. The inhibitor PD168393, fully restored the pHe values of IB3-1
37 cells, suggesting that the reduced pHe is mainly due to the increased EGFR activity and lactate. In
38 addition, in IB3-1 CF cells, the LDHA mRNA, protein expression, and activity are down-regulated
39 under EGFR inhibition. Thus, a constitutive EGFR activation seems to be responsible for the reduced
40 pHe in IB3-1 CF cells.

41 **Keywords:** CFTR; cystic fibrosis; EGFR; extracellular pH; IB3-1 cells; lactate; LDH

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45 **Introduction**

46 Cystic fibrosis (CF) is an autosomal recessive disease caused by mutations in the cystic
47 fibrosis transmembrane conductance regulator (CFTR) gene (Riordan et al. 1989). CFTR is an ATP-
48 gated chloride (Cl^-) channel, activated mainly through PKA phosphorylation (Chin et al. 2017),
49 which also participates directly or indirectly in the transport of bicarbonate (HCO_3^-) (Kunzelmann et
50 al. 2017), glutathione (GSH) (Kogan et al. 2003), and ATP (Egan 2002). Mutations in the CFTR
51 gene affect its expression and functionality leading to the complex CF phenotype (Lim et al. 2017;
52 Riordan 2008; Veit et al. 2016).

53 Several years ago, we postulated the hypothesis that the complex CFTR phenotype might be
54 the result of a differential gene expression between normal and CF cells (Cafferata et al. 1995).
55 Searching for possible CFTR-dependent genes by using differential display, we found different genes
56 which expression was altered in CF, such as *c-Src* (Gonzalez-Guerrico et al. 2002; Massip-Copiz et
57 al. 2017; Massip Copiz and Santa Coloma 2016), which in turn regulates MUC1 expression
58 (Gonzalez-Guerrico et al. 2002). This was the first signaling effector found for the CFTR signaling
59 pathway. We also found a reduced expression of MTND4, a mitochondrial protein encoded in the
60 mitochondrial DNA, essential for the assembly and activity of the mitochondrial Complex I
61 (Valdivieso et al. 2007; Valdivieso et al. 2012). Thus, we measured the mitochondrial complex I
62 (mCx-I) activity in CF cells and found that its activity was reduced (Clauzure et al. 2014; Valdivieso
63 et al. 2012)(reviewed in (Valdivieso and Santa-Coloma 2013)). Then, we thought that this mCx-I
64 failure could be the reason for the reduced extracellular pH reported in CF cells and tissues (Massip-
65 Copiz and Santa-Coloma 2018), through enhanced aerobic glycolysis. Confirming this hypothesis,
66 we found a decreased pHe, increased LDH activity and lactate secretion in intestinal Caco-2/pRS26
67 (Caco-2 colon carcinoma cells transfected with an shRNA-CFTR) compared to control Caco-
68 2/pRSCtrl cells, and also in lung IB3-1 CF cells compared to C38 cells (C38 are CFTR “rescue”
69 cells) (Valdivieso et al. 2019).

70 Interestingly, the inhibition of LDH with oxamate was able to completely revert the pHe
71 differences in Caco-2/pRS26 compared to control Caco-2/pRSCtrl cells, suggesting that, contrary to

72 what it is generally accepted (reviewed in (Massip-Copiz and Santa-Coloma 2018)), the increased
73 LDH and lactate secretion, and not a reduced bicarbonate secretion, was the preponderant mechanism
74 for the reduced pHe, at least in these colon epithelial cells and also in IB3-1 lung CF cells (Valdivieso
75 et al. 2019). On the other hand, the inhibition of c-Src with PP2 and JNK with SP600125 only
76 partially reverted the pHe differences between CF and control cells, suggesting that some additional
77 signaling events may be involved. In this regard, we found recently that CFTR regulates the
78 expression of EGFR ligands in Caco-2/pRS26 cells (shRNA for CFTR); in particular, epiregulin
79 (EREG) had the strongest response, which is upregulated through an IL-1 β autocrine loop (Clauzure
80 et al. 2014; Clauzure et al. 2017; Massip-Copiz et al. 2018; Massip-Copiz et al. 2017). This resulted
81 in an activated EGFR signaling (supplementary figures S1 and S2 in (Massip-Copiz et al. 2018)).
82 Noteworthy, JNK was a downstream effector in the EGFR signaling in these colon carcinoma cells.

83 Taken into account the activated EGFR signaling observed in CF cells (Burgel et al. 2007;
84 Kim et al. 2013; Martel et al. 2013; Massip-Copiz et al. 2018; Stolarczyk et al. 2018), this work
85 aimed to explore whether or not the EGFR is involved in pHe regulation and LDH expression of
86 IB3-1 CF cells. We used IB3-1 cells since these cells constitutively express IL-1 β and EGFR ligands
87 (Massip-Copiz et al. 2018), have an activated EGFR (Massip-Copiz et al. 2018), and a low pHe. We
88 found that the EGFR inhibitors AG1478 (reversible inhibitor) and PD168393 (irreversible inhibitor)
89 lead to an increased pHe in the conditioned media of the IB3-1 CF cells, reaching the pHe values of
90 CFTR “rescued” C38 cells. The effect of these inhibitors on the pHe was due to a decreased lactic
91 acid secretion in the conditioned media. The EGFR inhibitors also diminished the LDH activity and
92 reduced LDHA mRNA and protein expression. These results suggest that EGFR activity plays a key
93 role in regulating LDH expression and activity, lactate production, and secretion. Therefore, the
94 EGFR activity appears to be responsible for the extracellular pH reduction in the conditioned media
95 of IB3-1 CF cells, through lactate (lactic acid) hypersecretion.

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98 **MATERIALS AND METHODS**

99 **Reagents.** L(+)-lactic acid, free acid (Cat. # L1750-10G), protease inhibitor cocktail P2714, and
100 DMSO culture grade were purchased from Sigma-Aldrich (St. Louis, MO). AG1478 was from
101 Calbiochem (San Diego, CA) and PD168393 was from MedChem Express (MCE, Monmouth
102 Junction, NJ). All other reagents were analytical grade. The stock solutions of pathway inhibitors
103 were prepared at 1000 X in culture-grade DMSO. *Antibodies:* anti-rabbit antibody coupled to
104 horseradish peroxidase (polyclonal, W401B) was from Promega (Madison, WI); rabbit polyclonal
105 anti-actin antibody (A2066) was from Sigma-Aldrich, rabbit FITC-conjugated secondary antibody
106 (554020) was from BD Pharmingen, and rabbit monoclonal antibody anti-LDHA (C4B5, Cat. #3582)
107 and rabbit polyclonal antibody anti-phospho-LDHA (Cat. #8176) were from Cell Signaling
108 Technology (Danvers, MA).

109 **Cells.** IB3-1 (CRL-2777) and C38 (CRL-2779) cells were purchased from ATCC (www.atcc.org;
110 now available only at John Hopkins University). IB3-1 cells are bronchial epithelial cells derived
111 from a CF patient that had the most frequent mutation, $\Delta F508$, in one allele (Zeitlin et al. 1991).
112 These cells also have the non-sense mutation W1282X in the second allele (Gruenert et al. 2004),
113 which also determines by itself a severe disease (Shoshani et al. 1992). The IB3-1 cells have been
114 immortalized using the hybrid adenovirus adeno-12-SV40 (Zeitlin et al. 1991). C38 cells are IB3-1
115 cells transduced with an adeno-associated viral vector to stably express a functional CFTR, in a
116 truncated version of CFTR, which retains its Cl^- transport activity (Flotte et al. 1993). All cell lines
117 were cultured in DMEM/F12 with 14.29 mM HCO_3^- and 15.02 mM HEPES (Cat. No. 12500-096,
118 Life Technologies, GIBCO BRL, Rockville, MD) supplemented with 10 % FBS (Internegocios S.A.,
119 Mercedes, Buenos Aires, Argentina), 100 U/ml penicillin, 100 $\mu g/ml$ streptomycin, and 0.25 $\mu g/ml$
120 amphotericin B (Life Technologies, GIBCO BRL, Rockville, MD). Cultures were grown in a
121 humidified atmosphere containing 5 % CO_2 at 37 °C.

122 **Measurement of the extracellular pH (pHe).** Cells were cultured in p60 culture dishes (52.8 mm),
123 plated at 6×10^4 cells/cm² in 3 ml of culture medium (1×10^5 cells/dish). After incubation in serum-
124 free medium at the time indicated for each experiment, the pHe was measured in the conditioned

125 media by using a pH meter (UltraBasic UB-10, Denver Instrument, Bohemia, NY). To avoid pHe
126 changes in the culture medium due to atmospheric equilibration, each plate was maintained in the
127 incubator containing 5 % CO₂ until it was rapidly measured by using the pH meter.

128 ***Extracellular lactate measurement.*** Cells were cultured in p60 culture plates, seeded at a density of
129 4×10^4 cells/cm² in 3 ml of culture medium containing 5% FBS. Before treatments, cells were
130 incubated 24 h in 3 ml of serum-free medium to reach basal conditions, and then, the serum-free
131 medium was replaced by a new serum-free medium with or without treatments. After the second
132 incubation in serum-free medium, a sample (15 µl) of the medium was collected at 0, 24, and 48 h,
133 to measure lactate secretion. The samples were centrifuged at 600 x g for 10 min at 4 °C and the
134 lactate concentration was measured in the supernatants by using the Lactate kit from Roche (Cat. #
135 11822837 190, Roche Diagnostics GmbH, Mannheim, Germany), with some modifications to adapt
136 the measurements to a 96 wells plate spectrophotometer (Multiskan Spectrum, Thermo Fisher
137 Scientific, Vantaa, Finland) (Valdivieso et al. 2019). Briefly, the lactate calibration curve (5, 10, 20,
138 30, 40, 50, and 100 µM) was made by using a 150 mM lactate stock solution prepared from L(+)-
139 lactic acid dissolved in serum-free DMEM/F12 medium containing 15 mM HEPES and neutralized
140 to pH 7.4 using a 1 M KOH solution. The samples (conditioned media) were diluted 1:100 and 150
141 µl/well were loaded in 96 wells plates. The lactate kit reagents were mixed, 12.5 µl of R1 (this
142 solution is the hydrogen donor, ascorbate ≥ 30 U/ml, buffer) and 2.5 µl of R2 (4.9 mM 4-
143 aminoantipyrine, lactate oxidase (LOD, microorganisms) ≥ 15 U/ml, horseradish peroxidase ≥ 24
144 U/ml, buffer) per well, and 15 µl/well were added to the samples and the calibration curve. The
145 spectrophotometric measurement was performed at 660 nm after 5 min of incubation at RT.

146 ***Lactate dehydrogenase activity (LDH).*** Lactate Dehydrogenase Activity was determined
147 spectrophotometrically by measuring the oxidation of NADH during the reduction of pyruvate to
148 lactate (absorbance/min at 340 nm) as previously reported (Chen et al. 1989; Valdivieso et al. 2019).

149 ***LDHA reverse transcription and quantitative real-time PCR (qRT-PCR).*** qRT-PCR assays were
150 performed as previously described (Massip-Copiz et al. 2018; Valdivieso et al. 2012). Briefly, total

151 RNA was isolated by using a guanidinium thiocyanate-phenol-chloroform extraction solution
152 (Chomczynski and Sacchi 1987, 2006). The quality of RNA was checked by electrophoresis in
153 denaturing formaldehyde agarose gels (Sambrook J 1989), and measuring the ratios A260/A230
154 (greater than 2) and A260/A280 nm (over 1.7). Reverse transcription (RT) was performed by using
155 1 µg of total RNA, M-MLV reverse transcriptase (100 U, Promega, Madison, WI), and Oligo-dT12
156 in a 25 µl final reaction volume, according to manufacturer's instructions. The reaction was
157 performed for 90 min at 37 °C, 5 min at 75 °C, and then cooled to 4 °C. The synthesized cDNAs were
158 used immediately for PCR amplification or stored at -80 °C for later use. qRT-PCRs were performed
159 using an ABI 7500 real-time PCR system (Applied Biosystems Inc., Foster City, CA); the $\Delta\Delta C_t$
160 method was used for comparative quantification. TBP (Tata Box Binding Protein) was used as an
161 internal control. The cDNA samples were added to 25 µl of PCR reaction mixture containing a final
162 concentration of 2.5 mM MgCl₂, 0.4 mM deoxynucleotide triphosphates, 1 U of Pegasus Taq DNA
163 polymerase, 0.1 X EvaGreen (Biotium, Hayward, CA), 50 nM ROX as a reference dye, and 0.2 nM
164 of each primer. The qRT-PCR conditions were as follows: initial denaturation at 95 °C for 5 min,
165 followed by 30 cycles at 95 °C for 30 s, 60 °C for 30 s, and 72 °C for 30 s. The fluorescence signal
166 was acquired at the elongation step, at the end of each cycle. qRT-PCR reactions were carried out
167 in triplicates. The final quantification values were expressed as the mean of the Relative
168 Quantification (RQ) for each biological triplicate (n = 3). Primer sequences used in this paper were:
169 LDHA Fwd 5'-ATGGCAACTCTAAAGGATCAGC-3' Rv 5'- CCAACCCCAACA
170 GTAATCT-3' and TBP Fwd 5'-TGCACAGGAGCCAAGAGTGAA-3' Rv 5'-CACATCACAG
171 CTCCCCACCA-3'.

172 **Western Blots.** Cells were incubated as above indicated, washed twice with cold PBS, scraped with
173 cold extraction buffer (10 mM Tris pH 7.4, 100 mM NaCl, 0.1% SDS, 0.5% sodium deoxycholate,
174 1% Triton X-100, 10% glycerol) containing the protease inhibitor cocktail P2714 (5 mL of
175 cocktail/20 g of cell extract) plus phosphatase inhibitors (2 mM Na₃VO₄, 1 mM NaF, and 10 mM
176 Na₂PO₇), and centrifuged at 14 000 x g for 20 min at 4 °C. The supernatant was stored at -80 °C
177 until use. The protein concentration was measured by using the method of Lowry. SDS-PAGE and

178 Western blots were performed as previously described (Massip-Copiz et al. 2018). The band's
179 intensities were quantified by using the Image J software (<http://rsbweb.nih.gov>).

180 **Flow cytometry.** To obtain more accurate results, LDHA was also quantified by using flow cytometry
181 (Valdivieso et al. 2019). Briefly, IB3-1 cells were cultured as mentioned above, in p60 plates, at 37
182 °C in a humidified atmosphere containing 5 % CO₂. Cells were harvested with trypsin and washed
183 twice with PBS. Ice-cold samples containing 1 x 10⁶ cells per 150 µl of PBS were fixed by adding
184 50 µl of paraformaldehyde-sucrose 4X (paraformaldehyde 4 %-sucrose 4 % final concentration) to
185 each tube and incubated for 15 min at 4 °C. Cells were washed three times with PBS plus 0.2 %
186 Tween 20 (PBS-T) and centrifuged at 400 x g for 5 min. Unspecific binding sites were blocked with
187 5% BSA in PBS for 30 min and washed two times with PBS-T and centrifuged at 400 x g for 5 min.
188 Cells were incubated with 50 µl of the anti-LDHA rabbit antibody diluted 1:200 and incubated
189 overnight at 4 °C. Then, cells were washed three times with PBS-T and centrifuged at 400 x g for 5
190 min. Cells were incubated with 50 µl of the anti-rabbit-FITC antibody diluted 1:400, incubated 1 h
191 at RT, and washed three times with PBS-T. Cells were pelleted by centrifugation at 400 x g for 5
192 min, resuspended in 300 µl of PBS, and run on the flow cytometer (Accuri, BD Biosciences, San
193 José, CA). To quantify the LDHA content the mean of fluorescence intensity (MFI) was measured
194 and the results were expressed as mean ± SE of three independent experiments, each performed by
195 triplicates.

196

197 **Confocal immunohistochemistry (IHC).** IB3-1 and C38 cells were plated on coverslips in 24-well
198 plates (4 × 10⁴ cells/well; 2 × 10⁴ cells/cm²) and cultured for 48 h in 2 ml of DMEM-F12 without
199 serum 5%. Immunocytochemistry was performed as previously described (Valdivieso et al. 2019)
200 with some modifications. Briefly, cells were rinsed twice in ice-cold PBS and fixed with a freshly
201 prepared solution of 4% paraformaldehyde in PBS, for 20 min at RT. Then, cells were rinsed 3 times
202 in Tris-Buffered Saline 1× (TBS) and permeabilized with 0.1% Triton X-100 in TBS for 15 min at
203 RT. After three washes in ice-cold TBS, cells were blocked with 5% BSA-TBS for 1 h at room
204 temperature. Then, cells were incubated with a primary anti-LDH-A antibody, dilution 1:200 in 5%

205 BSA-TBS plus Tween-20 (0.05% v/v) and incubated overnight at 4 °C. After primary antibody
206 incubation cells were washed three times with TBS plus Tween-20 (0.05% v/v) and incubated with
207 FITC-conjugated secondary antibody, dilution 1:400 in the same buffer for 2 h, at RT.
208 Immunofluorescence images were captured with a Zeiss LSM 510 confocal microscope (Carl Zeiss,
209 Jena, Germany) using a 63X/1.2 NA (NA, numerical aperture) water-immersion objective. For FITC
210 (Ex/Em: 490/525), a 488-laser line was used for excitation and an LP 505 nm filter for emission
211 (green).

212

213 **Statistics.** Unless otherwise indicated, all the results corresponded to the average of three independent
214 experiments (mean \pm SD, n=3). One-way ANOVA and Tukey's tests were applied to determine
215 significant differences among samples ($p < 0.05$). In addition, we verified the significant differences
216 using the non-parametric Mann-Whitney or the Kruskal-Wallis (both using medians and CI instead
217 of means and SD). When differences exist in the significance between these tests, they are shown in
218 the figure legends. Significant differences between slopes were calculated by using the online
219 software Free Statistics Calculators (Sopper 2019), using the average slopes and SD values from n =
220 3 independent experiments (df = 2). The open circles in the graph bars are the average values of each
221 independent experiment.

222

223 **RESULTS**

224 **EGFR activity mediates hyperacidity in the conditioned medium of IB3-1 cells**

225 As shown in Figure 1 A, in agreement with our earlier results, IB3-1 cells have a more acidic
226 pHe compared to C38 cells (IB3-1 pHe = 7.19 ± 0.03 ; C38 pHe = 7.37 ± 0.04 (n = 3), $p < 0.05$
227 compared to IB3-1 cells). Also, a significant difference was observed in the lactic acid secreted to
228 the extracellular medium of C38 and IB3-1 cells (2.03 ± 0.34 mM vs 4.99 ± 0.32 mM (n = 3), $p < 0.05$)
229 incubated in serum-free medium for 48 h (Fig. 1B). As shown in Fig. 1C, there was a time-dependent
230 secretion of lactic acid that increased linearly in both cell lines (0, 24, and 48 h). The rate for lactate
231 production was significantly ($p < 0.05$, df = 2, $t = 4.62$) higher in IB3-1 cells (slope = 0.084 ± 0.009

232 (n=3), $R^2 = 0.99$) than in C38 cells (slope = 0.026 ± 0.006 (n=3), $R^2 = 0.95$). In agreement with these
233 results, the intracellular LDH activity (Fig. 1D) showed a significant rise ($p < 0.05$) in IB3-1 cells
234 ($154 \% \pm 13.0 \%$, n = 3) compared to control cells ($100 \% \pm 3.2$, n = 3).

235 To determine whether the EGFR receptor was involved in pHe regulation, IB3-1 and C38
236 cells were incubated 24 h in serum-free media, and then treated with the EGFR inhibitors AG1478
237 (tyrphostin AG-1478) and PD168393, for 48 h. These inhibitors were chosen since they are potent,
238 specific, and have different mechanisms of action, decreasing the probability of simultaneous
239 nonspecific off-target effects. AG1478 is a potent and reversible EGFR kinase inhibitor that blocks
240 EGFR tyrosine kinase activity and autophosphorylation (Levitzki and Gazit 1995; Osheroov and
241 Levitzki 1994); it also induces the formation of inactive, unphosphorylated EGFR dimers in the
242 presence or absence of the ligand (Arteaga et al. 1997). AG-1478 has an IC_{50} *in vitro* of 3 nM
243 (Levitzki and Gazit 1995) and an EC_{50} of 34.6 μ M in cultured U87MG cells (MG stands for
244 malignant glioma) (Han et al. 1996). At 20 μ M, AG1478 inhibits EGFR phosphorylation at Tyr1173
245 in MDA-MB-231 breast cancer cells (D'Anneo et al. 2013). AG1478 inhibited EGF-stimulated
246 mitogenesis without affecting PDGF-induced DNA synthesis and [3H]-Thymidine uptake stimulated
247 by FGF-2 was not affected by AG1478 at 100 μ M (Rice et al. 1999). The ED_{50} against HER2-
248 Neu and PDGFR is over 100 μ M (Levitzki and Gazit 1995).

249 On the other hand, PD168393 is a potent, irreversible kinase inhibitor of EGFR and ErbB2
250 that alkylate Cys-773 by docking into the ATP binding pocket of EGFR tyrosine kinase, in the
251 cytoplasmic side of the receptor; it showed an IC_{50} of 0.7 nM (purified EGFR kinase) and EC_{50} of
252 1.3 nM in HS-27 human fibroblasts, and an EC_{50} of 4.3 nM in A431 cells, for EGF-induced (100
253 ng/ml EGF, 5 min) EGFR autophosphorylation (2 μ M during 1 h preincubation to reach 100
254 irreversible inhibition) (Fry et al. 1998). it is inactive against insulin, platelet-derived growth factor,
255 and basic FGF receptor TKs, as well as protein kinase C at 50 μ M (Fry et al. 1998).

256 To make sure that we were using the adequate concentrations of inhibitors, dose-response
257 curves were obtained (Fig. 2A and Fig. 2B). IB3-1 cells were treated with increased concentrations

258 of AG1478 or PD168393 and the pHe was measured in conditioned media after 48 h incubation. As
259 shown in Figure 2A, AG1478 showed an $EC_{50} = 5.9 \pm 0.3$ (n=3) μ M, with a plateau starting at 10
260 μ M, which is the concentration used by Takai et al. with several ovarian cell lines (Takai et al. 2010).
261 On the other hand, as shown in Figure 2B, the inhibitor PD168393 showed an $EC_{50} = 2.1 \pm 0.5$ (n=3)
262 μ M, with a plateau at 10 μ M, which is the concentration previously used by Sun et al. in mouse
263 cardiomyocytes (Sun et al. 2015). The different mechanisms of action of these inhibitors decrease
264 the probability of similar off-target effects. Nevertheless, off-target effects have been observed in
265 other model systems for both inhibitors (Douglas et al. 2009; Shi et al. 2009). Also, other members
266 of the EGFR family, such as erbB2, could be involved in the observed effects; therefore, these results
267 should be taken with caution. We performed dose-response curves to determine the adequate doses
268 for these inhibitors that affect the pHe of IB3-1 cells. We selected a 10 μ M concentration since this
269 concentration already showed a plateau. This concentration of AG1478 was already used with IB3-
270 1 and C38 cells by Kim et al., showing these authors that 10 μ M inhibited the EGFR phosphorylation
271 at Y1068 in both IB3-1 and C38 cells (Kim et al. 2013). Thus, both inhibitors were then used at 10
272 μ M, which is considered a still acceptable concentration to avoid off-target effects (Knight and
273 Shokat 2005).

274 As shown in Figure 2C, there was a significant ($p < 0.05$) decrease in the pHe of IB3-1 cells
275 (7.19 ± 0.03 , n = 3) compared to the pHe of CF corrected cells C38 (7.37 ± 0.04 , n=3), which is a
276 value within the physiological range of 7.35-7.45 for blood; below 7.35 is considered acidemia
277 (Hopkins et al. 2020). When the cells were cultured in the presence of the EGFR inhibitor AG1478
278 (10 μ M, 48 h) there was a significant increment in the pHe compared with untreated control cells
279 (7.36 ± 0.01 vs 7.19 ± 0.03 , n = 3, $p < 0.05$). AG1478 slightly increased the pHe of C38 cells, although
280 the values did not reach a significant difference. Similarly, as shown in Figure 2D, IB3-1 cells treated
281 with the irreversible EGFR inhibitor PD168393 (10 μ M, 48 h) also increased their pHe (7.19 ± 0.03
282 vs 7.35 ± 0.02 , n = 3). However, as shown in Figure 2D, in contrast to AG1478, PD168393 did not
283 have any observable effect on C38 cells, and the pHe recovery was almost complete with this

284 inhibitor (7.35 ± 0.03 , $n=3$), compared to C38 cells (7.37 ± 0.04 , $n=3$). This full recovery in the pHe
285 suggests that the observed pHe reduction in IB3-1 cells is entirely due to EGFR activation.

286

287 **EGFR modulates LDH activity and lactic acid production**

288 Considering the effect of the EGFR in the pHe regulation shown above, we thought to
289 determine whether EGFR also regulates lactic acid secretion (measured as lactate) and LDH activity.
290 These parameters were measured in IB3-1 and C38 cells treated with the EGFR inhibitors AG1478
291 and PD168393. Fig 3A shows a decreased LDH activity in IB3-1 cells treated with AG1478
292 compared with control cells (154 ± 13 % vs. 120 ± 4.9 %, $n = 3$, $p < 0.05$). In the same way, as shown
293 in Fig. 3B, IB3-1 cells treated with PD168393 also decreased their LDH activity (154 ± 9.7 % vs 85
294 ± 5.1 %, $n = 3$, $p < 0.05$). Both inhibitors had no effects on the LDH activity of C38 cells. Regarding
295 lactate, as shown in Fig. 3C and 3D, after 48 h, these inhibitors also significantly ($p < 0.05$) decreased
296 the lactate secretion in IB3-1 cells. There is a time-dependent secretion of lactate that increased
297 linearly in IB3-1 cells with or without inhibitors; however, the rate for lactate production was
298 significantly ($p < 0.05$) lower in IB3-1 cells treated with AG1478 (slope = 0.066 ± 0.010 , $n=3$) (Fig.
299 3C) or PD168393 (slope = 0.048 ± 0.011 , $n=3$) (Fig. 3D) than in IB3-1 cells without treatment (slope
300 = 0.084 ± 0.009 , $n=3$). The effects on IB3-1 CF cells were stronger with the irreversible inhibitor
301 PD168393. However, although these inhibitors fully restored the pHe, only partially restored the
302 lactate levels compared to C38 cells after 48 h incubation (IB3-1 4.99 ± 0.32 mM ($n = 3$), $n=3$; IB3-
303 1 AG1478 3.68 ± 0.60 mM, $n=3$; IB3-1 PD168393 3.60 ± 0.75 mM, $n=3$; vs. C38 2.03 ± 0.24 mM;
304 $n=3$). There was always a basal lactate production independent of the EGFR activity.

305

306 **LDHA expression is upregulated in IB3-1 cells**

307 Then, we want to determine if the increased LDH activity and lactic acid secretion correlated
308 with LDHA expression in IB3-1 cells. We observed a significantly increased mRNA expression of

309 LDHA (Fig. 4A) in IB3-1 cells compared to C38 cells ($163 \pm 18.5\%$ vs. $100 \pm 5\%$, $n=3$). When we
310 analyzed the protein expression by Western blots (Fig. 4B), there was also an increased expression,
311 not only in total LDHA ($100 \pm 4.4\%$ vs. $146 \pm 9.7\%$, $n=3$) (Fig. 4C) but also in p-Tyr10-LDHA
312 ($100 \pm 4.5\%$ vs. $135 \pm 10\%$, $n=3$) (Fig. 4D). The expression of LDHA was also analyzed by confocal
313 microscopy. As shown in Figure E, IB3-1 cells showed an increased green FITC fluorescence signal
314 corresponding to the secondary Ab against anti-LDHA, compared to C38 cells. The differences in
315 LDHA expression observed by WBs and confocal microscopy were then quantified by using flow
316 cytometry, which provides a more reliable quantification, cell by cell (Fig. 4F-I). LDHA and p-
317 LDHA expression levels were expressed as Mean Fluorescence Intensity (MFI). The LDHA
318 expression ($100 \pm 7\%$ vs. $160 \pm 38\%$, $n=3$) (Fig. 4F, 4G) and the p- Tyr10-LDHA expression (100
319 $\pm 1\%$ vs. $195 \pm 9\%$, $n=3$) (Fig. 4H, 4I) were both significantly higher ($p<0.05$, $n=3$) in IB3-1 cells
320 than in C38 cells. The increased p-Tyr10-LDHA suggests that LDHA was not only overexpressed
321 in IB3-1 cells but also activated through tyrosine phosphorylation. However, the ratio p-Tyr10-
322 LDHA/LDHA remained approximately constant (the differences in the ratios were not significant;
323 results not shown), suggesting that the overexpressed LDHA is activated in the same proportion. The
324 kinase(s) responsible remains to be determined. These results suggest that LDHA and p-LDHA are
325 up-regulated in CF cells, in agreement with the observed increase in LDH activity and lactate
326 secretion.

327

328 **EGFR modulates LDHA expression**

329 Since *LDHA* expression (mRNA and protein) and activity increased in CF IB3-1 cells, we
330 wanted to know whether LDHA expression was also affected by the EGFR activity. To test this, IB3-
331 1 and C38 cells were incubated in the presence of the EGFR inhibitors and the LDHA mRNA
332 expression levels were measured by quantitative real-time PCR. As shown in Figure 5A, the EGFR
333 inhibitors significantly reduced the LDHA mRNA expression (IB3-1 $100 \pm 10\%$, AG1478 $52.9 \pm$
334 7.4% , PD168393 $65.3 \pm 1.8\%$, $n=3$). This was confirmed by flow cytometry, analyzing the
335 expression of total LDHA (Fig. 5B and 5C). LDHA protein expression levels were expressed as

336 Mean Fluorescence Intensity (MFI) (Fig. 5B). Representative cytometries are shown in Figure 5C,
337 plotted as FL1-A (Fluorescence intensity of green channel or FL1-A) vs FSC-A (forward scattering).
338 The results showed a significantly ($p < 0.05$, $n = 3$) decreased LDHA expression in IB3-1 cells treated
339 with the EGFR inhibitors AG1478 and PD 168393 (IB3-1 100 ± 10 %, AG1478 60 ± 18 %,
340 PD168393 80 ± 8 %, $n = 3$). Therefore, EGFR activity also regulates LDHA mRNA and protein
341 expression.

342

343 **DISCUSSION**

344 As a model system we used cultured IB3-1 airway CF cells, which have a mutated CFTR
345 ($\Delta F508/W1282X$ genotype), and C38 cells, which are IB3-1 “corrected cells”, transduced with a
346 viral vector expressing a truncated wt-CFTR, which resulted in a high basal CFTR activity (Flotte et
347 al. 1993). We used IB3-1 CF cells, cultured as nonconfluent monolayers since these cells, with
348 impaired CFTR activity, have a constitutive secretion of IL-1 β (Clauzure et al. 2014; Clauzure et al.
349 2017; Massip-Copiz et al. 2017), which in Caco-2/pRS26 cells activates the expression of EGF
350 ligands through JNK (but not c-Src) and keeps an activated EGFR signaling (Massip-Copiz et al.
351 2018), and inflammatory state (Yang et al. 2019). Thus, with IB3-1 cells there is not a need to add
352 external stimuli that can produce unnecessary off-target effects and complicate the interpretation of
353 the results. Also to avoid off-target effects of the dibutyryl-cAMP, isoproterenol, and the IBMX (3-
354 isobutyl-1-methylxanthine) used for CFTR stimulation, we decided to perform the cultures in the
355 absence of CFTR stimulation and use C38 (des-1-119-CFTR) cells instead of S9 (IB3-1 cells
356 expressing wt-CFTR) cells. We did not use S9 cells since these cells need CFTR stimulation to show
357 differences in activity compared to IB3-1 cells. On the other hand, C38 cells, without stimulation,
358 already have increased rates of Cl $^-$ efflux compared to IB3-1 cells (Flotte et al. 1993), and do not
359 necessarily need stimulation to show differences with IB3-1 cells. CFTR is overexpressed in C38
360 cells compared to IB3-1 cells (Andersson et al. 2008), and this might explain the increased basal
361 CFTR activity in C38 cells. When the activity of the mitochondrial Complex I (mCx-I) was
362 measured; differences between S9 and IB3-1 cells were observed only upon stimulation (Valdivieso
363 et al. 2012). Also, S9 cells need CFTR stimulation to show differences with IB3-1 cells in IL-1 β

364 secretion (Clazure et al. 2017). When the intracellular chloride was measured in S9 and IB3-1 cells,
365 it became evident that in the presence of a stimulation cocktail (200 mM dibutyryl cAMP, 200 mM
366 IBMX, and 20 mM isoproterenol), the IB3-1 cells have some residual CFTR activity, as occurs in
367 CF patients (Sermet-Gaudelus et al. 2002); the chloride increases in the presence of CFTR(inh)-172,
368 and S9 cells have less intracellular chloride than IB3-1 cells because the CFTR is functional
369 (Clazure et al. 2017). This was also reflected in the secretion of lactate by IB3-1 cells compared
370 with C38 cells(Valdivieso et al. 2019).

371 In previous studies, IL-1 β strongly acidified the conditioned media of Caco-2/pRSctrl cells
372 and mediated the increased NOX1/4 and ROS levels and decreased pHe, in Caco-2/pRSctrl and IB3-
373 1 cells, through an autocrine positive feedback signaling, in which c-Src and JNK were involved
374 (Clazure et al. 2014; Clazure et al. 2017; Massip-Copiz et al. 2017; Valdivieso et al. 2019). Also,
375 the IL-1 β autocrine loop induced EGFR ligands, particularly epiregulin. This resulted in an activated
376 p-EGFR in cells with impaired CFTR activity (Massip-Copiz et al. 2018). Then, we noted that
377 besides epiregulin (EREG) upregulation, c-Src and JNK may be downstream signaling effectors for
378 EGFR, opening the possibility that the observed IL-1 β effects on pHe were indirectly mediated by
379 EGFR signaling. Thus, using two pharmacological inhibitors of EGFR that act through different
380 mechanisms (one reversible and the other irreversible) we assessed this hypothesis.

381 In the present work, we first measured the pHe, lactate levels, and LDH expression and
382 activity in IB3-1 CF cells and C38. The results showed that a reduced pHe is present in the
383 conditioned media of IB3-1 cells compared to the pHe values of the CFTR-corrected C38 cells (Fig.
384 1), confirming our previous observations (Valdivieso et al. 2019). The LDHA was found
385 overexpressed in IB3-1 cells, as determined by WBs, confocal microscopy, and flow cytometry. The
386 cytometric results (fluorescence measured in each of 10.000 cells) suggests that the reduction in the
387 pHe and hypersecretion of lactate in IB3-1 cells should not only be attributed to possible accelerated
388 growth of IB3-1 cells compared to C38 cells but rather to an increased LDH expression in each cell.
389 The cells accumulated a large amount of lactate, reaching a concentration near 5 mM after 48 h. This
390 represents a huge amount of lactate, but still far from the buffering capacity of the DMEM-F12

391 medium with 15 mM HEPES and 15 mM bicarbonate. A key question is, therefore, which is the
392 buffer capacity of the ASL and the basolateral compartment. Only when the buffer capacity is
393 exceeded, we will see a drastic reduction in the pHe. This might explain some of the contradictory
394 results regarding the pHe in CF cells. Also, to measure lactate is a better measure than pHe, since
395 only when the buffer capacity is exceeded a drastic reduction on the pHe will be noted.

396 Incubation of IB3-1 CF cells with the reversible EGFR inhibitor AG1478 partially restored
397 the pHe levels of IB3-1 cells compared to the values of C38 control cells (C38 are IB3-1 CF cells
398 expressing wt-CFTR). AG1478 restore the IB3-1 cells to the values of untreated C38 cells, but basal
399 values of C38 cells were also increased by AG1478. On the contrary, the irreversible (alkylating)
400 EGFR inhibitor PD168393 did not affect basal values of C38 cells and fully restored the pHe of IB3-
401 1 cells compared to C38 cells. Furthermore, these EGFR inhibitors decreased LDHA expression,
402 LDH activity, and lactate production. These effects are in agreement with the results reported by
403 Coaxum et al. (Coaxum et al. 2009) in cultured renal glomerular podocytes stimulated with EGF, in
404 which the EGFR tyrosine kinase inhibitor AG1478 inhibited the extracellular acidification rate
405 (ECAR), although the ECAR effect was attributed to the EGF activation of Na⁺/H⁺ exchange. One
406 may think that at least partially the Na⁺/H⁺ exchanger may contribute to the decreased pHe in IB3-1
407 cells. However, our previous results with IB3-1 and C38 cells (Valdivieso et al. 2019) showed an
408 almost complete recovery of the pHe of IB3-1 cells, compared to C38 cells, by incubation with the
409 LDH inhibitor oxamate.

410 Our results regarding EGFR upregulation in IB3-1 cells are in agreement with previous
411 results obtained in CF bronchial epithelial CFBE41o(-) cells lacking functional CFTR cultured at the
412 air-liquid interface (ALI). This was confirmed by using differentiated primary human bronchial
413 epithelial cells (HBEC-ALI) (Stolarczyk and Scholte 2018; Stolarczyk et al. 2018). An activated
414 EGFR signaling pathway was also observed by Martel et al. (Martel et al. 2013) in CFTR ΔF508-
415 expressing airway epithelial cells exposed to *Pseudomonas aeruginosa*. On the other hand, our results
416 regarding the differences in LDHA expression levels between IB3-1 and C38 cells are in agreement

417 with a recent proteomic analysis of these cells (Ciavardelli et al. 2013); this LDHA upregulation has
418 been also observed in the bronchial and nasal epithelium from CF patients (Ogilvie et al. 2011;
419 Polineni et al. 2018). LDH shows a complex regulation and several transcription factors and kinases
420 regulates its activity (Mishra and Banerjee 2019). Increased expression of EGFR has been reported
421 by Burgel et al. in patients with cystic fibrosis (Burgel et al. 2007). Also, Martel et al. (Martel et al.
422 2013) observed an increased EGFR phosphorylation (at Tyr-1068) in the CF airway epithelium and
423 sub-epithelial inflammatory cells of CF lung biopsies (Martel et al. 2013). Furthermore, it was
424 reported that p-EGFR is increased in IB3-1 cells (CF) compared to C38 cells (Kim et al. 2013), and
425 recently, this was also observed in the human CF bronchial epithelial cell line CFBE41o- (Stolarczyk
426 et al. 2018) and in Caco-2/pRS26 cells (Fig. S1 in Massip-Copiz et al.(Massip-Copiz et al. 2018)).

427 Taken together, these results suggest that the EGFR receptor has a key role in regulating
428 LDHA expression and activity in IB3-1 CF cells, which in turn determines an increased acid lactic
429 secretion and decreased pHe. Taken into account our recent results with IB3-1 and Caco-2 cells
430 (Clauzure et al. 2014; Clauzure et al. 2017; Massip-Copiz et al. 2018; Massip-Copiz et al. 2017;
431 Valdivieso and Santa-Coloma 2019; Valdivieso et al. 2016; Valdivieso et al. 2019), it appears that
432 the mechanisms of hyperacidity in cells with impaired CFTR activity involve the CFTR → Cl⁻ → IL-
433 1β,c-Src → EREG → EGFR → c-Src, JNK → LDHA → lactate axis. We added here the role
434 of EGFR in this mechanism, which is crucial since its inhibition completely restores the pHe
435 to the values of control C38 cells, a physiological value of pHe=7.37. Noteworthy, the more
436 specific and irreversible EGFR inhibitor PD168393 had a zero influence on the pHe of C38
437 cells, reinforcing the important role of EGFR signaling in the reduced pHe observed here in
438 IB3-1 cells and previously in Caco-2/pRS26 cells. The relationships between the different
439 signaling pathways studied here are illustrated in Figure 6 as a working hypothesis that summarizes
440 and helps to understand the results obtained.

441 In conclusion, these results suggest that extracellular acidification of CF epithelial cells IB3-
442 1 is partially mediated by the activation of the EGFR signaling, which results in the upregulation of

443 LDH-A (mRNA and protein), increased LDH activity, and lactate secretion. The EGFR downstream
444 signaling involved in LDHA expression and activity, besides the previously found c-Src and JNK
445 (Valdivieso et al. 2019), is still to be determined. Increased intracellular and extracellular lactate
446 concentrations might have a strong physiological (Latham et al. 2012) and pathophysiological (Feng
447 et al. 2018; Lardner 2001; Martinez et al. 2007; Massip-Copiz and Santa-Coloma 2018)
448 consequences in cells in which the EGFR signaling is activated through the CFTR failure or in
449 conditions using a different mechanism to activate EGFR, such as those occurring in cancer. These
450 results indicate that EGFR signaling is a new effector in the CFTR and Cl⁻ signaling pathways.

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837 **Figure legends**

838 **Figure 1. The impairment of the CFTR activity in IB3-1 cells decreases the extracellular pH,**
839 **increases LDH activity, and lactic acid secretion.** IB3-1 (CF cells) and C38 cells (IB3-1 “rescued”
840 cells) were incubated in serum-free medium and the pHe measured. (A) pHe values in the conditioned
841 media after 48 h. (B) Lactic acid concentration in conditioned media from IB3-1 and C38 cells,
842 measured as lactate, after 48 h of incubation. (C) Lactic acid concentration in conditioned from IB3-
843 1 cells (black line) and C38 cells (gray line), at 0, 24, and 48 h, measured as lactate. The results were
844 fitted using linear regression. The slopes are shown and had a significant difference ($p < 0.05$, $n=3$).
845 * indicates significant differences compared to the untreated cells. (D) Intracellular LDH activity in
846 IB3-1 and C38 cells, measured after 48 h of incubation in serum-free medium; the values of C38 was
847 normalized to 100 % of activity. * indicates $p < 0.05$. IB3-1 cells show a significant reduction in
848 pHe, in conditioned media.

849 **Figure 2. EGFR inhibitors normalized the pHe of IB3-1 cells.** IB3-1 and C38 cells were cultured
850 in serum-free medium in the presence of EGFR inhibitors and the pHe was measured at 48 h. (A)

851 Dose-response curve corresponding to pH_e measured in conditioned media from IB3-1 cells treated
852 with different concentrations of the EGFR inhibitor AG1478; ED₅₀ = 5.9 ± 0.3 μM. (B) Dose-
853 response curve of IB3-1 cells treated with the EGFR inhibitor PD168393; ED₅₀ = 2.1 ± 0.5 μM. The
854 dose-response curves were fitted by using a sigmoidal function. (C) IB3-1 and C38 cells incubated
855 with 10 μM AG1478. (D) IB3-1 and C38 cells incubated with 10 μM PD168393. * indicates p <
856 0.05.

857 **Figure 3. LDH activity and lactic acid secretion are modulated by EGFR inhibitors.** IB3-1 and
858 C38 cells were cultured in serum-free medium in the presence of EGFR inhibitors. (A) Intracellular
859 LDH activity in C38 and IB3-1 cells treated with the EGFR inhibitor AG1478 (10 μM). LDH activity
860 was expressed as %, normalized to C38 as 100% activity. (B) Cells were incubated with the EGFR
861 inhibitor PD168393 (10 μM). (C) Lactate concentration in conditioned media of IB3-1 cells treated
862 with AG1478 at 0, 24, and 48 h. (D) Lactate concentration in conditioned media of IB3-1 cells treated
863 with PD168393 at 0, 24, and 48 h. * indicates p < 0.05.

864 **Figure 4. LDHA expression is upregulated in CF-cells.** IB3-1 and C38 cells were incubated 48 h
865 in serum-free medium. After incubation, total proteins or total RNA were extracted and the LDHA
866 protein levels and *LDHA* mRNA were determined by WB or real-time PCR. The same treatment was
867 applied to measure LDHA and p-LDHA by using flow cytometry. (A) Quantitative real-time RT-
868 PCR of *LDHA* mRNA expression levels in C38 and IB3-1 cells. (B) Representative WB
869 corresponding to LDHA and p-LDHA from whole-cell lysates of C38 and IB3-1 cells. (C)
870 Densitometric quantification of p-LDHA/actin (D) Densitometric quantification of LDHA/actin. (E)
871 Confocal image of LDHA in C38 and IB3-1 cells; (a) confocal fluorescent image with anti LDHA
872 antibodies (b) visible transmitted light image. (F) Representative cytometry of LDHA expression.
873 (G) average MFI (mean fluorescence intensity) values of F. (H) Representative cytometry of p-
874 LDHA expression. (I) MFI average values of data in H. MFI values are normalized to control values.
875 *indicates p < 0.05.

876 **Figure 5. LDHA expression is modulated by EGFR inhibitors.** IB3-1 cells were incubated 48 h
877 in serum-free medium and treated with EGFR inhibitors (AG1478 and PD168393). After incubation,
878 total RNA was extracted and the LDHA mRNA was determined by real-time PCR. Alternatively,
879 cells were collected and fixed with paraformaldehyde 4% to measure LDHA expression by flow
880 cytometry by using the anti-LDHA Ab. (A) Quantitative real-time RT-PCR of LDHA mRNA
881 expression levels in IB3-1 cells. (B) LDHA expression is shown as mean fluorescence intensity
882 (MFI) and normalized to control values. *indicates $p < 0.05$. (C) Representative dot plots of the
883 cytometries shown in B. FL1-A (fluorescence intensity in the green channel) vs FSC-A (forward
884 scattering). Plots are shown for IB3-1 cells treated with the EGFR inhibitors AG1478 and PD168393,
885 respectively. *indicates $p < 0.05$.

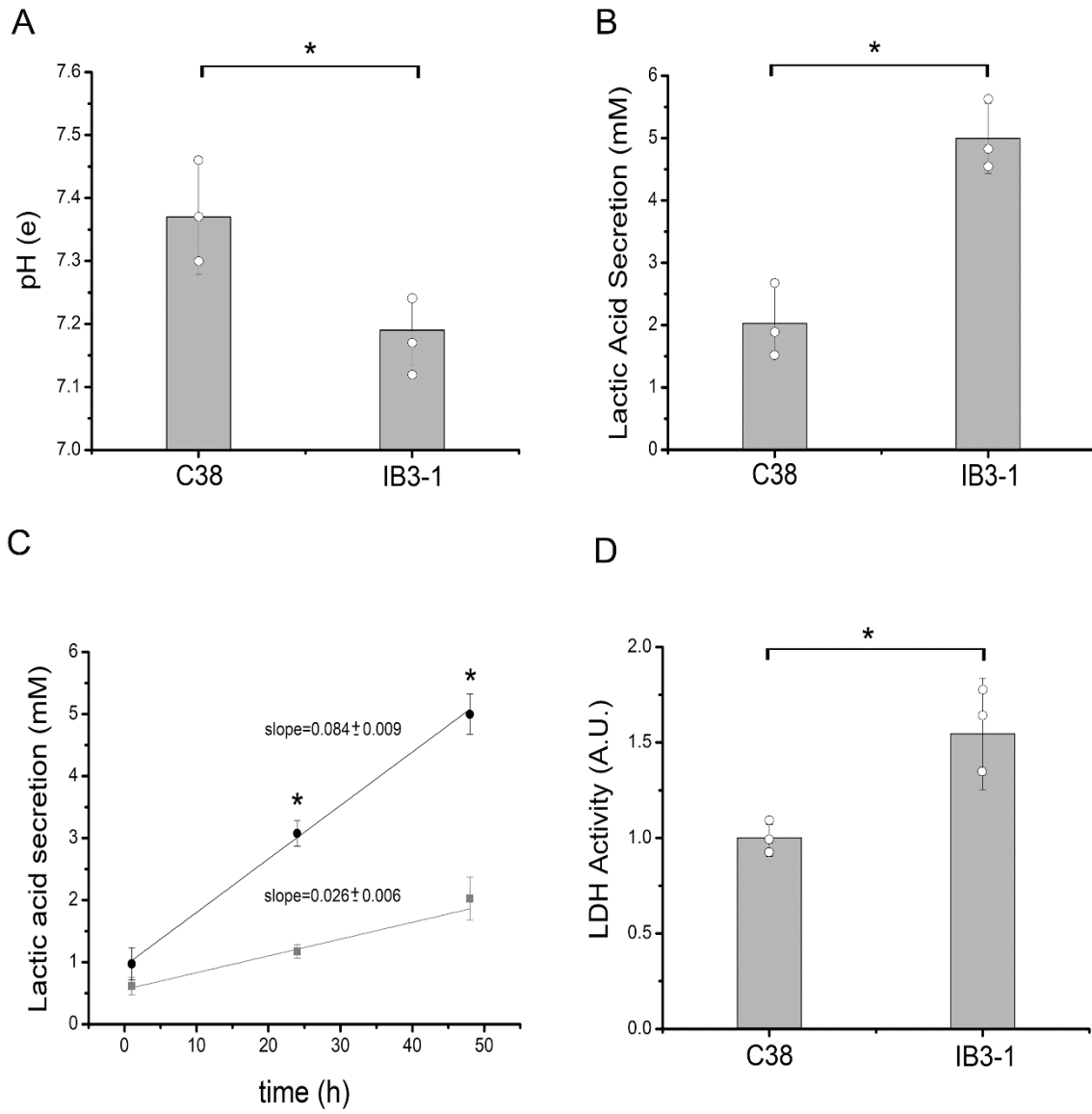
886 **Figure 6. Graphic summary.** The figure summarizes the pathways and interactions involved in
887 the results obtained here. It is just a working model to illustrate the interactions known so far. Several
888 interactions need further studies, in particular the intermediary effectors downstream of EGFR that
889 stimulate both, the LDHA expression and regulation by phosphorylation. The impairment of the
890 CFTR activity induces chloride accumulation that, acting as a second messenger, starts an autocrine
891 IL-1 β loop, with stimulated c-Src and JNK activities that modulate the secretion of lactate
892 {Valdivieso, 2019 #1129}. Here, EGFR seems to be an important player in regulating lactate
893 secretion. The activated EGFR in IB3-1 cells resulted in a higher LDH activity and production of
894 lactate, which is secreted (together with H⁺), reducing the extracellular pH (pHe). The signaling
895 mechanism between CFTR and EGFR, which includes Cl⁻, c-Src, IL-1 β , and EGFR ligands are still
896 ill-defined (dotted line). These results were obtained using IB3-1 lung epithelial CF cells *in vitro* and
897 may differ from results obtained *in vivo* or expressed only under stress conditions (infections or
898 injured tissues).

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Figures

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Figure 1

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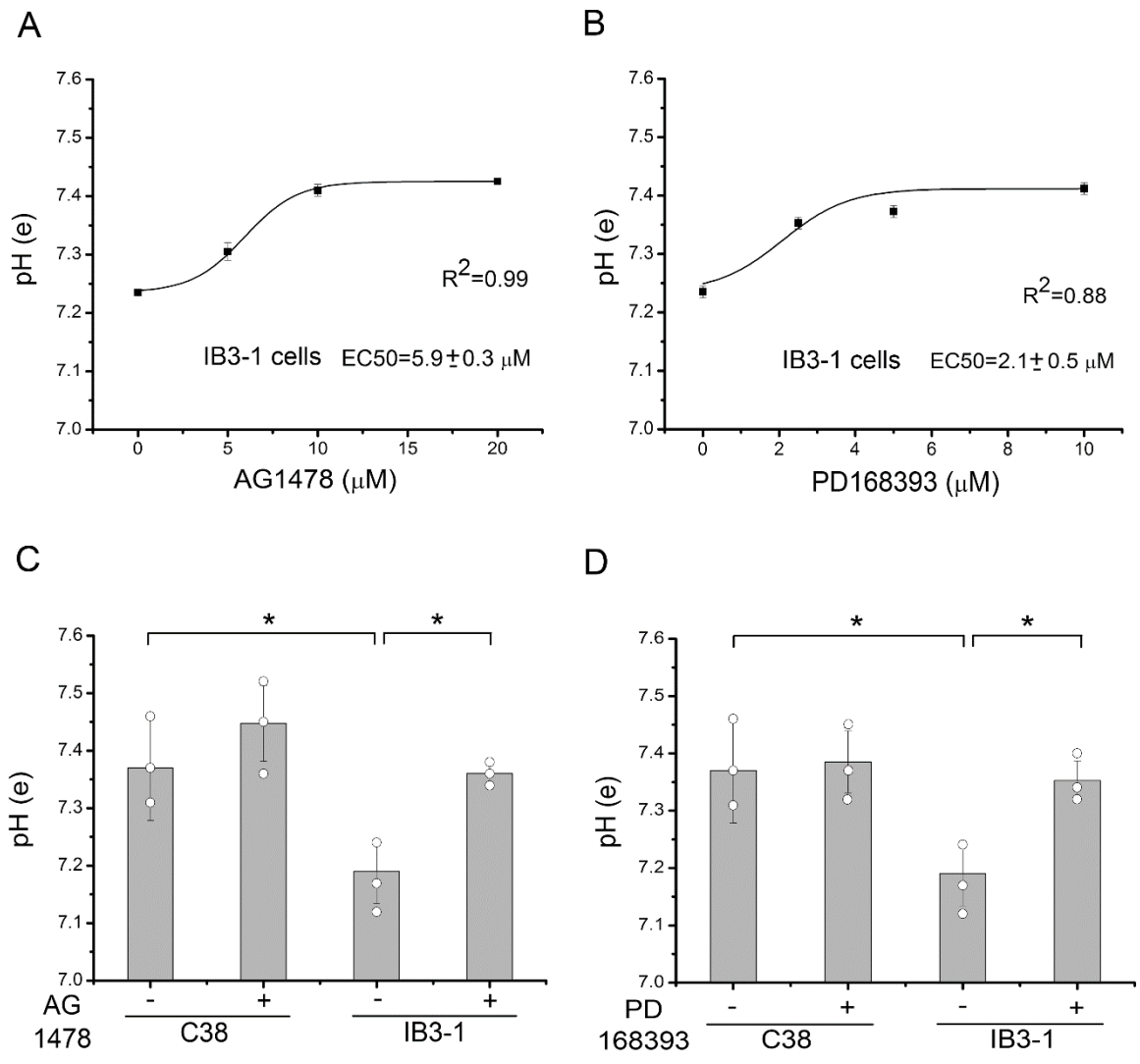
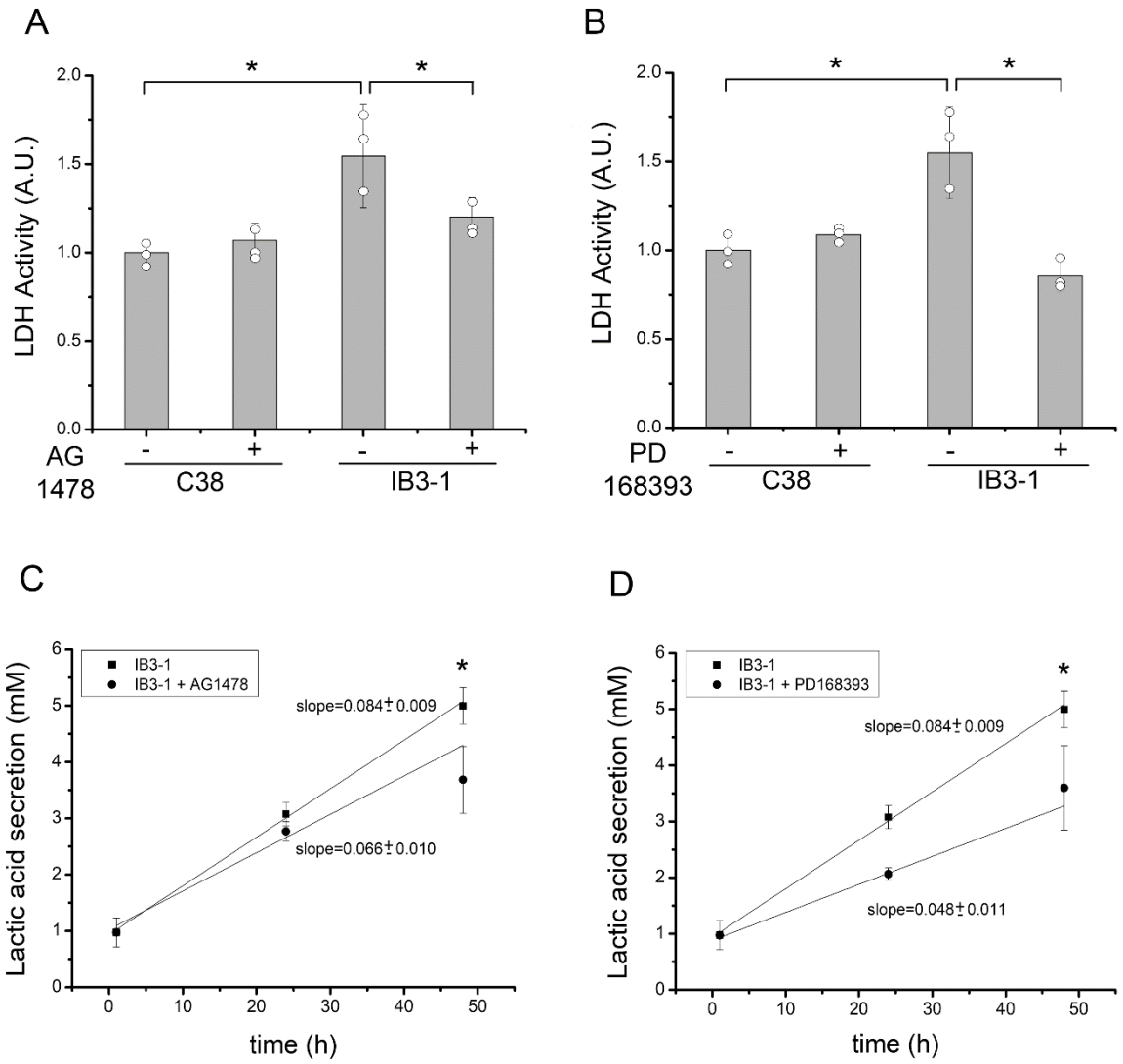
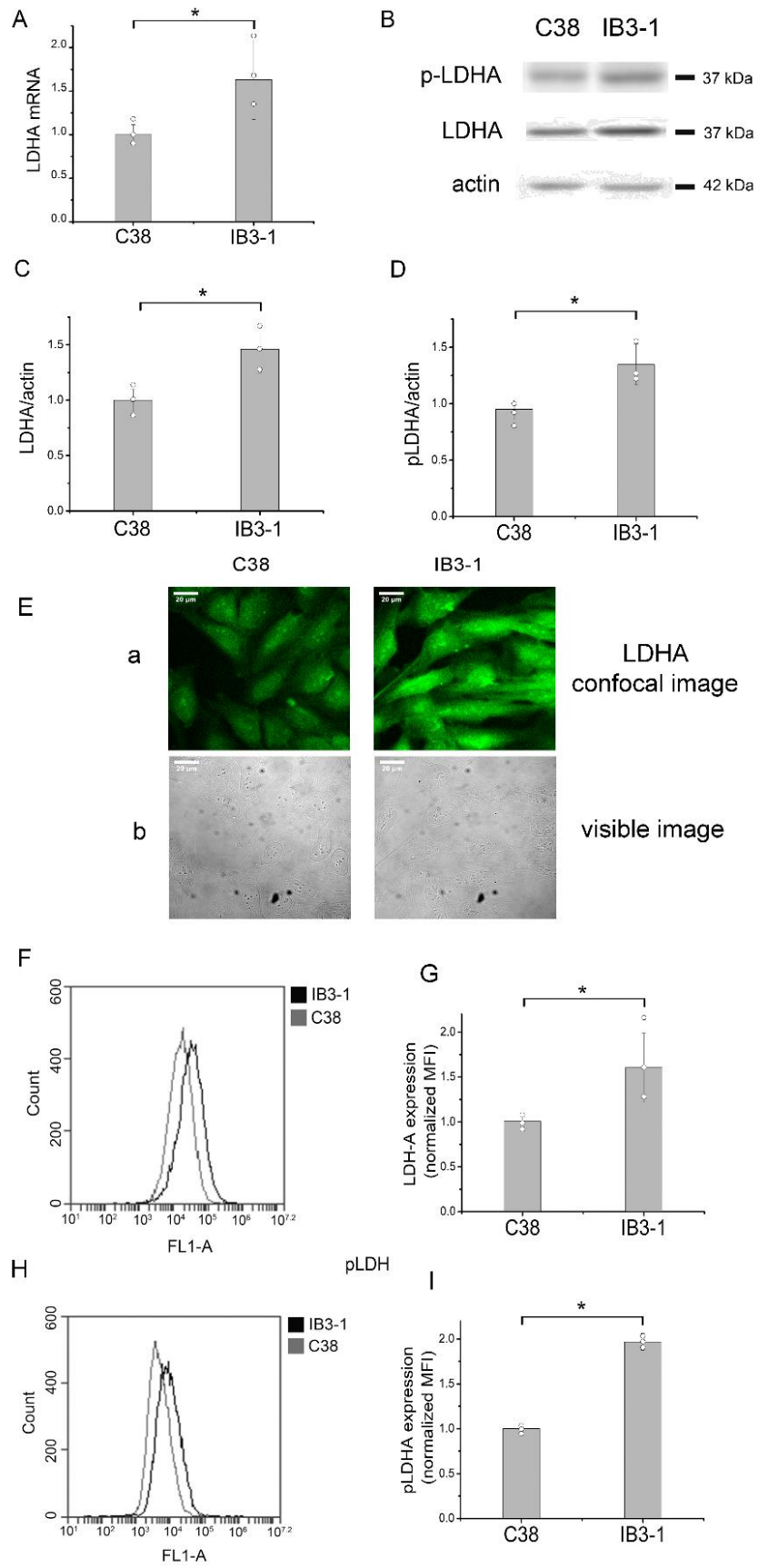


Figure 2



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Figure 3



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Figure 4

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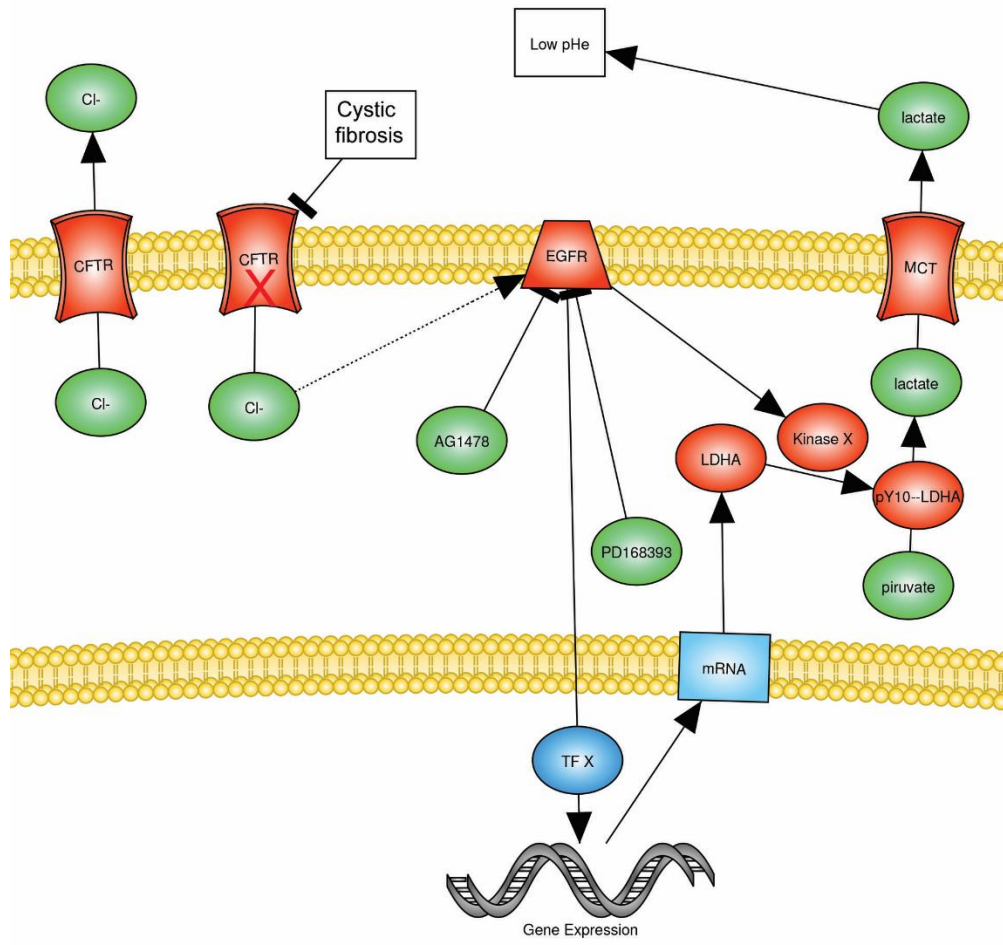
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Figure 5

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Figure 6